# The genus *Pyrenaearia* (Gastropoda, Helicoidea): Molecular and Morphological Systematics, Biogeography and Population Dynamics

# *Pyrenaearia* generoa (Gastropoda, Helicoidea): Sistematika Molekularra eta Morfologikoa, Biogeografia eta Populazio Dinamika


**PhD thesis**

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eman ta zabal zazu

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A thesis submitted by

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for the degree of Doctor of Philosophy,

under the supervision of

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Astiro igo,
barraskilotxo
Fuji mendia da hau!

*Kobayashi Issa*-ren haikua

To the little things that run the world

# Esker onak

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# Contents

# Preface

This thesis is divided into six chapters.

First, general framework is given in the introduction (**Chapter 1**) where the target genus, *Pyrenaearia*, is described in detail and its potential as invertebrate sentinel taxon is highlighted in a context of biodiversity crisis, where invertebrate conservation has been largely neglected. Since a multidisciplinary approach combining molecular, geometric morphometrics and capture-recapture techniques is proposed, the basis and development of each discipline are also briefly described in this chapter.

The general and specific goals of the thesis are gathered in **Chapter 2**.

**Chapter 3**, subdivided into three papers (Papers I-III), deals with the systematics and biogeography of the genus *Pyrenaearia* and its subfamily, Leptaxinae.

In **Chapter 4** population dynamics and basic biological traits of one of the genus species, *P. carascalensis*, are investigated through a capture-recapture study, which is summarized in Paper IV.

In **Chapter 5** morphological integration of the genus' shell is assessed applying a multilevel approach using geometric morphometrics. The results are gathered in Paper V.

**Chapter 6** listed the main conclusions that can be drawn from this PhD thesis.

Finally, the abstract of a paper addressing the molecular basis of *Cepaea nemoralis* polymorphism (Paper VI), derived from the PhD formation period but not directly related to *Pyrenaearia,* is included as an **Appendix**.

# Hitzaurrea

Tesi hau sei kapitulutan banatzen da.

Lehenbizi, testuinguru orokorra ezartzen da sarreran (**1. kapitulua**). Bertan xede-generoa, *Pyrenaearia*, xehetasunez deskribatzen da eta taxon ornogabe zelatari gisa daukan potentziala nabarmentzen da biodibertsitate krisi testuinguru batean, non ornogabeen kontserbazioak arreta gutxi jaso izan duen. Teknika molekularrak, morfometria geometrikoa eta harrapaketa-berrarrapaketa metodologia konbinatzen dituen hurbiltze integratzailea proposatzen denez, diziplina bakoitzaren oinarria eta garapena ere deskribatzen dira kapitulu honetan.

Tesiaren helburu orokorra eta helburu zehatzak **2. kapituluan** biltzen dira.

**3. kapituluan**, hiru artikulutan banatuta (I-III artikuluak), *Pyrenaearia* generoaren eta bere subfamiliaren, Leptaxinae-ren, sistematika eta biogeografia jorratzen dira.

**4. kapituluan** generoaren espezie baten, *P. carascalensis*-en, populazio dinamika eta oinarrizko ezaugarri biologikoak ikertzen dira, harrapaketa-berrarrapaketa ikerketa baten bidez, IV. artikuluan laburtzen dena.

**5. kapituluan** generoaren oskolaren integrazio morfologikoa ebaluatzen da, morfometria geometrikoarekin maila-anitzeko hurbiltzea aplikatuz. Emaitzak V. artikuluan biltzen dira.

**6. kapituluak** doktorego tesi honetatik eratorri daitezkeen ondorio nagusiak zerrendatzen ditu.

Azkenik, *Cepaea nemoralis*-en polimorfismoaren oinarri molekularra ikertzen duen artikulu baten laburpena (VI. artikulua) **Eranskin** gisa bildu da, doktorego honen formaziotik eratorri dena, baina zuzenean *Pyrenaearia*-rekin erlazionatuta ez dagoena.

# CHAPTER 1

## Introduction

# 1. Biodiversity: its importance and crisis

The origin of the term "biodiversity", created from the contraction of "*biological diversity*", is rather new. In 1980, T.E. Lovejoy introduced the term "biological diversity" for the first time to name a group of species conforming a community but without any neat definition. That same year, the marine biologists Norse and McManus (1980) proposed a more formal definition for biological diversity where genetic and ecological concepts were already included. However, the coining of the term "biodiversity" is set in 1988 when E.O. Wilson published a book under the name "Biodiversity" based on the "National Forum on BioDiversity" hosted in 1986 in USA (Haila and Kouki, 1994). Indeed, the release of this publication increased the use of the term not only in scientific literature but also in other society areas.

Thereafter, the meaning of the term biodiversity has changed. From being in the beginning a quantitative concept expressing species diversity only, it progressively gained complexity to become at the end in a multi-scalar concept that joins the diversity of life in all its manifestations and scales, including genes, species and ecosystems, as well as ecosystem processes that can be considered at different time and scales. Currently, since in order to plan conservation and management strategies a clear and specific conceptual framework is important, the formal definition proposed for biodiversity in the international Convention on Biological Diversity (CBD) in 1992 is taken as reference. This stated that "biological diversity or biodiversity means the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems" (CDB, 1992; Article 2).

Thus, biodiversity is multidimensional since any biota is characterized by its genetic, taxonomic and ecological diversity (MEA, 2005). In other words, three levels are enclosed within biological diversity: genetic diversity, organismal diversity and ecological diversity. The elements within each of these levels are tightly related in nested hierarchies and the levels are also closely linked with each other, even sharing some elements (Gaston, 2010). In addition, these dimensions of biodiversity vary throughout space and time. Thereby, we can speak about the biodiversity of a drop of water or in the entire Earth, but also about the biodiversity at present, at a given time or period in the past or future, or over the entire history of the Earth (Gaston, 2010).

Clearly, the complexity and scale of biodiversity is huge, and delimiting and understanding it very difficult, perhaps impossible. However, its complexity equates its importance. Indeed, biodiversity is the basis of ecosystems services that are fundamental for human welfare, because it holds and maintains ecosystem functions (MEA, 2005).

Ecosystem services are the set of ecosystem functions that sustain and fulfil human kind (Daily, 1997; Kremen, 2005) and can range from global scale to microscopic (Sekercioglu, 2005). Even though it has been long known that ecosystems sustain societies, the explicit recognition of ecosystem services is quite new (Ehrlich and Ehrlich 1981; Mooney and Ehrlich 1997). And yet, thereafter, plenty of works have been published about them (i.e., Costanza et al., 2014, 1997; Lin et al., 2018; Patterson and Coelho, 2009; Worm et al., 2006) with the global overview performed in the Millennium Ecosystem Assessment (2005) as reference. In this synthesis, ecosystem services are already classified into in four groups: provisioning, regulating, cultural and supporting services. Indeed, ecosystems clean the air and the water, generate oxygen and stabilize climate; some organisms decompose and detoxify detritus, transforming our residues; thousand of animals pollinate plants, protect them from pests and spread their seeds; other species create and maintain the soils on which we grow our food and also recycle their nutrients; and, of course, humans directly consume and use thousands of plants, animals and microorganisms (Sekercioglu, 2010).

However, currently, Earth is undergoing a deep biodiversity loss due to human activities (Cardinale et al., 2012; Cardoso et al., 2011; Purvis and Hector, 2000). It has been warned for years that the trend of species extinction rate was approaching to surpass the background extinction rate found in the fossil record (Hooper et al., 2012). Currently, scientists agree that Earth is already undergoing the sixth mass extinction, being the actual rate of species extinction 100 times higher than the "normal rate" through geological time (Ceballos et al., 2017, 2015; Ceballos and Ehrlich, 2018; Pimm et al., 2014). Thus, according to conservative estimates 3000 species go extinct yearly, that is eight species per day (González-Oreja, 2008; Wheeler et al., 2004). Biodiversity loss destabilizes ecosystem functions and leads to ecosystem services loss, endangering our welfare and, hence, being among the most important environmental problems (Balvanera et al., 2006). In fact, there is an industrialized human population of almost 8000 million people that completely depends on the ecosystem services that biodiversity provides (Dirzo et al., 2014; Hallmann et al., 2017). For example, the loss of a pollinator may decrease the productivity in many crops (Kremen et al., 2002), and the loss of groundwater fauna may disrupt water purification processes, leading to pollution problems (Boulton et al., 2008). Being responsible for the biodiversity loss, we have a moral obligation to stop it, but selfishly, if we want to ensure ecosystem services, preserving biodiversity is mandatory.

To overcome this crisis, effective conservation and management plans are required, as well as setting conservation priorities. To achieve this, improving our knowledge of biodiversity at all levels is indispensable, from species delimitation and biological and genetic characterization, to ecosystem functions. However, it will be specially

important, to identify areas that are particularly rich in species and/or present unique evolutive or ecological features (Myers et al., 2000), and also settling adequate monitoring systems (Catalan et al., 2017b).

## 2. The importance of invertebrates and the lack of proper knowledge about them

Despite the evidences about biodiversity loss are alarming, they are probably conservative estimates if we take into account the little information available for most taxonomic groups, especially for invertebrates.

Among multicellular organisms invertebrates are the dominant group in terms of diversity, abundance and biomass. About 80% of the described species are invertebrates, with beetle species alone making up 10 times more species than vertebrates (Cardoso et al., 2011). For instance, one hectare of Amazonas Forest can contain more than one billion of invertebrates (Wilson, 1987) and ant biomass in the Serengeti Plains is bigger than mammal biomass (Hölldobler and Wilson, 1990). Although the information about them is scarce, we know that without invertebrates ecosystems could not maintain their functions. Invertebrates may be saprophagous, phytophagous, endo-, ecto- or hiper-parasitoids, symbionts or the last predators of food chains. Some are cosmopolitan and others are restricted to minute areas. They live in terrestrial ecosystems and in aquatic ecosystems both of freshwater or saltwater. Furthermore, they can even be found in the air. With such diversity and variety of functions, invertebrates are essential to maintain ecosystem functions and, consequently their services. Not for nothing did E.O. Wilson (1987) describe invertebrates as "*the little things that run the world*".

In the ongoing biodiversity crisis, invertebrates suffer from the same extinction processes as larger and more familiar organisms but, in addition, they have to deal with some additional ones, such as co-extinction and the extinction of narrow habitat specialists (Dunn, 2005; Dunn et al., 2009). However, even though they are more vulnerable and fundamental for countless ecosystem services necessary for humans, invertebrates have largely been neglected in biodiversity conservation politics, prioritizing more emblematic and charismatic animals, that are vertebrates (Cardoso et al., 2011; Schuldt and Assmann, 2010). This bias is clearly reflected on the World Conservation Union's (IUCN) Red List (2018): while almost every vertebrate has been assessed, only 1% of the described arthropods have been assessed and only 10% in the case of the molluscs. In fact, currently, there is a significant disproportion between the number of protected invertebrate species and those of vertebrates at any scale, to the detriment of invertebrates.

The main responsible for this bias and what is hindering the effective protection of invertebrates, is the lack of proper knowledge about them. Indeed, Cardoso et al. (2011) identified seven impediments that are to blame for the lack of adequate invertebrate conservation: (1) both invertebrates and the ecological services they provide are mostly unknown to the general public (the public dilemma); (2) policy-makers and environmental managers are usually unaware of the conservation problems of invertebrates (the political dilemma); (3) basic science on invertebrates is scarce and, above all, underfunded (the scientific dilemma); (4) many species (most of them) have not yet been described (the Linnean shortfall); (5) there is no any information about the distribution of most of the described species (the Wallacean shortfall); (6) the abundance and the change in space and time of most species remain unknown (the Prestonian shortfall); and, finally, (7) there is a large lack of information about the species ways of life and about their sensitivity to habitat alteration (the Hutchinsonian shortfall).

Thereby, the problem is not limited to the protection of specific species alone. In fact, due to the little information about invertebrates, the most important international strategies for conservation, such as the identification of hot spots (Brooks et al., 2006), have been based on a few vertebrate and vascular plant species (i.e. Brummitt and Lughadha, 2003; Lamoreux et al., 2006; Myers et al., 2000). Nevertheless, some studies have already shown that these strategies are not suited for invertebrate conservation (Kerr, 1997; Schuldt and Assmann, 2010). In these studies the changes in the species richness patterns of different invertebrate groups were investigated and then compared with the patterns of vertebrates and vascular plants and it was showed that they did no match. This means that conservation strategies designed on the basis of vertebrate and vascular plant species data that aim to preserve as much species as possible are actually harming invertebrate conservation since they do not share the same hot spots. Therefore, it is imperative not only to advance in the identification of endangered invertebrate species, but also to integrate them into global conservation strategies.

Furthermore, in biodiversity assessment, invertebrates often provide information about environmental quality and processes at a more relevant scale than vertebrates and plants (Yen and Butcher, 1997). Moreover, invertebrates are often more sensitive to environmental perturbations due to their short generation times and reduced dispersion capacity and, therefore, they may act as excellent indicators to track environmental changes and to get early warnings of catastrophic processes (Kremen et al., 1993). Thus, we may find exceptional sentinel species among invertebrates, both for identifying threats and for assessing the effectiveness of conservation measures.

It is time for invertebrates to receive the attention they deserve. If we aim to properly protect these animals, progressing in their knowledge is imperative, which can only be achieved by significantly allocating funds and efforts towards their basic research.

## 3. Target genus: *Pyrenaearia* Hesse, 1921 (Gastropoda: Helicoidea)

*Pyrenaearia* is a land snail genus endemic to the northern Iberian Peninsula enclosed within the subfamily Leptaxinae Boettger, 1909 belonging to the highly diverse family Hygromiidae Tryon, 1866 (Neiber et al., 2017). The genus shows a discontinuous distribution patter across the Cantabrian Mountains, the Pyrenees, the Mount Moncayo and the pre-littoral mountain system of Catalonia, where it occupies almost exclusively calcareous areas (Ortiz de Zárate, 1956; Puente, 1994). The most remarkable feature of *Pyrenaearia* is that it is a narrow habitat specialist. On the one hand, it is strictly confined to shady rocky areas: it always inhabits in rock walls (sicked on the surface or sheltered in the crevices) or on screes (under the stones); but, in addition, the rocky area must be shady and, consequently, it is restricted to north hillsides, very steep areas and very narrow gorges (Gómez-Moliner et al., 2009). On the other hand, *Pyrenaearia* genus is considered a cold adapted taxon because most of its populations are located in alpine and subalpine zones (Ortiz de Zárate, 1956; Prieto, 1986; Puente, 1994). Nevertheless, there are some populations distributed in the montane zone and even a few bellow it (Puente, 1994).

Thereby, this genus is restricted to mountainous areas, which have remained quite well conserved even in densely populated areas. But long-range transport of contaminants and especially climate change are now affecting these areas (Catalan et al., 2017a). Moreover, mountainous areas are especially vulnerable to climate change because their steeply altitude change promotes very different environmental conditions in short distances (Becker and Bugmann, 2001; Lloret, 2017). Rocky habitats are important elements of mountainous areas both in extension and ecological relevance and, since they are the most abrupt areas, they will be among the most affected areas by climate change. Some of this rocky habitats are included in Annex I section 8$^{th}$ of the Habitats Directive in 2 categories: scree (COD. EU 81) and rocky slopes with chasmophytic vegetation (COD. EU 82). Because of their habitat requirements, *Pyrenaearia* populations are always located in these priority habitats and due to the inherent fragmentation of rocky areas *Pyrenaearia* populations are usually isolated. Furthermore, land snails have a low active dispersal capability and, therefore, their response capacity to colonize new

suitable habitats in different altitude or latitude when an extreme environmental change took place will be limited (Fernández-Chacón et al., 2011). As a result, the genus is vulnerable and, thus, its species might need management and conservation plans. But at the same time, because of these characteristics, it could be an excellent sentinel taxon to track the changes that are taking place in mountainous areas and specially in their rocky areas that are priority habitats.

Although the systematics of gastropod families and the delimitation of their genera have traditionally been based on the morphology of the reproductive system, molecular phylogenetic studies have indicated a high level of homoplasy in this morphological character, highlighting that its usefulness is limited (i.e. Chueca et al., 2018; Davison et al., 2005; Hirano et al., 2014; Neiber et al., 2017, 2018; Wade et al., 2007). However, the morphology of *Pyrenaearia*'s reproductive system is a distinguishing characteristic and it is useful to differentiate the genus from the rest of gastropod genera. According to the descriptions of different *Pyrenaearia* species made by Manga (1983), Ortiz de Zárate (1943; 1956), Prieto (1986), Puente (1994) and Vilella (1963) (**Figure 1**), the genus has a medium size penis narrowed at its end. The penis papilla is cylindrical and, in cross section, it shows a main central groove with 2–3 smaller adjacent grooves. The epiphallus, longer than the penis, curls over the vas deferens which is a distinctive feature of the genus. The vas deferens ties to the distal ends of the penis and the epiphallus by a conjunctive tissue. The flagellum is narrow and its length very variable, ranging from shorter than penis to two times its length. The vagina is slightly curved and well developed.

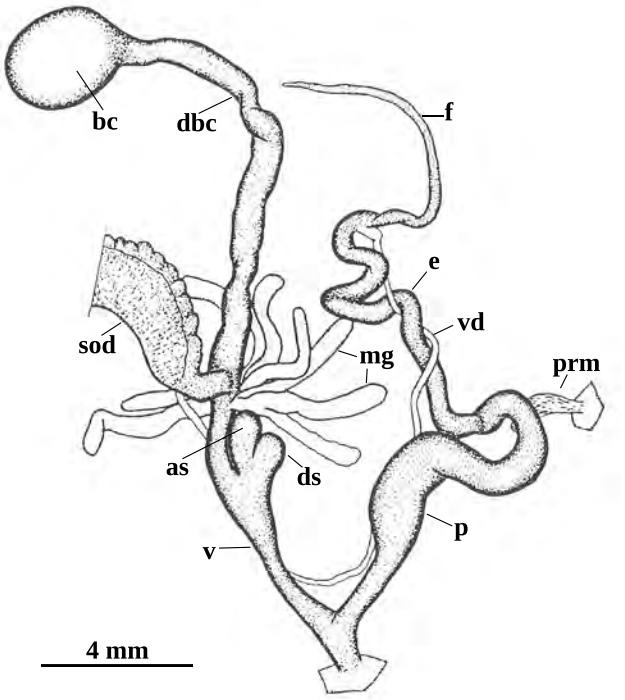


**Figure 1.** Genital anatomy of *Pyrenaearia*. Abbreviations, **bc**: bursa copulatrix, **dbc**: duct of bursa copulatrix; **sod**: spermoviduct; **mg**: mucus gland; **ds**: dart sac; **as**: accessory sac; **v**: vagina; **p**: penis; **prm**: penial retractor muscle; **vd**: vas deferens; **e**: epiphallus; **f**: flagellum. Modified from Prieto (1986).

The accessory copulatory system is formed by a single dart apparatus complex and four mucus glands located around the vagina. The dart apparatus complex consists of two sacks of similar size, with the accessory sac in the inner side and the dart-sac outside. The dart is small and conic, with circular section. Mucus glands are simple or bifurcated. Finally, the *bursa copulatrix* is rounded or oval and the length of its duct is similar or longer than penis length.

Even though the morphology of the genital anatomy undoubtedly diagnosed the genus, at species level the reproductive system lacks diagnostic characters

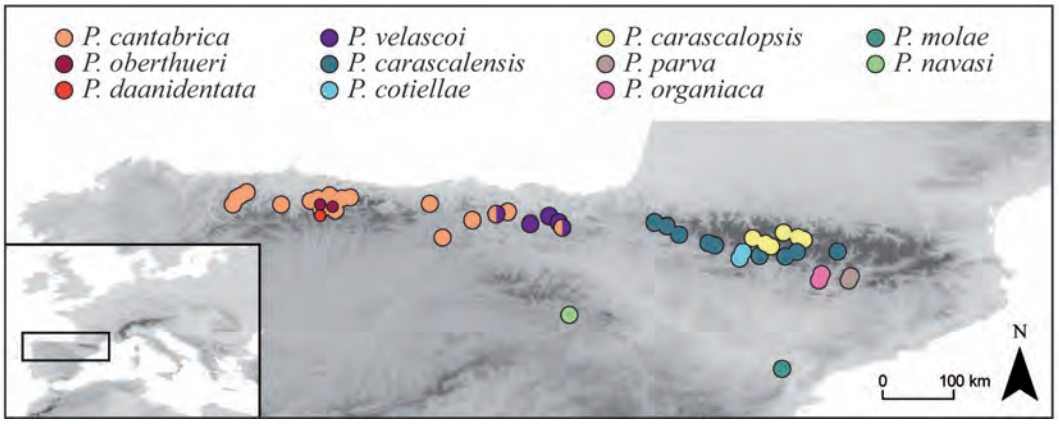


**Figure 2.** Distribution of the 11 species considered by Elejalde et al. (2009) derived from bibliographic citations.

(Ortiz de Zárate, 1956). In consequence, the taxonomy of *Pyrenaearia* has traditionally been based on shell characteristics such as shell colour, general shape and size, peristome shape, and the presence of hairs or teeth (Ortiz de Zárate, 1956; Puente, 1994). Nevertheless, many studies have shown that gastropod shell is tightly correlated with environmental factors, which may blur the establishment of adequate taxonomies (i.e. Fiorentino et al., 2008; Razkin et al., 2017; Stankowski, 2011, 2013).

To overcome this problem, Elejalde et al. (2009) carried out a phylogenetic study based on two mitochondrial loci (*COI* and *16S rRNA*) and one nuclear (*ITS1*) gene fragment in order to confirm the validity of the nominal species of the genus. In view of their results, they considered 11 species within *Pyrenaearia*. Four of them are endemic to the Cantabrian Mountains, *P. cantabrica*, *P. oberthueri*, *P. daanidentata* and *P. velascoi*; five are endemic to the Pyrenees, *P. carascalensis*, P. cotiellae, P. carascalopsis, P. parva and P. organiaca; P. molae lives in the pre-littoral mountain system of Catalonia; and, finally, *P. navasi* is restricted to Mount Moncayo. For each of these species considered by Elejalde et al. (2009) we provide below a short description of their shells and their distributions (species distribution is shown in **Figure 2**).

### *P. cantabrica* (Hidalgo, 1873) (Figure 3a–b)

*Locus typicus*: "Peña Abis, aux environs de Caldas de Oviedo" (Asturias).

*P. cantabrica* is distributed across the entire Cantabrian Mountains, on both the cantabrian and mediterranean watersheds, being particularly abundant in Picos de Europa. Its altitudinal distribution ranges from 40 m to 1800 m.

Its shell is lenticular, always convex bellow and ranging from convex to completely flat above. It has 4.5–5.5 whorls, being the last one 1.5–2 times wider than the previous, and the suture is deep (Manga, 1983; Ortiz de Zárate, 1956; Prieto, 1986). Maximum diameter ranges between 9.8–18 mm (Manga, 1983; Ortiz de Zárate, 1956; Prieto, 1986). The umbilicus is wide and round (1/6–1/4 of the maximum diameter) (Ortiz de Zárate, 1956; Prieto, 1986), which enables seeing internal whorls. The aperture is round and very oblique. The peristome is straight and it has a white thickening on the inside. White-greyish shell colour, with long radial garnet-brown stains and small spots. Periostracal hairs are common in young individuals that sometimes are kept in adulthood (Ortiz de Zárate, 1956; Prieto, 1986). The caryotype of this species is the only one known within the genus *Pyrenaearia*: 2n = 52 chromosomes (n = 26) (Aparicio, 1983).

The nominal species *P. schaufussi* (Kobelt in Rossmässler. 1876), *P. covadongae* Ortiz de Zárate, 1956 and *P. poncebensis* Ortiz de Zárate, 1956 are included within the synonymy of this species.

### *P. oberthueri* (Ancey, 1884) (Figure 3c)

*Locus typicus*: Engotable (Asturias).

The distribution of this species is restricted to the central and the western massifs of Picos de Europa (Raven, 1988), where it inhabits above 1600 m.

The general shell shape of *P. oberthueri* is similar to that of *P. cantabrica*. However, *P. oberthueri* is smaller (maximum diameter 7–10 mm) (Ortiz de Zárate, 1956) and, most importantly, it is more globose and striated. The umbilicus is also round and wide but narrower than the one of *P. cantabrica* (Ortiz de Zárate, 1956). The aperture is round, with a straight peristome that has a white thickening on the inside. The shell colour is greyish-bluish but at the aperture it becomes red-yellowish and there are reddish and bluish thin stains irregularly spread throughout the shell. Both Ortiz de Zárate (1956)

and Raven (1988) noticed a clinal shape variation following altitude for this species and for *P. cantabrica*, increasing shell height and decreasing shell diameter as altitude increased.

### *P. daanidentata* Raven, 1988 (Figure 3d)

*Locus typicus*: "Hoyo del Burro, 2.5 km S. of Peña Santa de Castilla, Picos del Cornión" (León).

The distribution of *P. daanidentata* is very restricted. It is only known from the south watershed of Peña Santa Mountain above 2000 m, in the western Picos de Europa (Gómez-Moliner et al., 2011a).

Its shell is thick and lenticular, convex both below and above. It has five whorls, with fine striae that grow regularly. Maximun diameter ranges between 8.2–9 mm and the umbilicus is wide, 1/5–1/4 of the maximum diameter (Raven, 1988). This species is undoubtedly distinguished due to the strong thickening inside the small and round aperture, just after the peristome, that in most individuals forms one or two teeth. The shell is grey, with oblique brown-greyish irregular lines. *P. daanidentata* is assessed as Vulnerable in the IUCN Red List.

### *P. velascoi* (Hidalgo, 1867) (Figure 3e)

*Locus typicus*: "Pico de Altamira, dans la Peña de Gorbea" (Bizkaia).

*P. velascoi* lives in the highest summits of the Basque Mountains, from the Gorbeia Massif to the Andia Mountain Range, above 1300 m.

Although it is endemic to the Cantabrian Mountains, morphologically is more similar to the Pyrenean species than to the above three species. It has a depressed shell, equally convex above and below and, besides being more globose than the three previous species, similarly to Pyrenean species, it never has periostracal hairs (Ortiz de Zárate, 1956). With marked and irregular striae, it has 4.75–5.25 whorls, being the last whorl 1.5–2 times wider than the penultimate (Prieto, 1986) and the maximum diameter ranges between 14.2–18.6 mm (Ortiz de Zárate, 1956; Prieto, 1986). The umbilicus is narrow and deep, partially covered by the columellar edge. The aperture oblique and oval. The peristome is straight, without inside thickening. White-greyish shell colour, with irregularly distributed thin

radial brownish lines. Some individuals have a white carenal band (Ortiz de Zárate, 1956; Prieto, 1986). *P. velascoi* is considered as Vulnerable in the IUCN Red List.

### *P. carascalensis* (Férussac, 1821) (Figure 3f)

*Locus typicus*: "Fôret de Carascal en Aragon".

This species is distributed throughout the entire Axial Pyrenees and, despite most of the populations lay above 1200 m, it has exceptionally been found bellow that altitude.

Its shell, depressed, is more convex below than above and it has conspicuous striae irregularly distributed, with a faint spiral microsculpture above. It has 4.25–5 whorls, the last whorl 2 times wider than the previous, and maximum diameter ranges from 13.4–16.5 mm (Faci, 1991; Prieto, 1986). The umbilicus is very narrow and the aperture very oblique and oval. The peristome is straight, without internal thickening, and its reflected columellar edge partially covers the umbilicus. Yellow-brownish or greyish shell colour, decorated with radial darker brownish lines and a whitish carenal band that is not present in all individuals.

*P. transfuga* (Fagot, 1885) was synonymized within this species by Elejalde et al. (2009).

### *P. cotiellae* (Fagot, 1906) (Figure 4a)

*Locus typicus*: "Cirque calcaire d'Armèna, sur le mont Cotiella" (Huesca).

The distribution of *P. cotiellae* is restricted to Cotiella Massif, in the north-east of Huesca. Two populations are known there, one in the Armeña Cirque and the other in the Sahún mountain pass, both above 1800 m (Puente, 1994).

This species is very similar to *P. carascalensis* in terms of general shape, striae, aperture, peristome and size (maximum diameter around 15 mm), but according to Fagot (1906) they can be easily distinguished because the shell of *P. cotiellae* is whitish and its umbilicus is wider.

### *P. carascalopsis* (Bourguignat in Fagot, 1884) (Figure 4b)

*Locus typicus*: "Port de Salau, sur les deux versants français et espagnol" (Lleida and Ariège).

This species is distributed in the north watershed of the Axial Pyrenees. Specifically, it has been found in the Maladetta Massif and the Esera Valley in the west of its distribution, in Aula and Salau mountain passes in the east and between these two regions in the Besiberri Massif and Vielha mountain pass, all above 1800 m (Gómez-Moliner et al., 2011b).

Considering morphological traits alone, the status and differentiation of this species have been subjects of debate. Some authors have synonymized it with *P. carascalensis*, while others have considered it as a variety or subspecies of this species (see Puente, 1994). Furthermore, based on morphological data the species *P. esserana* has been considered synonym, variety or subspecies of *P. carascalensis* and it has also been suggested that it may be very close to *P. carascalopsis* (Faci, 1991). However, the molecular work of Elejalde et al. (2009) undoubtedly supports that *P. esserana* is a synonym of *P. carascalopsis* and that *P. carascalopsis* is a valid species. Then, the morphology and colour of the shell are very similar to those of *P. carascalensis*: depressed shell, narrow umbilicus partially covered by the columellar edge and brownish background colour with darker lines. Nevertheless, according to some authors the umbilicus of this species is narrower and the aperture more round but, most importantly, its striae are thicker (Faci, 1991; Ortiz de Zárate, 1956). On the other hand, it seems that the spire height of *P. carascalopsis* is more variable. Indeed, for some individuals a flatter shell has been reported, while for others almost a trochoidal shell has been described (Ortiz de Zárate, 1956). Finally, the maximum diameter of this species ranges between 9.5–10.5 mm (Faci, 1991; Gómez-Moliner et al., 2011b; Ortiz de Zárate, 1956) and, hence, it is smaller than *P. carascalensis*. In the IUCN Red List the species is assessed as Vulnerable.

### *P. parva* Ortiz de Zárate, 1956 (Figure 4c)

*Locus typicus*: "Montaña Pedra-Forca de la Sierra del Calí" (*sic*) (Lleida).

*P. parva* is endemic to Cadí Mountain Range and Pedraforca Massif, where it lives above 2000 m (Altimira, 1963).

It is the smallest species from the Pyrenees with a maximum diameter ranging from 8.2–9.8 mm (Altimira, 1963; Ortiz de Zárate, 1956). The shell is depressed, with 4.5 whorls (Ortiz de Zárate, 1956). The umbilicus, deep and quite wide, enables seeing the internal whorls. The aperture is obliquely oval and the straight peristome is slightly reflected in its columellar edge, partially covering the umbilicus. *P. parva* can be easily discerned because it has very conspicuous irregular white striae, mainly in the last whorl. The spaces between striae are yellowish-brown. This species is considered as Vulnerable in the IUCN Red List.

### *P. organiaca* (Fagot, 1905) (Figure 4d)

*Locus typicus*: "Calcaires du défilé d'Organyá, vallée de la Segre" (Lleida).

Despite the species has been cited in the Noguera Pallaresa Valley, in the most recent samplings it has only been found in the Segre basin, specifically, in Organyá, Esplunvins and Pont de la Torre gorges (Gómez-Moliner et al., 2011c). Unlike the previous species, *P. organiaca* lives only at altitudes ranging between 500–700 m.

The shell of this species is also depressed, with 5–5.5 whorls, the last one wide (Gómez-Moliner et al., 2011c; Ortiz de Zárate, 1956). Among *Pyrenaearia* species it is one of the largest since its maximum diameter ranges between 12–18 mm (Altimira, 1963; Gómez-Moliner et al., 2011c). The aperture is obliquely oval. The peristome is slightly reflected outside and more prominently in its columellar edge, partially covering the narrow umbilicus. The shell is bright, brown-greyish and with numerous white and conspicuous striae, less marked in the inferior side, clearly discerning the species. In the IUCN Red List the species is assessed as Vulnerable.

### *P. molae* Haas, 1924 (Figure 4e)

*Locus typicus*: "Monte dico 'Mola de Falset'" (Tarragona).

The distribution of *P. molae* is restricted to the north faces of Montsant, Colldejou and Llavería Massifs, in the pre-littoral mountain system of Catalonia (Martínez-Ortí and Bros, 2012; Ortiz de Zárate, 1956; Vilella, 1963). It occupies the summits of these massifs, between 800 and 900 m above see level.

The maximum diameter of this species ranges between 11.5–16 mm (Gómez-Moliner et al., 2011d; Ortiz de Zárate, 1956; Vilella, 1963). Its shell is very similar to that of *P. organiaca*: depressed, with a wide last whorl, bright, with numerous white striae, obliquely oval aperture and the umbilicus partially covered by the peristome. However, there are some characteristics that undoubtedly allow discerning them. It has more whorls than *P. organiaca*, 5–6.25, and the umbilicus is narrower, 1/7–1/6 of the maximum diameter (Ortiz de Zárate, 1956; Vilella, 1963). It is less globose, being the spire of some individuals almost flat. Nevertheless, the most noticeable difference is that *P. molae* has more striae but less marked and consequently the shell appearance is whiter since it has less brownish lines. Moreover, most individuals have a white carenal band. *P. molae* is also considered as Vulnerable in the IUCN Red List.

### *P. navasi* (Fagot, 1907) (Figure 4f)

*Locus typicus*: "Moncayo. Nevera de San Miguel au sommet" (Zaragoza).

*P. navasi* is the only *Pyrenaearia* species inhabiting a siliceous emplacement. It is restricted to the north face of Moncayo Massif, in the north of the Iberian System. The populations of this species are located above 1600 m but they are more abundant above 2000 m (Faci, 1991; Gómez-Moliner et al., 2011e; Prieto, 1991). However, a population is known from a beech/oak forest at 1200 m, a completely atypical habitat for the genus (Prieto, 1991).

Perhaps because it lives on siliceous substrate, the shell of this species is the most different within the genus. With a subglobose shape, the shell is thin and fragile, almost translucent and uniformly brown. It has faint oblique striae and an oval aperture. The peristome is very fragile and its colour lighter; it is reflected in the columellar edge partially covering the narrow umbilicus. Maximum diameter usually ranges between 10–11.8 mm and it has 4.5–5 (Faci, 1991; Gómez-Moliner et al., 2011e; Ortiz de Zárate, 1956). Nevertheless, the individuals from the forest population are larger and more globose, with a maximum diameter ranging between 14.4–16.4 mm and 5–5.5 whorls (Prieto, 1991). Due to these differences two subspecies have been considered, the small *P. navasi navasi* and *P. navasi sylvatica* Prieto, 1991 living in the forest, but study of Elejalde et al. (2009) did not support their differentiation. *P. navasi* is assessed as Vulnerable in the IUCN Red List.

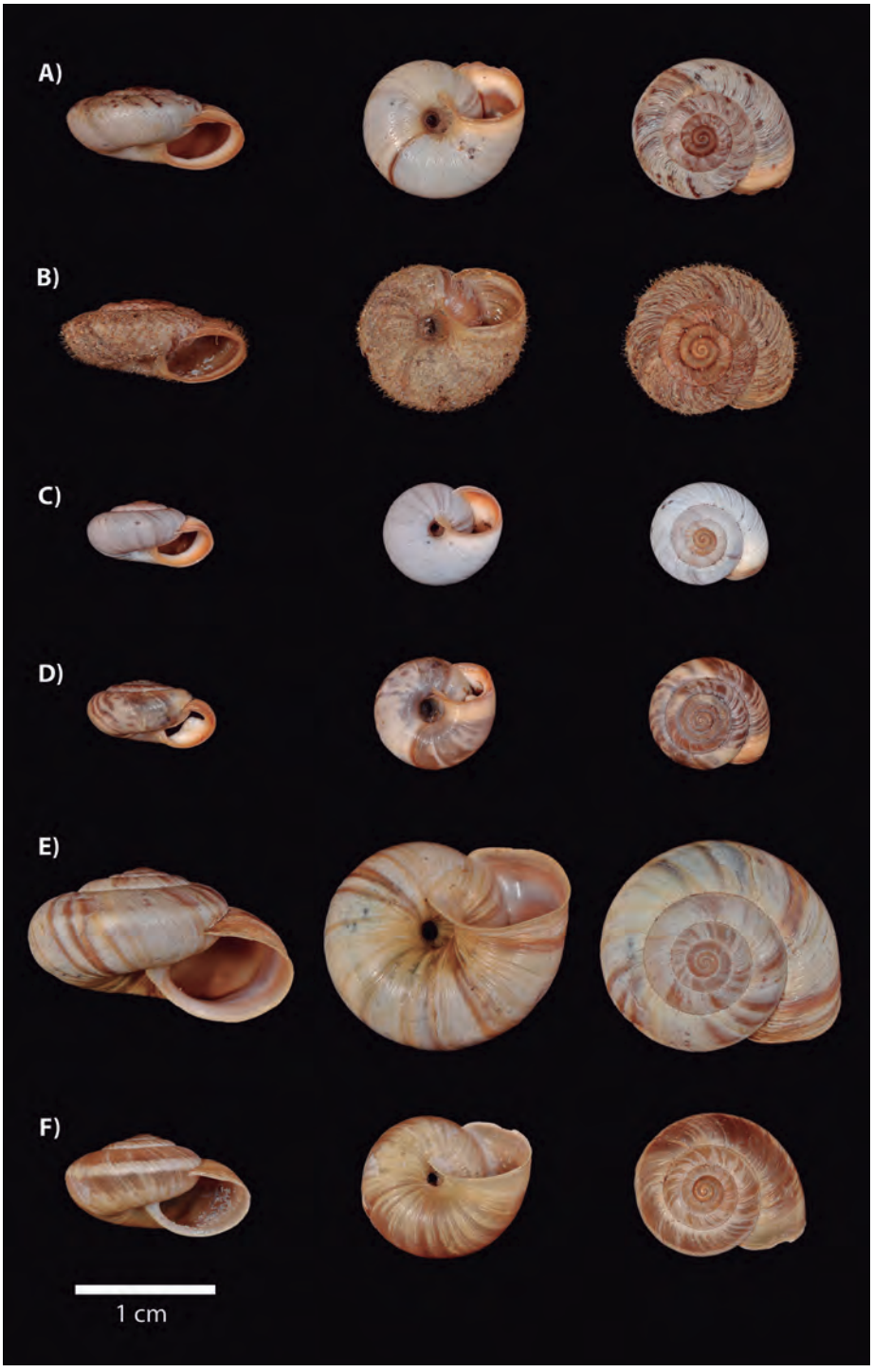


**Figure 3.** Frontal (left), inferior (middle) and superior (right) views of *Pyrenaearia* shells. A: *P. cantabrica* (Beriain, Nafarroa); B: *P. cantabrica* (Arenas de Cabrales – Poncebos: tuneles, Asturias); C: *P. oberthueri* (Vega Urriellu, Asturias); D: *P. daanidentata* (Canal del Perro – Collado del Burro, León); E: *P. velascoi* (Aizkorri, Gipuzkoa) and F: *P. carascalensis* (El Portalet, Pyrénées-Atlantiques).

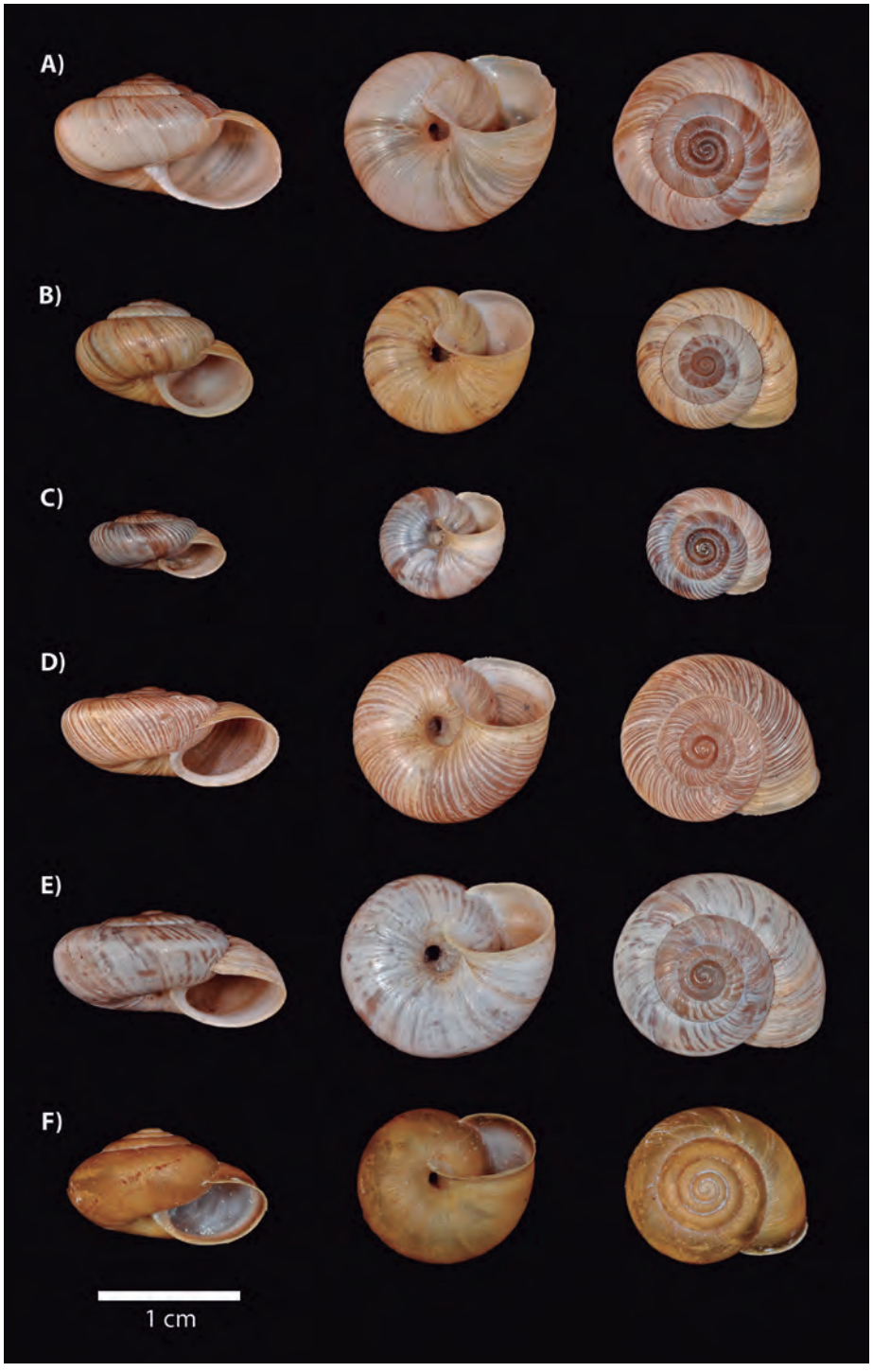


**Figure 4.** Frontal (left), inferior (middle) and superior (right) views of *Pyrenaearia* shells. A: *P. cotiellae* (Collado de Sahún, Huesca); B: *P. carascalopsis* (Port de Salau, Ariège); C: *P. parva* (Sierra de Cadí, Barcelona); D: *P. organiaca* (Congost del Pont de la Torre, Lleida); E: *P. molae* (Mola de Colldejou, Tarragona) and F: *P. navasi* (Fuente de San Gaudioso en Moncayo, Zaragoza).

We have taken these 11 species considered by Elejalde et. al. (2009) as starting point; however, the species delimitation approach used by these authors was limited to identifying monophyletic groups and comparing them with the nominal species recognized so far and, since their phylogeny was not fully resolved, the validity of some of the species described above remains unclear. Indeed, the phylogenetic relationships between *P. carascalensis*, *P. cotiellae*, *P. molae* and *P. velascoi* were not resolved and, although the last three species formed monophyletic groups, *P. carascalensis* turned out paraphyletic within the clade that joined the four species. In addition, *P. cantabrica*, *P. daanidentata* and *P. oberthueri* formed also a polytomy.

Nevertheless, its taxonomy is not the only unclear aspect of the systematics of *Pyrenaearia*: the systematics of its subfamily, Leptaxinae, remains also unresolved. Currently, without neat diagnostic morphological differences, the status of Leptaxinae is based solely on molecular information and includes three disjunctly distributed tribes: Leptaxini, Cryptosaccini (which includes *Pyrenaearia*) and Metafruticicolini (Neiber et al., 2017). However, the phylogenetic relationships between these tribes are not resolved and, moreover, the clustering of some of the genera to the tribes is not statistically supported. Therefore, the phylogenetic relationships between *Pyrenaearia* and its closest genera are unresolved and without that information the inference of the evolutionary history of the genus is not possible (Weir and Schluter, 2008).

Furthermore, the basic biological traits, ecology and population demographics of *Pyrenaearia* are completely unknown and population monitoring has never been carried out in the genus. This information is essential to understand how the genus interacts with the environment and other organisms and to assess whether it is in a decline process and determine its causes (Duangchantrasiri et al., 2016; Esteban et al., 2016; Guimarães et al., 2014; McGill et al., 2006). Moreover, a proper understanding of these traits is necessary to forecast the future responses of the species.

This huge lack of information on the genus, therefore, calls into question its adequate conservation. Indeed, without even knowing which species constitute the genus, it is difficult to establish if any of them is involved in a process of decline. In fact, in every conservation and management plan, it is essential to determine which is the biological unit to which the efforts are directed. On the other hand, launching appropriate conservation and management plans is not possible without any information about species biology, ecology and demography. In addition, monitoring *Pyrenaearia* populations could help understanding the processes that are taking place in the rocky areas of mountainous regions that are priority EU habitats. Hence, their monitoring may enable launching appropriate conservation and management measures, and allocating resources adequately, being maybe crucial to preserve mountain biodiversity. But the lack of knowledge on the genus makes also impossible using *Pyrenaearia* as a sentinel taxon. Here, unlike previous

works about *Pyrenaearia*, we propose an integrative approach to gather information about all the facets of the genus in order to ensure its conservation and to enable its use as a sentinel taxon. This way, in this Ph.D. Thesis, we have combined molecular phylogenetics, geometric morphometrics and capture-recapture methodologies.

Currently, molecular data have become an integral part of biological research. Molecular phylogenetics uses the information contained on molecular data to reconstruct phylogenetic relationships, i.e., to infer phylogenies (San Mauro and Agorreta, 2010). According to modern evolutionary theory, all organisms descent form a common ancestor which means that any species, extant or extinct, is related; this pattern of historical relationships is called phylogeny (Page and Holmes, 1998). In the late 20$^{th}$ century, the advent of powerful computers, PCR technique and Sanger sequencing lead to an exponential growth of molecular phylogenies. Now, with the development of high-throughput sequencing that has increased enormously the quantity and rate of molecular data collection cheaply, the field of phylogenetics has entered a new era (Mardis, 2008; Metzker, 2010). Concomitantly to these technological advances, molecular phylogenetics has grown, as have its applications (Yang and Rannala, 2012). Besides to reconstruct the relationships among species on the tree of life and to understand their evolution, molecular phylogenetics can currently be used to delimit and characterize species (Jones, 2017; Yang and Rannala, 2010) or to investigate the demographic changes and migration patterns of species (Hohenlohe et al., 2018), among others. Furthermore, it has become an indispensable tool for genome comparisons and, for example, it can be used to classify metagenomic sequences (Alloui et al., 2015) or to reconstruct ancestral genomes (Vialle et al., 2018) among some other applications.

The advent of geometric morphometrics and its posterior development has provoked a radical change in the study of biological forms (Adams et al., 2004, 2013). Geometric morphometrics merges shape data from landmark coordinates located on points, curves and surfaces over biological structures with a statistical theory for shape analysis. This approach allows for the use of multivariate analyses to test biological hypothesis concerning morphology while preserving the original geometry of the landmark configuration throughout the analyses and, thereby, permits to represent statistical results as actual shapes (Mitteroecker and Gunz, 2009). This technique has permitted measuring and quantifying shape variations that were not detectable with classic morphometrics, showing better performance (Evin et al., 2008). But in addition, it has allowed integrating morphological data with data from other sources, such as molecular ones, comprehensively, which has extended significantly the questions about shape variation and evolution that can be addressed (e.g. Klingenberg, 2014, 2016; Savriama et al., 2012).

Finally, gathering information about basic biological traits (e.g. maturity age or lifespan) and estimating demographic parameters (e.g. survival, reproductive success or dispersal) involve the analysis of individual monitoring data which usually depends on direct observation of populations (Gimenez and Choquet, 2010). In the wild, these data are obtained from capture-recapture technique. In this method individuals are directly captured or indirectly encountered (e.g. by camera traps or non-invasive genetics sampling), individually marked and released to their environment so that they can be observed over time (Royle et al., 2018). The statistical analysis of these data has been an active field of research for 50 years and today it remains a trending topic (Lebreton et al., 1999; Pradel, 2005; Royle et al., 2018). Since these methods account for detection uncertainty and, more recently, for spacial heterogeneity, they have become very important in the study and understanding of wild population demographics (Karanth et al. 2006, Pradel et al. 2010), in enhancing ecological theory (Cooch et al. 2002) and in informing wildlife conservation and management practices (Nichols and MacKenzie 2004; Gimenez and Choquet, 2010). Therefore, they have become standard sampling and analytical framework for the study of population processes and for gathering information about basic biological traits (Williams et al. 2002).

# References


Adams, D.C., Rohlf, F.J., Slice, D.E., 2013. A field comes of age: Geometric morphometrics in the 21st century. Hystrix, Ital. J. Mammal. 24, 7–14. https://doi.org/10.4404/hystrix-24.1-6283

Adams, D.C., Rohlf, F.J., Slice, D.E., 2004. Geometric morphometrics: Ten years of progress following the 'revolution.' Ital. J. Zool. 71, 5–16. https://doi.org/10.1080/11250000409356545

Alloui, T., Bousseboughi, I., Chaoui, A., Nouar, A.Z., Chettah, M.C., 2015. Usearch: A Meta Search Engine based on a New Result Merging Strategy, in: 7th International Joint Conference on Knowledge Discovery, Knowledge Engineering and Knowledge Management (IC3K). pp. 531–536.

Altimira, C., 1963. Notas malacológicas. Misc. Zool. 1, 15–25.

Aparicio, M.T., 1983. Estudio morfológico y citotaxonómico de algunos Helicidos de la fauna española, en especial de la región central. Madrid, PhD thesis, Universidad Complutense de Madrid, Spain.

Balvanera, P., Pfisterer, A.B., Buchmann, N., He, J.S., Nakashizuka, T., Raffaelli, D., Schmid, B., 2006. Quantifying the evidence for biodiversity effects on ecosystem functioning and services. Ecol. Lett. 9, 1146–1156. https://doi.org/10.1111/j.1461-0248.2006.00963.x

Becker, A., Bugmann, H., 2001. Global change and mountain regions. The mountain research initiative, IGBP report 49. IGBP Secretariat, Stockholm, Sweden.

Boulton, A.J., Fenwick, G.D., Hancock, P.J., Harvey, M.S., 2008. Biodiversity, functional roles and ecosystem services of groundwater invertebrates. Invertebr. Syst. 22, 103–116. https://doi.org/10.1071/IS07024

Brooks, T.M., Mittermeier, R.A., da Fonseca, G.A.B., Gerlach, J., Hoffmann, M., Lamoreux, J.F., Mittermeier, C.G., Pilgrim, J.D., Rodrigues, A.S.L., 2006. Global Biodiversity Conservation Priorities. Science 313, 58–62. https://doi.org/10.1126/science.1127609

Brummitt, N., Lughadha, E.N., 2003. Biodiversity: Where's hot and where's not. Conserv. Biol. 17, 1442–1448.

Cardinale, B.J., Duffy, J.E., Gonzalez, A., Hooper, D.U., Perrings, C., Venail, P., Narwani, A., MacE, G.M., Tilman, D., Wardle, D.A., Kinzig, A.P., Daily, G.C., Loreau, M., Grace, J.B., Larigauderie, A., Srivastava, D.S., Naeem, S., 2012. Biodiversity loss and its impact on humanity. Nature 486, 59–67. https://doi.org/10.1038/nature11148

Cardoso, P., Erwin, T.L., Borges, P.A. V, New, T.R., 2011. The seven impediments in invertebrate conservation and how to overcome them. Biol. Conserv. 144, 2647–2655. https://doi.org/10.1016/j.biocon.2011.07.024

Catalan, J., Ninot, J.M., Aniz, M.M., 2017a. The high mountain conservation in a changing world, in: Catalan, J., Ninot, J.M., Aniz, M.M. (Eds.), High Mountain Conservation in a Changing World. Springer Nature, Cham, Switzerland, pp. 3–36.

Catalan, J., Ninot, J.M., Aniz, M.M., 2017b. High Mountain Conservation in a Changing World. Springer Nature, Cham, Switzerland.

CDB (Secretariat of the Convention on Biological Diversity), 1992. Convention on biological diversity, Montreal.

Ceballos, G., Ehrlich, P.R., 2018. The misunderstood sixth mass extinction. Science 360, 1080–1081.

Ceballos, G., Ehrlich, P.R., Barnosky, A.D., García, A., Pringle, R.M., Palmer, T.M., 2015. Accelerated modern human–induced species losses: Entering the sixth mass extinction. Sci. Adv. 1, e1400253.

Ceballos, G., Ehrlich, P.R., Dirzo, R., 2017. Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. Proc. Natl. Acad. Sci. U.S.A. 114, E6089–E6096. https://doi.org/10.1073/pnas.1704949114

Chueca, L.J., Gómez-Moliner, B.J., Madeira, M.J., Pfenninger, M., 2018. Molecular phylogeny of *Candidula* (Geomitridae) land snails inferred from mitochondrial and nuclear markers reveals the polyphyly of the genus. Mol. Phylogenet. Evol. 118, 357–368. https://doi.org/10.1016/j.ympev.2017.10.022

Cooch, E.G., Cam, E., Link, W., 2002. Occam's shadow: Levels of analysis in evolutionary ecology--where to next? J. Appl. Stat. 29, 19–48. https://doi.org/10.1080/02664760120108421

Costanza, R., D'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R. V, Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., 1997. The value of the world's ecosystem services and natural capital. Nature 387, 253–260.

Costanza, R., de Groot, R., Sutton, P., van der Ploeg, S., Anderson, S.J., Kubiszewski, I., Farber, S., Turner, R.K., 2014. Changes in the global value of ecosystem services. Glob. Environ. Chang. 26, 152–158. https://doi.org/10.1016/j.gloenvcha.2014.04.002

Daily, G.C., 1997. Nature's services: societal dependence on natural ecosystems. Island Press, Washington DC, USA.

Davison, A., Wade, C.M., Mordan, P.B., Chiba, S., 2005. Sex and darts in slugs and snails (Mollusca: Gastropoda: Stylommatophora). J. Zool. 267, 329–338. https://doi.org/10.1017/S0952836905007648

Dirzo, R., Young, H.S., Galetti, M., Ceballos, G., Isaac, N.J.B., Collen, B., 2014. Defaunation in the Anthropocene. Science 345, 401–406.

Duangchantrasiri, S., Umponjan, M., Simcharoen, S., Pattanavibool, A., Chaiwattana, S., Maneerat, S., Kumar, N.S., Jathanna, D., Srivathsa, A., Karanth, K.U., 2016. Dynamics of a low-density tiger population in Southeast Asia in the context of improved law enforcement. Conserv. Biol. 30, 639–648. https://doi.org/10.1111/cobi.12655

Dunn, R.R., 2005. Modern insect extinctions, the neglected majority. Conserv. Biol. 19, 1030–1036. https://doi.org/10.1111/j.1523-1739.2005.00078.x

Dunn, R.R., Harris, N.C., Colwell, R.K., Koh, L.P., Sodhi, N.S., 2009. The sixth mass coextinction: Are most endangered species parasites and mutualists? Proc. R. Soc. B Biol. Sci. 276, 3037–3045. https://doi.org/10.1098/rspb.2009.0413

Ehrlich, P., Ehrlich, A., 1981. Extinction: the causes and consequences of the disappearance of species. Ballantine Books, New York, USA.

Elejalde, M.A., Madeira, M.J., Prieto, C.E., Backeljau, T., Gómez-Moliner, B.J., 2009. Molecular Phylogeny, Taxonomy, and Evolution of the Land Snail Genus *Pyrenaearia* (Gastropoda, Helicoidea). Am. Malacol. Bull. 27, 69–81. https://doi.org/10.4003/006.027.0206

Esteban, R., Verborgh, P., Gauffier, P., Giménez, J., Guinet, C., de Stephanis, R., 2016. Dynamics of killer whale, bluefin tuna and human fisheries in the Strait of Gibraltar. Biol. Conserv. 194, 31–38. https://doi.org/10.1016/j.biocon.2015.11.031

Evin, A., Baylac, M., Ruedi, M., Mucedda, M., Pons, J.-M., 2008. Taxonomy, skull diversity and evolution in a species complex of *Myotis* (Chiroptera: Vespertilionidae): a geometric morphometric appraisal. Biol. J. Linn. Soc. 95, 529–538.

Faci, G., 1991. Contribución al conocimiento de diversos moluscos terrestres y su distribución en la Comunidad Autónoma Aragonesa. Zaragoza, PhD thesis, University of Zaragoza, Spain.

Fagot, P., 1906. Mollusca nova. Provinciae Aragoniae. Bol. Soc. Arag. Cienc. Nat. 5, 171–173.

Fernández-Chacón, A., Bertolero, A., Amengual, A., Tavecchia, G., Homar, V., Oro, D., 2011. Spatial heterogeneity in the effects of climate change on the population dynamics of a Mediterranean tortoise. Glob. Chang. Biol. 17, 3075–3088. https://doi.org/10.1111/j.1365-2486.2011.02469.x

Fiorentino, V., Salomone, N., Manganelli, G., Giusti, F., 2008. Phylogeography and morphological variability in land snails: the Sicilian *Marmorana* (Pulmonata, Helicidae). Biol. J. Linn. Soc. 94, 809–823. https://doi.org/10.1111/j.1095-8312.2008.01023.x

Gaston, K.J., 2010. Biodiversity, in: Sodhi, N.S., Ehrlich, P.R. (Eds.), Conservation Biology for All. Oxford University Press, Oxford, UK, pp. 27–44.

Gimenez, O., Choquet, R., 2010. Individual heterogeneity in studies on marked animals using numerical integration: capture-recapture mixed models. Ecology 91, 951–957.

Gómez-Moliner, B.J., Elejalde, M.A., Madeira, M.J., Prieto, C.E., Puente, A.I., Altonaga, K., 2011a. *Pyrenaearia daanidentata* Raven, 1988, in: Verdú, J.R., Numa, C., Galante, E. (Eds.), Atlas y Libro Rojo de Los Invertebrados Amenazados de España (Especies Vulnerables). Dirección General de Medio Natural y Política Forestal, Ministerio de Medio Ambiente, Medio rural y Marino, Madrid, Spain, pp. 908–910.

Gómez-Moliner, B.J., Elejalde, M.A., Madeira, M.J., Prieto, C.E., Puente, A.I., Altonaga, K., 2011b. *Pyrenaearia carascalopsis* Fagot, 1884, in: Verdú, J.R., Numa, C., Galante, E. (Eds.), Atlas y Libro Rojo de Los Invertebrados Amenazados de España (Especies Vulnerables). Dirección General de Medio Natural y Política Forestal, Ministerio de Medio Ambiente, Medio rural y Marino, Madrid, Spain, pp. 903–907.

Gómez-Moliner, B.J., Elejalde, M.A., Madeira, M.J., Prieto, C.E., Puente, A.I., Altonaga, K., 2011c. *Pyrenaearia organiaca* Fagot, 1905, in: Verdú, J.R., Numa, C., Galante, E. (Eds.), Atlas y Libro Rojo de Los Invertebrados Amenazados de España (Especies Vulnerables). Dirección General de Medio Natural y Política Forestal, Ministerio de Medio Ambiente, Medio rural y Marino, Madrid, Spain, pp. 919–923.

Gómez-Moliner, B.J., Elejalde, M.A., Madeira, M.J., Prieto, C.E., Puente, A.I., Altonaga, K., 2011d. *Pyrenaearia molae* Haas, 1924, in: Verdú, J.R., Numa, C., Galante, E. (Eds.), Atlas y Libro Rojo de Los Invertebrados Amenazados de España (Especies Vulnerables). Dirección General de Medio Natural y Política Forestal, Ministerio de Medio Ambiente, Medio rural y Marino, Madrid, Spain, pp. 911–914.

Gómez-Moliner, B.J., Elejalde, M.A., Madeira, M.J., Prieto, C.E., Puente, A.I., Altonaga, K., 2011e. *Pyrenaearia navasi* Fagot, 1907, in: Verdú, J.R., Numa, C., Galante, E. (Eds.), Atlas y Libro Rojo de Los Invertebrados Amenazados de España (Especies Vulnerables). Dirección General de Medio Natural y Política Forestal, Ministerio de Medio Ambiente, Medio rural y Marino, Madrid, Spain, pp. 915–918.

Gómez-Moliner, B.J., Madeira, M.J., Elejalde, M.A., Cabria, M.T., Ruiz-González, A., Chueca, L.J., Vergara, M., Razkin, O., 2009. Los estudios del ADN como apoyo a la biología de la conservación de fauna. Rev. Int. los Estud. Vascos, RIEV 54, 351–386.

González-Oreja, J.A., 2008. The Encyclopedia of Life vs. the Brochure of Life: Exploring the relationships between the extinction of species and the inventory of life on Earth. Zootaxa 1965, 61–68.

Guimarães, M., Munguía-Steyer, R., Doherty, P.F., Martins, M., Sawaya, R.J., 2014. Population dynamics of the critically endangered golden lancehead pitviper, *Bothrops insularis*: Stability or decline? PLoS One 9, e95203. https://doi.org/10.1371/journal.pone.0095203

Haila, Y., Kouki, J., 1994. The phenomenon of biodiversity in conservation biology. Ann. Zool. Fennici 31, 5–18.

Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., de Kroon, H., 2017. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PLoS One 12, e0185809.

Hirano, T., Kameda, Y., Kimura, K., Chiba, S., 2014. Substantial incongruence among the morphology, taxonomy, and molecular phylogeny of the land snails *Aegista*, *Landouria*, *Trishoplita*, and *Pseudobuliminus* (Pulmonata: Bradybaenidae) occurring in East Asia. Mol. Phylogenet. Evol. 70, 171–181. https://doi.org/10.1016/j.ympev.2013.09.020

Hohenlohe, P.A., Hand, B.K., Andrews, K.R., Luikart, G., 2018. Population Genomics Provides Key Insights in Ecology and Evolution, in: Rajora, O. (Ed.), Population Genomics. Springer, Cham, Switzerland, pp. 483–510.

Hölldobler, B., Wilson, E.O., 1990. The Ants. Harvard University Press, Cambridge, Massachusetts, USA.

Hooper, D.U., Adair, E.C., Cardinale, B.J., Byrnes, J.E.K., Hungate, B.A., Matulich, K.L., Gonzalez, A., Duffy, J.E., Gamfeldt, L., O'Connor, M.I., 2012. A global synthesis reveals biodiversity loss as a major driver of ecosystem change. Nature 486, 105–108. https://doi.org/10.1038/nature11118

Jones, G., 2017. Algorithmic improvements to species delimitation and phylogeny estimation under the multispecies coalescent. J. Math. Biol. 74, 447–467. https://doi.org/10.1007/s00285-016-1034-0

Karanth, K.U., Nichols, J.D., Kumar, N.S., Hines, J.E., 2006. Assessing tiger population dynamics using photographic capture-recapture sampling. Ecology 87, 2925–2937.

Kerr, J.T., 1997. Species richness, endemism, and the choice of areas for conservation. Conserv. Biol. 11, 1094–1100. https://doi.org/10.1046/j.1523-1739.1997.96089.x

Klingenberg, C.P., 2016. Size, shape, and form: concepts of allometry in geometric morphometrics. Dev. Genes Evol. 226, 113–137. https://doi.org/10.1007/s00427-016-0539-2

Klingenberg, C.P., 2014. Studying morphological integration and modularity at multiple levels: concepts and analysis. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 369, 2010249. https://doi.org/10.1098/rstb.2013.0249

Kremen, C., 2005. Managing ecosystem services: What do we need to know about their ecology? Ecol. Lett. 8, 468–479. https://doi.org/10.1111/j.1461-0248.2005.00751.x

Kremen, C., Colwell, R.K., Erwin, T.L., Murphy, D.D., Noss, R.F., Sanjayan, M.A., 1993. Terrestrial arthropod assemblages: their use in conservation planning. Conserv. Biol. 7, 796–808.

Kremen, C., Williams, N.M., Thorp, R.W., 2002. Crop pollination from native bees at risk from agricultural intensification. Proc. Natl. Acad. Sci. U.S.A. 99, 16812–16816. https://doi.org/10.1073/pnas.262413599

Lamoreux, J.F., Morrison, J.C., Ricketts, T.H., Olson, D.M., Dinerstein, E., McKnight, M.W., Shugart, H.H., 2006. Global tests of biodiversity concordance and the importance of endemism. Nature 440, 212–214. https://doi.org/10.1038/nature04291

Lebreton, J.D., Almeras, T., Pradel, R., 1999. Competing events, mixtures of information and multistratum recapture models. Bird Study 46, S39-46. https://doi.org/10.1080/00063659909477230

Lin, S., Wu, R., Yang, F., Wang, J., Wu, W., 2018. Spatial trade-offs and synergies among ecosystem services within a global biodiversity hotspot. Ecol. Indic. 84, 371–381. https://doi.org/10.1016/j.ecolind.2017.09.007

Lloret, F., 2017. Trade-offs in high mountain conservation, in: Catalan, J., Ninot, J.M., Aniz, M.M. (Eds.), High Mountain Conservation in a Changing World. Springer Nature, Cham, Switzerland, pp. 37–59.

Manga, M.Y., 1983. Los Helicidae (Gatropoda, Pulmonata) de la provincia de León. León, PhD thesis, Institución "Fray Bernardino de Sahagun", Spain.

Mardis, E.R., 2008. The impact of next-generation sequencing technology on genetics. Trends Genet. 24, 133–141. https://doi.org/10.1016/j.tig.2007.12.007

Martínez-Ortí, A., Bros, V., 2012. Nuevos datos sobre la distribución geográfica de *Pyrenaearia molae* (Hass, 1924) (Mollusca, Gastropoda, Hygromiidae). Noticiario SEM 58, 31–33.

McGill, B.J., Enquist, B.J., Weiher, E., Westoby, M., 2006. Rebuilding community ecology from functional traits. Trends Ecol. Evol. 21, 178–185. https://doi.org/10.1016/j.tree.2006.02.002

MEA (Millennium Ecosystem Assessment), 2005. Ecosystems and Human Well-being: Biodiversity Synthesis. World Resources Institute, Washington DC, USA.

Metzker, M.L., 2010. Sequencing technologies - the next generation. Nature 11, 31–46.

Mitteroecker, P., Gunz, P., 2009. Advances in Geometric Morphometrics. Evol. Biol. 36, 235–247. https://doi.org/10.1007/s11692-009-9055-x

Mooney, H.A., Ehrlich, P.R., 1997. Ecosystem services: a fragmentary history, in: Daily, G.C. (Ed.), Nature's Services: Societal Dependence on Natural Ecosystems. Island Press, Washington DC, USA, pp. 11–19.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853–858. https://doi.org/10.1038/35002501

Neiber, M.T., Razkin, O., Hausdorf, B., 2017. Molecular phylogeny and biogeography of the land snail family Hygromiidae (Gastropoda: Helicoidea). Mol. Phylogenet. Evol. 111, 169–184. https://doi.org/10.1111/zsc.12218

Neiber, M.T., Walther, F., Hausdorf, B., 2018. Phylogeny and reclassification of the Caucasigenini radiation from the Caucasus region (Gastropoda, Hygromiidae). Zool. Scr. 47, 54–62. doi: 10.1111/zsc.12259

Nichols, J.D., Mackenzie, D.I., 2004. Abundance estimation and Conservation Biology. Anim. Biodivers. Conserv. 27, 437–439.

Norse, E.A., McManus, R.E., 1980. Ecology and living resources: biological diversity, in: Environmental Quality 1980: The Eleventh Annual Report of the Council on Environmental Quality. U.S. Government Printing Office, Washington DC, USA, pp. 31–80.

Ortiz de Zárate, A., 1956. Observaciones anatómicas y posición sistemática de varios Helícidos españoles. IV. Género *Pyrenaearia* Hesse, 1921. Bol. R. Soc. Esp. Hist. Nat. 54, 35–61.

Ortiz de Zárate, A., 1943. Observaciones anatómicas y posición sistemática de varios Helícidos españoles. Bol. R. Soc. Esp. Hist. Nat. 41, 61–83.

Page, R.D.M., Holmes, E.C., 1998. Molecular evolution: a phylogenetic approach. Blackwell Science, Oxford, UK.

Patterson, T.M., Coelho, D.L., 2009. Ecosystem services: Foundations, opportunities, and challenges for the forest products sector. For. Ecol. Manage. 257, 1637–1646. https://doi.org/10.1016/j.foreco.2008.11.010

Pimm, S.L., Jenkins, C.N., Abell, R., Brooks, T.M., Gittleman, J.L., Joppa, L.N., Raven, P.H., Roberts, C.M., Sexton, J.O., 2014. The biodiversity of species and their rates of extinction, distribution, and protection. Science 344, 1246752. https://doi.org/10.1126/science.1246752

Pradel, R., 2005. Multievent: An Extension of Multistate Capture–Recapture Models to Uncertain States. Biometrics 61, 442–447. https://doi.org/10.1111/j.1541-0420.2005.00318.x

Pradel, R., Choquet, R., Lima, M.A., Merritt, J., Crespin, L., 2010. Estimating population growth rate from capture-recapture data in presence of capture heterogeneity. J. Agric. Biol. Environ. Stat. 15, 248–258. https://doi.org/10.1007/s13253-009-0008-8

Prieto, C.E., 1991. *Pyrenaearia navasi sylvatica* ssp.nov., una subespecie originada por una regresion glaciar cuaternaria en el macizo del Moncayo. Kobie 20, 69–75.
Prieto, C.E., 1986. Estudio sistemático y biogeográfico de los Helicidae *sensu* Zilch, 1959-60 (Gastropoda: Pulmonata: Stylommatophora) del País Vasco y regiones adyacentes. Leioa, PhD thesis, University of the Basque Country (EHU/UPV), Spain.
Puente, A.I., 1994. Estudio taxonómico y biogeográfico de la superfamilia Helicoidea Rafinesque, 1815 (Gastropoda: Pulmonata: Stylommatophora) de la Península Ibérica e Islas Baleares. Leioa, PhD thesis, University of the Basque Country (EHU/UPV), Spain.
Purvis, A., Hector, A., 2000. Getting the measure of well-being. Nature 405, 212–219. https://doi.org/10.1038/35012221
Raven, J.G., 1988. *Pyrenaearia daanidentata* spec. nov. (Helicidae), a toothed species from the Cantabrian mountains, Spain. Basteria 52, 121–123.
Razkin, O., Gómez-Moliner, B.J., Vardinoyannis, K., Martínez-Ortí, A., Madeira, M.J., 2017. Species delimitation for cryptic species complexes: Case study of *Pyramidula* (Gastropoda, Pulmonata). Zool. Scr. 46, 55–72. https://doi.org/10.1111/zsc.12192
Royle, J.A., Fuller, A.K., Sutherland, C., 2018. Unifying population and landscape ecology with spatial capture-recapture. Ecography 41, 444–456. https://doi.org/10.1111/ecog.03170
San Mauro, D., Agorreta, A., 2010. Molecular systematics: A synthesis of the common methods and the state of knowledge. Cell. Mol. Biol. Lett. 15, 311–41. https://doi.org/10.2478/s11658-010-0010-8
Savriama, Y., Gómez, J.M., Perfectti, F., Klingenberg, C.P., 2012. Geometric morphometrics of corolla shape: Dissecting components of symmetric and asymmetric variation in *Erysimum mediohispanicum* (Brassicaceae). New Phytol. 196, 945–954. https://doi.org/10.1111/j.1469-8137.2012.04312.x
Schuldt, A., Assmann, T., 2010. Invertebrate diversity and national responsibility for species conservation across Europe – A multi-taxon approach. Biol. Conserv. 143, 2747–2756. https://doi.org/10.1016/j.biocon.2010.07.022
Sekercioglu, C.H., 2010. Ecosystem functions and services, in: Sodhi, N.S., Ehrlich, P.R. (Eds.), Conservation Biology for All. Oxford University Press, Oxford, UK, pp. 45–72.
Stankowski, S., 2013. Ecological speciation in an island snail: evidence for the parallel evolution of a novel ecotype and maintenance by ecologically dependent postzygotic isolation. Mol. Ecol. 22, 2726–2741. https://doi.org/10.1111/mec.12287
Stankowski, S., 2011. Extreme, continuous variation in an island snail: local diversification and association of shell form with the current environment. Biol. J. Linn. Soc. 104, 756–769. https://doi.org/10.1111/j.1095-8312.2011.01748.x
Vialle, R.A., Tamuri, A.U., Goldman, N., 2018. Alignment modulates ancestral sequence reconstruction accuracy. Mol. Biol. Evol. 35, 1783–1797. https://doi.org/10.1093/molbev/msy055
Vilella, M., 1963. Notas malacológicas I. Contribución al conocimiento de *Pyrenaearia molae* Hass. Misc. Zool. 1, 27–32.
Wade, C.M., Hudelot, C., Davison, A., Naggs, F., Mordan, P.B., 2007. Molecular phylogeny of the helicoid land snails (Pulmonata: Stylommatophora: Helicoidea), with special emphasis on the Camaenidae. J. Molluscan Stud. 73, 411–415. https://doi.org/10.1093/mollus/eym030
Weir, J., Schluter, D., 2008. Calibrating the avian molecular clock. Mol. Ecol. 17, 2321–2328.
Wheeler, Q.D., Raven, P.H., Wilson, E.O., 2004. Taxonomy: Impediment or expedient? Science 303, 285. https://doi.org/10.1126/science.303.5656.285
Williams, B.K., Nichols, J.D., Conroy, M.J., 2002. Analysis and management of animal populations. Academic Press, San Diego, USA.
Wilson, E.O., 1987. The little things that run the world (The importance and conservation of invertebrates). Conserv. Biol. 1, 344–346.
Worm, B., Barbier, E.B., Beaumont, N., Duffy, J.E., Folke, C., Halpern, B.S., Jackson, J.B.C., Lotze, H.K., Micheli, F., Palumbi, S.R., Sala, E., Selkoe, K.A., Stachowics, J.J., Watson, R., 2006. Impacts of biodiversity loss on ocean ecosystem services. Science 314, 787–791.
Yang, Z., Rannala, B., 2012. Molecular phylogenetics: principles and practice. Nature 13, 303–314. https://doi.org/10.1038/nrg3186
Yang, Z., Rannala, B., 2010. Bayesian species delimitation using multilocus sequence data. Proc. Natl. Acad. Sci. U.S.A. 107, 9264–9269. https://doi.org/10.1073/pnas.0913022107
Yen, A.L., Butcher R., 1997. An overview of the conservation of non-marine invertebrates in Australia. Endangered Species Program, Environment Australia.

# 1. KAPITULUA

## Sarrera

# 1. Biodibertsitatea: bere garrantzia eta krisia

"Biodibertsitate" hitzaren jatorria, "*biological diversity*" ("dibertsitate biologiko") laburtzetik sortua, nahiko berria da. 1980ean T.E. Lovejoy-k "dibertsitate biologiko" terminoa erabili zuen lehenengo aldiz komunitate bat osatzen zuen espezie taldea izendatzeko, definizio argirik eman gabe. Urte horretan bertan, Norse eta McManus-ek (1980), itsas biologian adituak, definizio formalagoa proposatu zuten dibertsitate biologikoarentzat eta bertan dagoeneko dibertsitate genetiko eta dibertsitate ekologiko kontzeptuak kontsideratu zituzten. Baina "biodibertsitate" hitzaren formalizazioa, 1988ean gertatu zela kontsideratzen da, E.O. Wilson-ek 1986ean AEBetan egin zen "National Forum on BioDiversity" foroaren akta "Biodiversity" izenpean argitaratu zuenean; izan ere, argitalpen horrek hitzaren erabilpena izugarri zabaldu zuen, bai literatura zientifikoan, bai gizartearen esparru ezberdinetan ere (Haila eta Kouki, 1994).

Orduz geroztik, biodibertsitate hitzaren esanahia aldatuz joan zen. Hasieran espezie aberastasuna bakarrik adierazten zuen kontzeptu kuantitatiboa izatetik, pixkanaka konplexutasuna lortzen joan zen, azkenean bizitzaren aniztasuna bere manifestazio eta eskala guztietan bere gain hartzen dituen kontzeptu multieskalar bilakatuz, denbora eta espazio eskala ezberdinetan kontsideratu daitezkeen gene, espezie eta ekosistemak zein prozesu ekologikoak bilduz bere barne. Egun, Dibertsitate Biologikoari Buruzko Hitzarmenak (CDB) 1992an biodibertsitatearentzat proposaturiko definizioa hartzen da erreferentzia gisa, izan ere, kontserbazioaren ikuspuntutik, biodibertsitatearen kudeaketa estrategiak planifikatzerako orduan, garrantzitsua da esparru kontzeptual argi eta zehatz bat izatea. Honen arabera, "dibertsitate biologikoa edo biodibertsitatea iturri orotako organismo bizidunen aniztasuna da, izan lurrekoak, itsasokoak edo beste ur ekosistemetakoak *inter alia*, eta organismo horiek parte hartzen duten konplexu ekologikoak ere barne hartuz; honek espezie barruko aniztasuna, espezie artekoa eta ekosistemena biltzen dituelarik" (CDB, 1992; 2. artikulua).

Biodibertsitatea multidimentsionala da beraz, edozein biota bere dibertsitate genetikoak, taxonomikoak eta ekologikoak ezaugarritzen baitute (MEA, 2005). Hau da, aniztasun biologikoaren barruan hiru maila bereizten dira: dibertsitate genetikoa, organismoen dibertsitatea eta dibertsitate ekologikoa. Maila hauetako bakoitzaren barruko elementuak estuki erlazionatuta daude elkar gurutzatzen diren hierarkietan, eta mailak ere haien artean estuki erlazionatzen dira, elementu batzuk partekatzeraino (Gaston, 2010). Biodibertsitatearen dimentsio hauek, gainera, espazioan eta denboran aldatu egiten dira. Horrela, ur tanta baten biodibertsitateaz edo Lur osoarenaz hitz egin daiteke eta, halaber, orainaldiko biodibertsitateaz hitz egin daiteke, iraganeko edo etorkizuneko une zehatz batekoaz edo Lurraren historia osoarenaz (Gaston, 2010).

Argi dago biodibertsitatea oso konplexua dela, guztiz zedarritzea eta ulertzea oso zaila delarik, ezinezkoa agian. Baina bere konplexutasuna bere garrantziaren parekoa da. Izan ere, biodibertsitatea gizakiaren ongizaterako beharrezkoak diren zerbitzu ekosistemikoen oinarria da, ekosistemen funtzioak eusten eta mantentzen dituelako (MEA, 2005).

Zerbitzu ekosistemikoak gizakia sostengatzen duten ekosistema funtzio sorta dira (Daily, 1997; Kremen, 2005), eskala global batetik eskala mikroskopiko bateraino aurkitu daitezkeenak (Sekercioglu, 2010). Nahiz eta aspaldidanik jakin izan den ekosistemek gizarteak mantentzen dituztela, zerbitzu ekosistemikoen aitortze esplizitua nahiko berria da (Ehrlich eta Ehrlich 1981; Mooney eta Ehrlich 1997). Eta, hala ere, orduz geroztik haiei buruzko lan eskerga argitaratu izan da (adibidez, Costanza et al., 2014, 1997; Lin et al., 2018; Patterson eta Coelho, 2009; Worm et al., 2006), Milurteko Ekosistemen Ebaluazioan egindako sintesi globala (2005) erreferente delarik. Bertan, besteak beste, zerbitzu ekosistemikoak lau multzotan bereizten dira: hornitze-zerbitzuak, erregulazio-zerbitzuak, kultura-zerbitzuak eta euskarri-zerbitzuak. Izan ere, ekosistemek airea eta ura garbitzen dituzte, oxigenoa sortu eta klima egonkortu; organismo batzuek detritua deskonposatzen eta desintoxikatzen dute gure hondakinei irtenbidea emanez; milaka animaliak landareak polinizatzen dituzte, izurrietatik babestu eta haien haziak dispertsatu; beste espezie batzuek gure janaria hazteko ezinbestekoak diren zoruak sortzen eta mantentzen dituzte eta baita nutrienteak berritu ere; eta, jakina, gizakiok era zuzenean kontsumitzen eta erabiltzen ditugu milaka landare, animalia eta mikroorganismo (Sekercioglu, 2010).

Baina, gaur egun, Lurra biodibertsitate galera sakon bat jasaten ari da giza jardueren eraginez (Cardinale et al., 2012; Cardoso et al., 2011; Purvis eta Hector, 2000). Urtetan zehar ohartarazi izan da espezieen iraungitze tasaren joera erregistro fosilean aurkitutako iraungitze tasa basala gainditzetik gero eta gertuago zegoela (Hooper et al., 2012). Gaur, zientzialariak ados daude Lurra dagoeneko seigarren iraungipen masiboa jasaten ari dela, momentu honetan espezieen iraungipen tasa historia geologikoan zeharreko tasa normala baino 100 aldiz handiagoa delarik (Ceballos et al., 2017, 2015; Ceballos eta Ehrlich, 2018; Pimm et al., 2014). Hala, kalkulu zuhurrenek urtero 3000 espezie desagertzen direla diote, hau da, egunean 8 espezie (González-Oreja, 2008; Wheeler et al., 2004). Biodibertsitatearen galera ingurumen arazo garrantzitsuenen artean dago, ekosistemen funtzioak ezegonkortzen dituelako eta zerbitzu ekosistemikoen galera dakarrelako, gizakien ongizatea kolokan jarriz (Balvanera et al., 2006). Kontuan hartu behar da ia 8000 milioi pertsonaz osaturiko populazio industrializatua dagoela, biodibertsitateak ahalbidetzen dituen zerbitzu ekosistemikoen erabat menpekoa dena (Dirzo et al., 2014; Hallmann et al., 2017). Adibidez, polinizatzaile baten galerak uzta askoren ekoizpena jaitsi dezake (Kremen

et al., 2002), eta lurpeko uren fauna galerak uraren garbiketa prozesuak eten ditzake kutsadura arazoak sortuz (Boulton et al., 2008). Gizakiok, biodibertsitatearen galeraren eragileak izanik, betebehar moral bat daukagu prozesu hau gelditzeko; eta, bestalde, berekoiki zerbitzu ekosistemikoak bermatu nahi baditugu, biodibertsitatea mantentzea derrigorrezkoa da, noski.

Krisi hau gainditzeko kontserbazio eta kudeaketa plan eraginkorrak behar dira, eta lehentasunak zehaztu. Baina horretarako ezinbestekoa da biodibertsitatearen ezagutzan aurrera egitea, bere maila guztietan, espezieen zedarritzetik eta karakterizazio biologiko zein genetikotik ekosistema funtzioetara. Bereziki garrantzitsua izango da, hala ere, espezietan oso aberatsak diren eta/edo ezaugarri ebolutibo zein ekologiko esklusiboak dituzten inguruak identifikatzea (Myers et al., 2000) eta baita jarraipen sistema egokiak finkatzea ere (Catalan et al., 2017b).

## 2. Ornogabeen garrantziaz eta haiekiko ezjakintasunaz

Biodibertsitatearen galeraren inguruan bildutako datuak oso kezkagarriak diren arren, litekeena da kalkuluak oso motz gelditzea, kontuan hartzen baldin badugu talde taxonomiko gehienen inguruan daukagun informazio eskasa, bereziki ornogabeen inguruan.

Organismo multizelularren artean, bai aberastasun aldetik, bai kopuru aldetik eta, askotan, baita biomasari dagokionez ere, ornogabeak nagusi dira. Deskribatutako espezieen %80 inguru ornogabeak dira, kakalardoak bakarrik ornodunak baino 10 aldiz espezie gehiagoz osatuta daudelarik (Cardoso et al., 2011). Amazonako Oihanaren hektarea batean, adibidez, mila milioi ornogabe baino gehiago egon daitezke (Wilson, 1987) eta Serengetiko lautadetan inurrien biomasa ugaztunena baino handiagoa da (Hölldobler eta Wilson, 1990). Haien inguruko informazioa eskasa den arren, badakigu ornogaberik gabe ekosistemek ezingo lituzketela haien funtzioak mantendu. Izan ere, ornogabeak saprofagoak, fitofagoak, endo-, ekto- edo hiper-bizkarroiak, sinbionteak edo kate luze baten azken harrapakariak izan daitezke. Badaude kosmopolitak eta badaude oso eremu txikitara mugatuta daudenak. Ekosistema lurtarretan eta ur-ekosistema gezetan zein gazitan bizi dira. Are, airean ere aurkitu daitezke. Horrelako espezie dibertsitate handiarekin eta funtzio anitzekin, ornogabeak ezinbestekoak dira ekosistemen funtzioak eta, ondorioz, haien zerbitzuak mantentzeko. Ez da harritzekoa E.O. Wilson-ek (1987) ornogabeak "mundua mugitzen duten gauza txikiak" ("*the little things that run the world*") bezala definitu izana.

Murgilduta gauden biodibertsitate krisian, ornogabeek handiagoak eta ezagunagoak diren beste organismoek pairatzen dituzten iraungitze prozesu berberak jasateaz

gain, beste batzuei ere aurre egin behar diete, hala nola ko-iraungitzeari eta habitat espezialista zorrotzen iraungitzeari (Dunn, 2005; Dunn et al., 2009). Hala ere, nahiz eta zaurgarriagoak izan eta gizakiontzat funtsezkoak diren zerbitzu ekosistemiko zenbatezinen oinarri izan, gehienetan ornogabeak ez dira aintzat hartzen biodibertsitate kontserbazio politiketan eta normalean animalia enblematiko eta karismatikoei ematen zaie lehentasuna, ornodunei alegia (Cardoso et al., 2011; Schuldt eta Assmann, 2010). Errealitate hau argi islatzen da Natura eta Baliabide Naturalak Kontserbatzeko Nazioarteko Batasunaren (NKNB) Zerrenda Gorrian (2018), non ia ornodun guztiak ebaluatuak izan diren bitartean, deskribatuta dauden artropodo espezieen %1 baino ez baitaude ebaluatuta, eta moluskuen kasuan %10. Egun, edozein eskalatan, alde nabarmena dago babestutako ornodun kopuruaren eta ornogabe kopuruaren artean, ornogabeen kaltetan.

Alde honen arrazoia, ornogabeen kontserbazioa oztopatzen duena, haien inguruan dagoen ezjakintasuna da eta Cardoso et al.-ek (2011) nabarmendu zutenez, zazpi oztopok eragiten dute ezjakintasun hau: (1) bai ornogabeak eta bai haiek eskainitako zerbitzu ekosistemikoak guztiz ezezagunak dira publiko orokorrarentzat (dilema publikoa); (2) politikariek eta ingurumenaren kudeatzaileek gehienetan ez dituzte ornogabeen kontserbazio arazoak ezagutzen (dilema politikoa); (3) ornogabeen inguruko oinarrizko zientzia urria da eta, batez ere, diru gutxi bideratzen da hauek ikertzeko (dilema zientifikoa); (4) espezie asko (gehienak) oraindik ez dira deskribatu (Linneo-defizita); (5) ez dago informaziorik espezie gehienen banaketaren inguruan (Wallace-defizita); (6) ez dakigu nola aldatzen den espezieen abundantzia denboran eta espazioan (Preston-defizita); eta (7) espezieen bizimoduaren eta habitat aldaketekiko sentikortasunaren inguruko informazioa falta da (Hutchinson-defizita).

Hala, arazoa ez da bakarrik espezie zehatzen babesera mugatzen. Izan ere, informazio falta hau dela eta, kontserbazio estrategia internazional garrantzitsuenak, biodibertsitate puntu beroen identifikazioa esaterako (Brooks et al., 2006), landare kormodun eta ornodun gutxi batzuetan oinarritzen dira (adibidez Brummitt eta Lughadha, 2003; Lamoreux et al., 2006; Myers et al., 2000) eta hainbat ikerketek dagoeneko frogatu dutenez, estrategia hauek ez dute balio ornogabeak babesteko (Kerr, 1997; Schuldt eta Assmann, 2010). Izan ere, ikerketa horietan ornogabe talde ezberdinen espezie aberastasun patroien aldaketak aztertzerakoan eta ornodunen eta landare kormodunen patroiekin alderatzerakoan, patroiak bat ez zetozela ikusi zuten. Hau da, ornodun eta landare kormodunen datuekin espezie kopuru handiena babesteko diseinatutako kontserbazio estrategiek ornogabeak kaltetzen dituzte, ez baitituzte puntu bero berberak partekatzen. Premiazkoa da, beraz, ez bakarrik arriskuan dauden ornogabeen identifikazioan aurrera egitea, baizik eta kontserbazio estrategia globaletan ere integratzea.

Are, biodibertsitate ebaluazioetan, ingurumen kalitatea eta prozesuak aztertzeko, ornogabeek sarritan ornodunek eta landareek baino informazio esanguratsuagoa gordetzen dute (Yen eta Butcher, 1997). Ez hori bakarrik, haien belaunaldi-denbora laburra eta dispertsio eskasagatik, ornogabeak askotan ingurumen aldaketekiko sentikorragoak dira eta ondorioz oso egokiak dira ingurumen aldaketak jarraitzeko eta prozesu katastrofikoen abisu goiztiarrak lortzeko (Kremen et al., 1993). Beraz, ornogabeen artean espezie zelatari bikainak aurkitu ahal ditugu, bai arriskuen berri emateko eta baita kontserbazio neurrien eraginkortasuna aztertzeko ere.

Bada garaia ornogabeek merezi duten arreta jaso dezaten. Gizakiaren ongizaterako hain garrantzitsuak diren animalia hauek babestu nahi baditugu, haien inguruko ezjakintasunari aurre egin behar zaio eta, horretarako, haien oinarrizko ikerketarako dirua eta ahaleginak era esanguratsuan bideratzea premiazkoa da.

## 3. Xede-generoa: *Pyrenaearia* Hesse, 1921 (Gastropoda: Helicoidea)

*Pyrenaearia* Iberiar Penintsulako iparraldean endemikoa den lur barraskilo genero bat da, Leptaxinae Boettger, 1909 subfamilian kokatzen dena, Hygromiidae Tryon, 1866 gasteropodo familia izugarri dibertsoaren baitan (Neiber et al., 2017). Bertan, banaketa patroi eten batekin, Kantauriar Mendietan, Pirinioetan, Moncayo Mendian eta Kataluniako Kostaldeko Mendietan zehar hedatzen da (Ortiz de Zárate, 1956; Puente, 1994). Ia beti karedunak diren inguru menditsuetan bizi den genero hau, habitat eskakizun oso zehatzak dituelako da berezia. Alde batetik, eremu harritsuetara hertsiki lotuta dago: beti bizi da arroka-hormetan (itsatsita edo arrakaletan babestuta) edo hartxingadietan harrien azpian; gainera, inguru harritsu hori laiotzean egoteko beharra dauka eta, ondorioz, ipar magaletara, eremu oso malkartsuetara eta haizpitarte oso estuetara mugatuta dago (Gómez-Moliner et al., 2009). Bestalde, *Pyrenaearia* generoa hotzera egokitutako taxon gisa hartu izan da, bere populazio gehienak estai subalpetarrean eta alpetarrean kokatzen direlako (Ortiz de Zárate, 1956; Prieto, 1986; Puente, 1994). Dena den, badaude estai menditarretik banatzen diren populazioak eta baita bertatik beherako gutxi batzuk ere (Puente, 1994).

Hala, generoa inguru menditsuetara mugatuta dago, zeinak nahiko ondo kontserbatu izan diren dentsitate handiko inguruetan ere. Baina kutsatzaileen urrutirako garraioak eta batez ere aldaketa klimatikoak inguru hauek kaltetzen hasi dira (Catalan et al., 2017a). Zinez, inguru menditsuak bereziki zaurgarriak dira aldaketa klimatikoaren aurrean, haien altitude aldaketa azkarra dela eta distantzia tarte txiki batean ingurumen baldintza oso ezberdinak egoten direlako (Becker eta Bugmann, 2001; Lloret, 2017). Habitat harritsuak inguru menditsuetako

elementu garrantzitsuak dira bai haien hedapenari dagokionez eta bai haien garrantzi ekologikoari dagokionez ere eta, eremu malkartsuenak izanik, aldaketa klimatikoak gehien kaltetuko dituen inguruen artean egongo dira. Habitat harritsu hauetako batzuk Habitat Zuzentarauaren I. Eranskinaren baitan 8. atalean biltzen dira, 2 kategoriatan: lur-jausi arrokatsuak (COD. UE 81) eta landaredi kasmofitikoa duten malda arrokatsuak (COD. UE 82). Haien habitat eskakizunak direla eta, *Pyrenaearia* populazioak lehentasunezko habitat horietan kokatzen dira beti eta, inguru harritsuek berezko fragmentazioa izaten dutenez, *Pyrenaearia* populazioak isolatuta egoten dira. Honi, lur barraskiloek dispertsio gaitasun eskasa daukatela batu behar zaio eta, beraz, ingurumen aldaketa bortitzen aurrean haien erantzun gaitasuna mugatua izango da beste latitude edo altitudeetako eremu egokiak kolonizatzeko (Fernández-Chacón et al., 2011). Honen guztiaren ondorioz generoa zaurgarria da eta, beraz, bere espezieek kudeaketa eta kontserbazio planen beharra izan dezakete. Baina aldi berean, ezaugarri hauek direla eta, taxon zelatari paregabea izan daiteke inguru menditsuetan eta bereziki lehentasunezko habitatak diren eremu harritsuetan gertatzen diren aldaketak jarraitzeko.

Gasteropodo familia askoren sistematikaren finkapena eta generoen zedarritzea ugaltze-aparatuaren morfologian oinarritu izan diren arren, ikerketa filogenetiko molekularrek aparatu honen ezaugarri anatomikoetan homoplasiak ohikoak direla frogatu dute, karaktere honen erabilerak mugak dituela agerian utziz (adibidez Chueca et al., 2018; Davison et al., 2005; Hirano et al., 2014; Neiber et al., 2017, 2018; Wade et al., 2007). Hala ere, *Pyrenaearia*-ren ugaltze-aparatuaren morfologia bereizgarria da eta bere espezieak gainerako gasteropodo generoetatik desberdintzeko balio du. Manga (1983), Ortiz de Zárate (1943; 1956), Prieto (1986), Puente (1994) eta Vilella-k (1963) *Pyrenaearia* espezie ezberdinen inguruan egindako deskribapenetan oinarrituz (**1. irudia**), generoak muturretan estutzen den tamaina ertaineko zakila dauka. Honek zakil-papila zilindriko bat dauka, zeharkako ebakiduran ildaska zentral bat eta 2–3 erreten (altzo) bereizi daitezkeelarik. Zakila baino luzeagoa den epifaloa hodi-deferentearen gainean kiribiltzea ezaugarri bereizgarria da. Hodi-deferentea zakilaren eta epifaloaren mutur distaletara ehun konjuntibo bidez lotzen da. Flageloa estua da eta bere luzera oso aldakorra, zakilaren luzera baino txikiagotik bere bikoitzerainokoa izan daitekeelarik. Ondo garatutako bagina dauka, pixka bat kakotuta. Aparatu akuilatzailea azkon-aparatu bakun batez eta baginaren inguruan kokatzen diren lau muki-guruinez osatuta dago. Azkon-aparatua antzeko tamaina daukaten bi zakuk osatzen dute, azkon-zakua kanpoaldean eta zaku-osagarria barnealdean. Azkona txikia eta konikoa da, sekzio borobilarekin. Muki-guruinak sinpleak izan daitezke edo bitan adarkatuak egon (gutxitan hirutan). Azkenik, *bursa copulatrix*-ak forma borobildua edo obalatua dauka eta bere hodiak

zakilaren antzeko luzera edo handiagoa izan dezake.

Nahiz eta ugaltze-aparatuaren morfologiak generoa zalantzarik gabe bereizten duen, espezie mailan ez du inolako aldaketarik aurkezten (Ortiz de Zárate, 1956). Ondorioz, urtetan zehar *Pyrenaearia*-ren taxonomia oskolaren ezaugarrietan oinarritu izan da, kolorea, forma, tamaina, peristomaren forma eta ileen edo hortzen presentzia kontuan hartuz besteak beste (Ortiz de Zárate, 1956; Puente, 1994). Baina ikerketa askok frogatu dutenez, gastropodoen oskola estuki korrelazionatzen da ingurumen faktoreekin, zenbaitetan taxonomia egokiak ezartzea zailduz (adibidez Fiorentino et al., 2008; Razkin et al., 2017; Stankowski, 2011, 2013).

Arazo honi aurre egin nahian, Elejalde et al.-ek (2009) ikerketa filogenetiko bat burutu zuten generoaren espezie nominalen baliozkotasuna egiaztatze aldera, bi gene mitokondrial (*COI* eta *16S rRNA*) eta nuklear bat (*ITS1*) erabiliz. Haien emaitzen arabera, *Pyrenaearia* 11 espeziek osatzen dute. Lau Kantabriar Mendietan endemikoak dira, *P. cantabrica*, *P. oberthueri*, *P. daanidentata* eta *P. velascoi*; bost Pirinioetan endemikoak, *P. carascalensis*, *P. cotiellae*, *P. carascalopsis*, *P. parva* eta *P. organiaca*; Kataluniako Kostaldeko Mendietan dago *P. molae*; eta, azkenik, *P. navasi* Moncayo Mendian bakarrik ezagutzen da. Jarraian Elejalde et al.-ek (2009) kontsideratutako espezie hauetako bakoitzaren oskolaren deskribapen motz bat eta haien banaketa zehazten dira (espezieen banaketa **2. irudian** erakusten da).

### *P. cantabrica* (Hidalgo, 1873) (3. irudia a–b)

*Locus typicus*: "Peña Abis, aux environs de Caldas de Oviedo" (Asturias).

*P. cantabrica* Kantauriar Mendikate osoan zehar banatzen da, bai kantauriar isurialdean bai mediterranearrean, eta bereziki ugaria da Picos de Europa inguran. 40–1800 m bitarteko altitude-tartean aurkitu daiteke.

Bere oskola lentikularra da, azpialdean beti ganbila eta gainaldean ganbiletik guztiz zapalerainoko gradientea izan dezake. 4,5–5,5 espira buelta ditu, azkenengo buelta azken aurrekoa baino 1,5–2 aldiz zabalagoa delarik, eta sutura oso nabarmena da (Manga, 1983; Ortiz de Zárate, 1956; Prieto, 1986). Bere diametro maximoa 9,8–18 mm bitartekoa da (Manga, 1983; Ortiz de Zárate, 1956; Prieto, 1986). Zilbor zabal eta borobila dauka (diametro maximoaren 1/6–1/4) (Ortiz de Zárate, 1956; Prieto, 1986), barruko kiribila ikustea ahalbidetzen duena. Irekiera borobildua eta oso zeiharra da. Peristoma zuzena da eta bere barnealdean kolore zuriko loditzea dauka. Oskolak kolore zuri-grisaxka dauka, kolore nabarreko orban luzexka erradial eta orban borobil txikiekin apaindua. Ohikoa da

gazteek ile periostrakalak izatea, batez ere gainaldean, eta kasu batzuetan heldutasunean ere mantentzen dituzte (Ortiz de Zárate, 1956; Prieto, 1986). *Pyrenaearia* generoaren barruan, espezie honen kariotipoa bakarrik ezagutzen da: 52 kromosoma ditu (kromosoma kopuru haploidea n = 26) (Aparicio, 1983).

Espezie honen sinonimiaren barruan *P. schaufussi* (Kobelt in Rossmässler. 1876), *P. covadongae* Ortiz de Zárate, 1956 eta *P. poncebensis* Ortiz de Zárate, 1956 espezie nominalak sartzen dira.

### *P. oberthueri* (Ancey, 1884) (3. irudia c)

*Locus typicus*: Engotable (Asturias).

Espezie honen banaketa Picos de Europa ingurura mugatzen da, erdialdeko eta mendebaldeko mendiguneetara (Raven, 1988), non 1600 m-tatik gora agertzen den.

*P. oberthueri*-ren oskol forma orokorra *P. cantabrica*-ren antz handia dauka, baina espezie honek aurrekoak baino tamaina txikiagoa dauka (diametro maximoa 7–10 mm bitartekoa) (Ortiz de Zárate, 1956) eta, batez ere, bere egitura globosoagoa delako eta ildaskak dituelako ezberdintzen da. *P. oberthueri*-k ere zilbor borobil eta zabala dauka, baina *P. cantabrica*-rena baino estuagoa da (Ortiz de Zárate, 1956). Irekiera borobildua da, peristoma zuzen batekin, zeinak barnealdean kolore zuriko loditzea daukan. Oskolak kolore grisaxka-urdinxka dauka eta gorri-horixka da irekieran. Hazkuntzaren noranzkoan, irregularki, orban mehe gorri-urdinxkak ditu. Bai Ortiz de Zárate-k (1956) eta bai Raven-ek (1988) espezie honetan eta *P. cantabrica*-n altitudearen araberako bariazio morfologiko klina bat behatu zuten, altitudea handitu ahala oskolen altuera handituz eta diametroa txikituz.

### *P. daanidentata* Raven, 1988 (3. irudia d)

*Locus typicus*: "Hoyo del Burro, 2.5 km S. of Peña Santa de Castilla, Picos del Cornión" (León).

*P. daanidentata*-ren banaketa oso mugatuta dago. Picos de Europa Mendien mendebaldeko mendiguneko Peña Santa tontorraren hegoaldeko isurialdean bakarrik aurkitu da, 2000 m-tatik gora (Gómez-Moliner et al., 2011a).

Bere oskola lodia eta lentikularra da, bai azpialdean eta bai gainaldean ganbila. Bost espira buelta ditu, erregularki hasten diren ildaska xumeekin. Diametro maximoa 8,2–9 mm bitartekoa da eta zilborra zabala da, diametro honen 1/5–1/4 betetzen duelarik (Raven, 1988). Espezie hau nahasezina da bere irekiera txiki borobilean, justu peristoma eta gero loditze sendo bat daukalako, norbanako gehienetan hortz zabal bat edo bi osatuz. Oskola grisa da, zeharkako marra marroi-grisaxka irregularrekin. NKNB-ren Zerrenda Gorrian *P. daanidentata* Ahula kategorian sartzen da.

### *P. velascoi* (Hidalgo, 1867) (3. irudia e)

*Locus typicus*: "Pico de Altamira, dans la Peña de Gorbea" (Bizkaia).

*P. velascoi* Euskal Mendietako gailurrik altuenetan bizi da, Gorbeia ingurutik Andia Mendilerroraino, 1300 m-tatik gora.

Nahiz eta Kantauriar Mendikatean endemikoa den, morfologikoki Pirinioetako espezieen antz handiagoa dauka aurreko hiruena baino. Dortsobentralki zapaldutako oskola dauka, gainaldean eta azpialdean neurri berdinean galgatua, eta aurreko hiru espezieena baino globosoagoa izateaz gain, ez du inoiz ile periostrakalik izaten (Ortiz de Zárate, 1956), Pirinioetako espezieen antzera. Ildaska nabarmen eta irregularrekin, 4,75–5,25 espira buelta ditu, azkenengo buelta azken aurrekoa baino 1,5–2 aldiz handiagoa delarik (Prieto, 1986) eta bere diametro maximoa 14,2–18,6 mm bitartekoa da (Ortiz de Zárate, 1956; Prieto, 1986). Zilborra estua eta sakona da, kolumelaren ertzak partzialki estalita. Irekiera zeiharra eta obalatua dauka. Peristoma zuzena da eta ez dauka barnealdeko loditzerik. Oskola zuri-grisaxka da, era irregularrean banatzen diren kolore arreko marra radial estuekin. Norbanako batzuetan banda karenal txuri bat agertu daiteke (Ortiz de Zárate, 1956; Prieto, 1986). *P. velascoi* NKNB-ren Zerrenda Gorrian Ahula kategorian sartzen da.

### *P. carascalensis* (Férussac, 1821) (3. irudia f)

*Locus typicus*: "Fôret de Carascal en Aragon".

Espezie hau Pirinio Axialen hedapen osoan zehar hedatzen da eta populazio gehienak 1200 m-tatik gora kokatzen diren arren, salbuespenez, kota baxuagoetan ere aurkitu da.

Bere oskola, dortsobentralki zapaldua, galgatuagoa da azpialdean gainaldean baino eta era irregularrean banatzen diren ildaska nabarmenak ditu, gainaldean mikroeskultura espiral ahul batekin. 4,25–5 espira bueltekin, azkenengo buelta azken aurrekoa baino 2 aldiz handiagoa da eta 13,4–16,5 mm bitarteko diametro maximoa dauka (Faci, 1991; Prieto, 1986). Zilborra oso estua da eta irekiera oso zeiharra eta obalatua. Peristoma zuzena da, barne loditzerik gabea, eta bere ertz kolumelar tolestuak zilborra partzialki estaltzen du. Kolorea hori-arrexka edo grisaxka da, marra radial arre ilunagoek apaindua eta banda karenal zurixka batekin, norbanako guztietan agertzen ez dena.

Elejalde et al.-ek (2009) *P. transfuga* (Fagot, 1885) espezie honekin sinonimizatu zuten.

### *P. cotiellae* (Fagot, 1906) (4. irudia a)

*Locus typicus*: "Cirque calcaire d'Armèna, sur le mont Cotiella" (Huesca).

*P. cotiellae*-ren banaketa Cotiella Mendigunera mugatuta dago, Huesca ipar-ekialdean. Bertan bi populazio ezagutzen dira, bata Armeña zirkuan eta bestea Sahún mendi-lepoan, biak 1800 m-tatik gora (Puente, 1994).

Espezie hau *P. carascalensis*-en oso antzekoa da, bai forma orokorrari, ildaskei, irekierari eta peristomari dagokionez eta baita tamainari dagokionez ere (15 mm inguruko diametro maximoa), baina Fagot-en (1906) arabera elkarrengandik erraz desberdindu daitezke *P. cotiellae*-k oskol zurixka daukalako eta bere zilborra zabalagoa delako.

### *P. carascalopsis* (Bourguignat in Fagot, 1884) (4. irudia b)

*Locus typicus*: "Port de Salau, sur les deux versants français et espagnol" (Lleida eta Ariège).

Espezie hau Pirinio Axialen ipar isurialdearen erdialdean banatzen da. Hala, bere banaketaren mendebaldean Maladetta Mendigunean eta Esera Haranean aurkitu da, ekialdean Aneu Haraneko Aula eta Salau Mendateetan eta bi gune horien artean Besiberri Mendigunean eta Vielha Mendatean, denak 1800 m-taik gora (Gómez-Moliner et al., 2011b).

Ezaugarri morfologikoak kontuan hartuz, espezie honen estatusa

eta desberdintzapena oso eztabaidatuak izan dira. Autore batzuek *P. carascalensis*-en sinonimian sartu dute, beste batzuek espezie horren barietate edo subespezie gisa hartu duten bitartean (ikusi Puente, 1994). Are gehiago, datu morfologikoekin *P. esserana* (Bourguignat in Fagot, 1888) espeziea ere *P. carascalensis*-en sinonimo, barietate eta subespezie kontsideratu izan da, batzuetan *P. carascalopsis*-en oso gertukoa izan zitekeela aipatu delarik (Faci, 1991). Elejalde et al.-en (2009) lan molekularrak ordea argi utzi du *P. esserana P. carascalopsis*-en sinonimo dela eta, aldi berean, *P. carascalopsis* baliozko espeziea dela berretsi du. Oskolaren morfologia eta kolorea, beraz, *P. carascalensis*-enaren oso antzekoak dira: dortsobentralki zapaldutako formarekin, zilbor estua ertz kolumelarrak partzialki estalita eta kolorez arre argia marra ilunagoekin. Hala ere, zenbait autoreen arabera espezie honek zilbor estuagoa eta irekiera borobilagoa dauzka eta, batez ere, bere ildaskak lodiagoak dira (Faci, 1991; Ortiz de Zárate, 1956). Bestalde, *P. carascalopsis*-ek espiraren altuerari dagokionez aldakortasun handiagoa daukala dirudi. Izan ere, norbanako batzuentzat *P. carascalensis*-ena baino espira zapalduagoa deskribatu da, baina beste batzuetan gainaldetik globosoena dela esan da, ia forma trokoideoa izateraino (Ortiz de Zárate, 1956). Azkenik, espezie honen diametro maximoa 9,5–10,5 mm bitartekoa da (Faci, 1991; Gómez-Moliner et al., 2011b; Ortiz de Zárate, 1956) eta, beraz, *P. carascalensis* baino txikiagoa. NKNB-ren Zerrenda Gorrian Ahula bezala katalogatuta dago.

### *P. parva* Ortiz de Zárate, 1956 (4. irudia c)

*Locus typicus*: "Montaña Pedra-Forca de la Sierra del Calí" (*sic*) (Lleida).

*P. parva* Cadí Mendizerran eta Pedraforca Mendigunean endemikoa da, non 2000 m-tatik gora bizi den (Altimira, 1963).

Pirinioetako espezierik txikiena da, 8,2–9,8 mm bitarteko diametro maximoarekin (Altimira, 1963; Ortiz de Zárate, 1956). Oskolak, dortsobentralki zapaldua, 4,5 espira buelta ditu (Ortiz de Zárate, 1956). Zilborra sakona eta nahiko zabala da, barruko kiribila ikustea ahalbidetzen duena. Irekiera zeharka obalatua da eta peristoma zuzena, bere ertz kolumelarrean xumeki tolestuz eta zilborra pixka bat estaliz. *P. parva* erraz bereizi daiteke ildaska txuri irregular oso nabarmenak dituelako, batez ere, azken bueltan. Ildasken arteko tartea horixka-arrea da. Espezie hau, NKNB-ren Zerrenda Gorrian, Ahula kategorian sartzen da.

### *P. organiaca* (Fagot, 1905) (4. irudia d)

*Locus typicus*: "Calcaires du défilé d'Organyá, vallée de la Segre" (Lleida).

Noguera Pallaresa Haranean aipatu bada ere, azkenengo laginketetan espezie hau Segre ibaiaren arroan bakarrik aurkitu da, Organyá, Espluvins eta Pont de la Torre haizpitarteetan (Gómez-Moliner et al., 2011c). Aurreko espezieak ez bezala, *P. organiaca* 500–700 m bitarteko altitudeetan bakarrik bizi da.

Espezie honen oskolak, dortsobentralki zapaldua baita ere, 5–5,5 espira buelta ditu, azkenengo buelta handi batekin (Gómez-Moliner et al., 2011c; Ortiz de Zárate, 1956). *Pyrenaearia* espezie handienen artean dago, diametro maximoaren neurria 12–18 mm bitartekoa baita (Altimira, 1963; Gómez-Moliner et al., 2011c). Irekiera zeharka obalatua da. Peristoma, zuzena, kanpoaldean xumeki tolesten da eta nabarmenago ertz kolumelarrean, zilbor estua partzialki estaliz. Oskola distiratsua da, kolore arre-grisaxkarekin eta ildaska txuri nabarmen ugarirekin, zilbor-aldean leunagoak direnak, espeziea argi bereiziz. NKNB-ren Zerrenda Gorrian Ahula bezala katalogatuta dago.

### *P. molae* Haas, 1924 (4. irudia e)

*Locus typicus*: "Monte dico 'Mola de Falset'" (Tarragona).

*P. molae*-ren banaketa Montsant, Colldejou eta Llavería Mendiguneen ipar isurialdera mugatzen da, Kataluniako Kostaldeko Mendietan (Martínez-Ortí eta Bros, 2012; Ortiz de Zárate, 1956; Vilella, 1963). Mendigune hauetako tontorrean bizi da, 800 eta 900 m-ko altitudeen artean.

Espezie honen diametro maximoa 11,5–16 mm bitartekoa da (Gómez-Moliner et al., 2011d; Ortiz de Zárate, 1956; Vilella, 1963). Bere oskolak *P. organiaca*-ren oskolaren antz handia dauka: dortsobentralki zapaldua, azkenengo espira buelta handiarekin, distiratsua, ildaska zuri ugarirekin, irekiera zeharka obalatuarekin eta peristoma zuzena zilbor estua partzialki estaliz. Hala ere, zalantzarik gabe desberdintzea ahalbidetzen duten hainbat ezberdintasun ditu. *P. organiaca*-k baino espira buelta gehiago ditu, 5–6,25 bitartean, eta zilborra estuagoa da, diametro maximoaren 1/6–1/7 (Ortiz de Zárate, 1956; Vilella, 1963). Ez dira hain globosoak, norbanako batzuk espira guztiz zapala ere badaukatelarik. Haien arteko desberdintasun nabarmenena, ordea, *P. molae*-k ildaska zuri

gehiago dituela eta hauek askoz xumeagoak direla da, orokorrean itxura zurixkagoa daukalarik kolore arreko marra gutxiago dituelako. Gainera, gehienek banda karenal zuri bat daukate. *P. molae* ere Ahula kategorian sartzen da NKNB-ren Zerrenda Gorrian.

### *P. navasi* (Fagot, 1907) (4. irudia f)

*Locus typicus*: "Moncayo. Nevera de San Miguel au sommet" (Zaragoza).

*P. navasi* inguru silizeo batean bizi den *Pyrenaearia* espezie bakarra da. Moncayo Mendigunearen ipar isurialdera mugatzen da, Iberiar Sistemaren iparraldean. Espezie honen populazioak 1600 m-tik gora kokatzen dira, 2000 m-tatik gora ugariagoak diren arren (Faci, 1991; Gómez-Moliner et al., 2011e; Prieto, 1991). Hala ere, 1200 m-tan, pago-ametz baso batean, populazio bat ezagutzen da, generoarentzat guztiz ezohikoa den habitata (Prieto, 1991).

Agian inguru silizeoan bizitzeagatik, espezie honen oskola genero osoan ezberdinena da. Forma subglobosoarekin, oso mehea eta hauskorra da, ia zeharrargia, kolore marroi uniformearekin. Oso nabariak ez diren ildaska zeiharrak ditu eta irekiera obalatua. Peristomak kolore argiagoa dauka eta oso hauskorra da; ertz kolumelarrean asko tolesten da, zilbor estua partzialki estaliz. Normalean 10–11,8 mm bitarteko diametro maximoa dauka eta 4,5–5 espira buelta (Faci, 1991; Gómez-Moliner et al., 2011e; Ortiz de Zárate, 1956). Hala ere, basoko populazioko norbanakoak handiagoak eta globosoagoak dira, 14,4–16,4 mm-ko diametro maximoarekin eta 5–5,5 espira bueltarekin (Prieto, 1991). Ezberdintasun hauek direla eta, bi subespezie desberdindu dira, *P. navasi navasi* txikia eta basoko *P. navasi sylvatica* Prieto, 1991, baina Elejalde et al.-ek (2009) banaketa hau gezurtatu dute. NKNB-ren Zerrenda Gorrian *P. navasi* Ahula bezala katalogatuta dago.

Elejalde et al.-ek kontsideratutako 11 espezie hauek gure abiapuntu izan dira; hala ere, autore hauen espezie zedarritzea talde monofiletikoak identifikatzera eta ordura arte kontuan hartzen ziren espezie nominalekin konparatzera mugatzen zen eta, haien filogenia guztiz ebatzita ez zegoenez, goiko zerrendan deskribaturiko espezie batzuen baliozkotasuna oraindik ez dago argi. Izan ere, *P. carascalensis*, *P. cotiellae*, *P. molae* eta *P. velascoi*-ren erlazio filogenetikoak ez ziren ebatzi eta, nahiz eta azken hiru espezie horiek talde monofiletikoak osatu zituzten, *P. carascalensis* parafiletiko

agertu zen lau espezieek osaturiko kladoan. Gainera, *P. cantabrica*, *P. daanidentata* eta *P. oberthueri*-k ere politomia bat osatzen zuten.

Baina generoaren taxonomia ez da *Pyrenaearia*-ren sistematikaren inguruan argitzeke dagoen alderdi bakarra: bere subfamiliaren sistematika ere, Leptaxinae-rena, ez dago ebatzita. Gaur egun Leptaxinae-ren estatusa datu molekularretan bakarrik oinarritzen da, ezberdintasun morfologiko bereizgarririk ez daukalako, eta hiru tribu bereizten dira: Leptaxini, Cryptosaccini (*Pyrenaearia* barne hartzen duena) eta Metafruticicolini (Neiber et al., 2017). Baina tribu hauen arteko erlazio filogenetikoak ez daude argi eta, are gehiago, genero batzuk sostengu estatistikorik gabe bildu dira tribuen baitan. Ondorioz, *Pyrenaearia*-ren eta berarekin hertsikien erlazionaturiko generoen arteko erlazio filogenetikoak ez dira ezagutzen eta informazio hori gabe ezin da generoaren eboluzioa nolakoa izan den inferitu (Weir eta Schluter, 2008).

Ez hori bakarrik, generoaren oinarrizko ezaugarri biologikoak, ekologia eta populazio demografia guztiz ezezagunak dira eta inoiz ez da bere populazioen jarraipenik egin. Informazio hau ezinbestekoa da ingurumenarekin eta beste organismoekin elkar nola eragiten duten ulertzeko, eta baita gainbehera prozesu batean dauden ebaluatzeko eta horren kausak identifikatzeko ere (Duangchantrasiri et al., 2016; Esteban et al., 2016; Guimarães et al., 2014; McGill et al., 2006). Are, ezaugarri horien inguruko ezagutza beharrezkoa da espezieek etorkizunean nola erantzungo duten iragartzeko.

Generoaren inguruan dagoen informazio falta handi honek, beraz, bere kontserbazioa kolokan jartzen du. Izan ere, zein espeziek osatzen duten ere jakin gabe, baten bat gainbeheran dagoen zehaztea ez da erraza. Hain zuzen ere, edozein kudeaketa edo kontserbazio programa martxan jartzeko ezinbestekoa da kontserbatu edo kudeatu nahi den unitate biologikoa definitzea. Bestalde, espezieen biologia, ekologia eta demografia ezagutu ezean, kontserbazio eta kudeaketa plan egokiak ere ezin dira martxan jarri. Gainera, *Pyrenaearia*-ren jarraipenak inguru menditsuetako lehentasunezko habitat harritsuetan gertatzen ari diren prozesuak ulertzen lagundu dezake eta, horrela, inguru hauetan kontserbazio eta kudeaketa neurri egokiak martxan jartzea eta baliabideak era egokian erabiltzea ahalbidetu, agian erabakigarri izanez mendietako biodibertsitatea kontserbatzeko. Baina generoaren inguruko ezagutza faltak ezinezko egiten du *Pyrenaearia* taxon zelatari gisa jardutea. Hemen, *Pyrenaearia*-ri buruzko aurreko lanetan ez bezala, ikuspegi integratzaile bat proposatzen dugu generoari buruzko informazioa biltzeko aspektu guztietan, horrela bere kontserbazioa bermatzeko eta espezie zelatari gisa erabiltzea ahalbidetzeko. Horretarako tesi honetan filogenetika

molekularra, morfometria geometrikoa eta harrapaketa-berrarrapaketa teknika konbinatu ditugu.

Datu molekularren erabilera gaur egun guztiz errotuta dago biologia ikerketetan. Filogenetika molekularrak datu molekularretan jasotako informazioa erabiltzen du erlazio filogenetikoak eraikitzeko, hau da, filogeniak inferitzeko (San Mauro eta Agorreta, 2010). Eboluzio teoria modernoaren arabera, organismo guztiak arbaso komun batetik eratorri dira, edozein espezie, bizirik dirauen edo iraungitutakoa, erlazionatuta dagoela esan nahi duena; erlazio historiko hauen patroiari filogenia deritzo (Page eta Holmes, 1998). XX. mendearen amaieran, potentzia handiko ordenagailuen, PCR-aren eta Sanger sekuentziazioaren agerpenak filogenia molekularren hazkunde esponentziala eragin zuen. Orain, errendimendu handiko sekuentziazio genomikoaren garapenarekin, filogenetika molekularra aro berri batean sartu da, datu molekularrak kopuru handitan eta era azkarrean biltzea ahalbidetu duelako arrazoizko prezioan (Mardis, 2008; Metzker, 2010). Aurrerapen teknologiko hauekin batera, filogenetika molekularra haziz joan da eta baita bere aplikazioak ere (Yang eta Rannala, 2012). Hala, filogenetika molekularra bizitzaren zuhaitzean espezieen arteko erlazioak eraikitzeko eta haien eboluzioa ulertzeko erabiltzeaz gain, gaur egun baliagarria da, besteak beste, espezieak zedarritzeko eta ezaugarritzeko (Jones, 2017; Yang eta Rannala, 2010) edota espezieen aldaketa demografikoak eta migrazio patroiak ikertzeko (Hohenlohe et al., 2018). Are, ezinbesteko tresna bilakatu da konparazio genomikoetan, beste aplikazio askoren artean, adibidez, sekuentzia metagenomikoak sailkatzeko (Alloui et al., 2015) edota antzinako genomak berreraikitzeko (Vialle et al., 2018) erabili baitaiteke.

Morfometria geometrikoaren agerpenak eta ondoren jasan zuen garapenak aldaketa erradikala ekarri du forma biologikoak aztertzerakoan (Adams et al., 2004, 2013). Morfometria geometrikoak egitura biologikoetan zehar banatzen diren puntu, kurba edo azaleretan kokatzen diren *landmark* koordenatuen forma informazioa teoria estatistikoarekin elkartzen du. Honek aldagai anitzeko analisien erabilera ahalbidetzen du formaren inguruko hipotesi biologikoak testatzeko prozesuan *landmark*-en jatorrizko konfigurazio geometrikoa mantentzen den bitartean eta, ondorioz, emaitza estatistikoak benetako forma gisa irudikatzea ahalbidetuz (Mitteroecker eta Gunz, 2009). Teknika honek, alde batetik, morfometria klasikoarekin antzemanezinak ziren forma aldaketak kuantifikatzea eta neurtzea posible egin du, errendimendua hobea izanik (Evin et al., 2008). Baina gainera, bestetik, datu morfologikoak beste iturri batzuetako datuekin sakonki integratzea ahalbidetu du, molekularrekin adibidez, nabarmenki handituz formaren aldaketaren

eta eboluzioaren inguruan erantzun daitezkeen galderak (adib., Klingenberg, 2014, 2016; Savriama et al., 2012).

Azkenik, oinarrizko ezaugarri biologikoak ezagutzeko (adib., heldutasun adina edo bizi-itxaropena) eta parametro demografikoak kalkulatzeko (adib., biziraupena, ugalketa arrakasta edo dispertsioa), beharrezkoa da populazioen zuzeneko behaketa, horrela norbanakoen banan-banako jarraipen datuak lortzeko (Gimenez eta Choquet, 2010). Landan, datu hauek harrapaketa-berrarrapaketa teknikaren bidez lortzen dira. Teknika honetan norbanakoak era ez zuzenean erregistratzen dira (adib., kamera-tranpekin edo laginketa genetiko ez-inbaditzaileekin) edota zuzenean harrapatu, era bereizgarrian markatu eta berriro inguruan askatzen dira, denboran zehar behatzeko (Royle et al., 2018). Datu hauen tratamendu estatistikoa ikerketa eremu aktiboa izan da 50 urtez eta gaur egun ere pil-pilean dago (Lebreton et al., 1999; Pradel, 2005; Royle et al., 2018). Metodo hauek norbanakoak atzemateko ziurgabetasunari eta, berriki, heterogeneotasun espazialari ere aurre egiteko gai direnez, garrantzi izugarria izan dute populazio basatien demografiaren ikerketan eta ezagutzan (Karanth et al. 2006, Pradel et al. 2010), teoria ekologikoaren aurrerapenean (Cooch et al. 2002) eta baita faunaren kontserbazio eta kudeaketa praktikak gidatzerakoan ere (Nichols eta MacKenzie 2004; Gimenez eta Choquet, 2010). Ondorioz, populazio prozesuak ikertzeko eta oinarrizko ezaugarri biologikoen informazioa biltzeko laginketa eta analisi esparru estandar bihurtu dira (Williams et al. 2002).

# CHAPTER 2

## Aims of the thesis

The main aim of this thesis is to gain insights into the systematics, the biogeography, and the population dynamics of the land snail genus *Pyrenaearia* through a multidisciplinary approach, combining molecular, geometric morphometrics and capture-recapture techniques, to improve the scientific knowledge of the genus and to ensure its conservation and enable its use as a sentinel taxon. The specific goals are to:

## 1. Reconstruct the phylogeny of *Pyrenaearia*

1.1. Construct the phylogenetic relationships for Pyrenaearia based on both mitochondrial and nuclear rRNA gene sequences.

## 2. Delimit species of *Pyrenaearia*

2.1. Apply multilocus species delimitation methods.

2.2. Apply 3D geometric morphometrics on the shell to stablish morphological groupings.

2.3. Integrate multilocus species delimitation methods with shell 3D geometric morphometrics to delimit *Pyrenaearia* species.

## 3. Review the taxonomy of *Pyrenaearia*

3.1. Compare the achieved delimitation scheme with previous taxonomy of the genus to determine species validity and establish synonymies.

3.2. Provide formal description for new species.

## 4. Infer phylogeographic patterns within *Pyrenaearia*

4.1. Use the new species delimitation scheme and the recovered phylogenetic relationships to infer phylogeographic patterns within the genus.

## 5. Investigate the variation and evolution of shell morphology within *Pyrenaearia*

5.1. Assess the contribution to shape variation of both population history and selection in different environments.

5.2. Determine the effect of allometry in shell shape.

5.3. Study morphological integration of the shell by analysing shape variation at different levels and covariation between suture and peristome morphology.

## 6. Reconstruct the phylogenetic relationships of the subfamily Leptaxinae and their position within Hygromiidae

6.1. Reconstruct the phylogeny of Leptaxinae and a representative taxa of Hygromiidae based on two mitochondrial and eight nuclear gene fragments.

## 7. Revise the classification of Leptaxinae

7.1. Compare the obtained molecular phylogeny with previous classifications of the subfamily.

## 8. Reconstruct the temporal and geographical framework of the diversification of Leptaxinae

8.1. Estimate divergence times by fossil calibration from a molecular phylogeny.

8.2. Estimate ancestral areas based on the calibrated tree.

## 9. Shed light upon basic biological traits of *Pyrenaearia carascalensis*

9.1. Investigate growth, maturity age, lifespan and dispersion habits of one population of P. carasc*alensis*.

## 10. Analyse *P. carascalensis* population demographics

10.1. Investigate the population dynamics across years and year-round.

10.2. Assess the correlation between life-history parameters and local climate variability.

## 11. Lay the foundation of a long-tern monitoring of *P. carascalensis* to monitor changes in mountainous areas

11.1. Establish optimal monitoring strategies.

2

# 2. KAPITULUA

## Tesiaren helburuak

Tesi honen helburu nagusia *Pyrenaearia* lur barraskilo generoaren sistematikaren, biogeografiaren eta populazio dinamikaren inguruko informazioa biltzea da teknika molekularrak, morfometria geometrikoa eta harrapaketa-berrarrapaketa metodologia konbinatzen dituen hurbiltze integratzailea erabiliz, horrela generoaren inguruko ezagutza zientifikoa hobetzeko eta bere kontserbazioa bermatzeko zein espezie zelatari gisa erabiltzea ahalbidetzeko. Helburu zehatzak ondorengoak dira:

## 1. *Pyrenaearia*-ren filogenia eraikitzea

1.1. *Pyrenaearia*-ren erlazio filogenetikoak eraiki sekuentzia mitokondrial eta nuklearretan oinarrituz.

## 2. *Pyrenaearia* espezieak zedarritzea

2.1. Espezie zedarritze metodo multilocus-ak aplikatu.

2.2. Oskolean 3D morfometria geometrikoa aplikatu talde morfologikoak zehazteko.

2.3. Espezie zedarritze metodo multilocus-ak eta oskolaren 3D morfometria geometrikoa integratu espezieak zedarritzeko.

## 3. *Pyrenaearia*-ren taxonomia berrikustea

3.1. Lortutako espezie zedarritzea generoak aurretik zeukan taxonomiarekin alderatu espezieen baliozkotasuna egiaztatzeko eta sinonimiak ezartzeko.

3.2. Espezie berriak formalki deskribatu.

## 4. *Pyrenaearia*-ren barruko patroi filogeografikoak ondorioztatzea

4.1. Espezie zedarritze berria eta lortutako erlazio filogenetikoak erabili generoaren barruko patroi filogeografikoak ondorioztatzeko.

## 5. Oskolaren morfologiaren bariazioa eta eboluzioa ikertzea *Pyrenaearia*-ren barruan

5.1. Oskolaren forman populazio historiak eta inguru ezberdinetan jazotako hautespenak daukaten ekarpena ebaluatu.

5.2. Alometriak oskolaren forman daukan eragina zehaztu.

5.3. Oskolaren integrazio morfologikoa ikertu, forma aldaketa maila ezberdinetan analizatuz eta suturaren eta peristomaren morfologiaren kobariazioa aztertuz.

## 6. Leptaxinae subfamiliaren erlazio filogenetikoak eraikitzea eta Hygromiidae-ren barruan daukan posizioa argitzea

6.1. Hygromiidae-ren hainbat taxon ordezkariren eta Leptaxinae-ren filogenia eraiki bi gene mitokondrial eta zortzi nuklear erabiliz.

## 7. Leptaxinae-ren sailkapena berrikustea

7.1. Lortutako filogenia molekularra subfamiliaren aurreko sailkapenekin alderatu.

## 8. Leptaxinae-ren dibertsifikazio tenporala eta geografikoa eraikitzea

8.1. Filogenia molekular batetik dibertsifikazio datak kalkulatu fosil bidezko kalibrazio bidez.

8.2. Kalibratutako zuhaitzean oinarrituz arbasoen banaketa estimatu.

## 9. *Pyrenaearia carascalensis*-en oinarrizko ezaugarri biologikoen inguruko informazioa lortzea

9.1. *P. carascalen*sis populazio baten hazkuntza, heldutasun adina, bizi-itxaropena eta dispertsio ohiturak ikertu.

## 10. *P. carascalensis*-en populazio demografia analizatzea

10.1. Populazio dinamika urteetan zehar zein urtean zehar aztertu.

10.2. Bizitza-historia parametroen eta tokiko aldakortasun klimatikoaren arteko korrelazioa ebaluatu.

## 11. *P. carascalensis*-en epe luzerako jarraipen estrategia martxan jartzea mendi inguruetako aldaketak jarraitzeko

11.1. Jarraipen estrategia hoberenak finkatu.

# CHAPTER 3

## Taxonomy, phylogeny & biogeography

# 3. KAPITULUA

## Taxonomia, filogenia & biogeografia

PAPER I

# Integrating multilocus DNA data and 3D geometric morphometrics to elucidate species boundaries in the case of *Pyrenaearia* (Pulmonata: Hygromiidae)

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# Integrating multilocus DNA data and 3D geometric morphometrics to elucidate species boundaries in the case of *Pyrenaearia* (Pulmonata: Hygromiidae)


## Abstract

To accurately delimit species the use of multiple character types is essential as all speciation processes are not equally reflected in different data (e.g. morphological, molecular or ecological characters). With the introduction of geometric morphometrics methods and advances in 3D technology, a comprehensive combination of molecular and morphological data has been enabled in groups where exhaustively quantifying and measuring morphological shape change was not possible before such as gastropod shells. In this study, we combined multilocus coalescent species delimitation methods with 3D geometric morphometrics of shell shape to delimit species within the land snail genus *Pyrenaearia*. A new taxonomic scheme was constructed for the genus identifying ten species. Two nominal species were synonymized and a hitherto unrecognized cryptic species was identified. Our findings support the importance of combining multiple lines of evidence as molecular and morphological data on their own do not yield the same information. Further, the integration of morphological and molecular data shows the importance of allometry in shell shape and suggests a combined effect of population history and selection in different environments on shells morphological variation. Our new taxonomy and phylogenetic reconstruction suggest that, besides the glacial cycles of the Pleistocene, passive dispersal and rock substrate complexity could also have been involved in the speciation of the genus.



## Keywords

*Pyrenaearia*; species delimitation; 3D; geometric morphometrics; molecular phylogeny; integrative taxonomy

# 1. Introduction

In numerous fields of science such as ecology, evolution, biogeography and conservation biology, species are the fundamental units of analysis (Sites and Marshall, 2003). To achieve a better understanding of the evolutionary processes and mechanisms involved in speciation, it is needed to delimit species into operational taxonomic units as the fundamental entity of study. Thus, accurate delimitation of species boundaries is crucial.

Species identification and description have been traditionally based solely on morphological traits. It is now known, however, that morphology-based taxonomy is frequently hindered by homoplasy, cryptic speciation or phenotypic plasticity (Agapow et al., 2004) and could lead to inflated or underestimated species numbers. On the other hand, the use of molecular markers as tools for species delimitation has drastically risen in the last few decades. In response to the increased availability of multilocus datasets and coalescent-based approaches that can handle gene tree conflicts, molecular methods of species delimitation have proliferated (e.g. Ence and Carstens, 2011; Hausdorf and Hennig, 2010; Jones, 2017; Knowles and Carstens, 2007; O'Meara, 2010; Yang and Rannala, 2010) and molecular approaches are now widely used. Moreover, empirical applications relying solely on genetic information are on the increase (e.g. Blair et al., 2015; Leaché and Fujita, 2010; Rato et al., 2015; Satler et al., 2013; Toussaint et al., 2015). Nevertheless, with a purely genetic approach, recent speciation events may go undetected because vast amounts of data are needed to recover species due to incomplete lineage sorting and/or introgression (Edwards and Knowles, 2014). Neutral loci may also be unable to detect lineages that speciated by natural selection (Solís-Lemus et al., 2015). Moreover, it has been shown that molecular delimitation methods are prone to confuse population genetic structure with species boundaries (Lohse, 2009; Sukumaran and Knowles, 2017) meaning that molecular methods may also inflate the number of species.

Considering species as separately evolving metapopulation lineages (de Queiroz, 2007), the criteria proposed by former species definitions (e.g. reciprocal monophyly, reproductive isolation or phenotypic diagnosability) have become alternative lines of evidence and not necessary characteristics that are relevant to determine lineage boundaries. Indeed, since the use of a single line of evidence to unravel species limits may result in erroneous species delimitations, the use of different character types (e.g. niche differences, morphology or molecular markers) has been increasingly recognized as an indispensable approach to delimit species (Padial et al., 2010). Notwithstanding, combining molecular data and morphology comprehensively has been challenging in some groups such as molluscs due to difficulties to exhaustively quantifying and measuring morphological variation. The emergence and later development of geometric morphometrics has provoked a radical shift in the

study of biological forms (Adams et al., 2004, 2013). This approach has allowed measuring and quantifying shape variations that were not detectable with classic morphometrics, showing a better performance (Evin et al., 2008). Geometric morphometrics combines shape data from landmark coordinates located on points, curves and surfaces over biological structures with a statistical theory for shape analysis. This allows for the use of multivariate analyses to test biological hypotheses involving morphology while preserving the original geometry of the landmark configuration throughout the analyses and thus permits to represent statistical results as actual shapes (Mitteroecker and Gunz, 2009). Further, the expansion of 3D technology is making the acquisition of three-dimensional data cheaper and more widely available. This has meant the extension of geometric morphometrics to the study of objects for which this had not yet been possible (e.g. very small or large objects, or internal structures). More importantly, we are now able to investigate structures whose main characteristic is precisely their 3D configuration such as vertebrate skulls (Cardini, 2014) or hard invertebrate structures such as gastropod shells (Scalici et al., 2016).

The land snail genus *Pyrenaearia* is endemic to the northern Iberian Peninsula, where it shows a discontinuous distribution pattern across the Cantabrian Mountains, Pyrenees, pre-littoral mountain system of Catalonia and Mount Moncayo (Ortiz de Zárate, 1956; Puente, 1994). *Pyrenaearia* species live only on rock walls or under stones in mountainous areas mainly comprised of calcareous rocks. The genus species are also strictly confined to shady environments, and populations are thus limited to north-facing surfaces or very abrupt terrains and mountain cliffs (Prieto, 1986; Puente, 1994). This discontinuous distribution of their suitable habitat and the limited active dispersal capabilities of land snails, means that species occur in strict allopatry. Effectively, just two sympatric populations are known (Puente, 1994) suggesting that their speciation must have been mainly allopatric.

*Pyrenaearia* species lack diagnostic anatomical characters (Ortiz de Zárate, 1956). Thus, taxonomy of the genus has been based on shell characters such as shell size, colour and general shape, peristome colour and morphology, and the presence or absence of hairs and teeth (Ortiz de Zárate, 1956; Puente, 1994). However, many studies have shown that gastropod shell is highly correlated with environmental factors, giving rise to local adaptations, which may blur taxonomy (e.g. Fiorentino et al., 2008; Razkin et al., 2017; Stankowski, 2011, 2013). Further, incomplete lineage sorting may also be hindering species delimitation in *Pyrenaearia* because of the recent speciation of the genus (Elejalde et al., 2009).

As a first attempt to resolve species delimitation in *Pyrenaearia*, Elejalde et al. (2009) conducted a phylogenetic study of the genus based on two mitochondrial (*COI*, *16S rRNA*) and one nuclear (*ITS1*) gene fragments. They considered 11 species

within the genus. Four are endemic to the Cantabrian Mountains, *P. cantabrica* (Hidalgo, 1873), *P. oberthueri* (Ancey, 1884), *P. daanidentata* Raven, 1988 and *P. velascoi* (Hidalgo, 1867). Five species are endemic to the Pyrenees, *P. carascalensis* (Férussac, 1821), *P. cotiellae* (Fagot, 1906), *P. carascalopsis* (Bourguignat in Fagot, 1884), *P. parva* Ortiz de Zárate, 1956 and *P. organiaca* (Fagot, 1905). *P. molae* Haas, 1924 is endemic in the south of the pre-littoral mountain system of Catalonia. Finally, *P. navasi* (Fagot, 1907) is endemic to Mount Moncayo, the only siliceous location. The delimitation approach used by Elejalde et al. (2009) was limited to identifying monophyletic groups within the genus and comparing these with the nominal species recognized. However, their phylogeny was not fully resolved and the status of some of the species remains uncertain. Thus the phylogenetic relationships between *P. carascalensis*, *P. cotiellae*, *P. molae* and *P. velascoi* were not resolved and, although the last three species were recovered as monophyletic groups, *P. carascalensis* was found paraphyletic within the clade that joined these species. *P. cantabrica*, *P. daanidentata* and *P. oberthueri*, formed also a polytomy. In addition, the relationships between the four main well-supported clades that they recovered were not resolved.

The main objective of the present study was, therefore, to update the taxonomy of the *Pyrenaearia* genus through the combined use of new molecular markers and 3D geometric morphometrics techniques whose application has been limited in the study of molluscs. Our specific goals were: (1) to clarify the phylogeny of the genus, (2) to delimit species by integrating molecular and morphological data, (3) to examine how shell shape has evolved, and (4) to define the impacts of the characters used, morphological versus molecular, on species delimitation.

# 2. Materials and Methods

## 2.1. Taxonomic sampling

The sampling strategy was designed to include not only all the species within the genus but also the maximum molecular and morphological variability of each of them. Thus, apart from covering the complete distribution ranges of each species, we also tried including *Pyrenaearia* populations from different altitudes, cardinal orientation and type of substrate. Extending the samples examined by Elejalde et al. (2009), 132 specimens representing 61 localities preserved in 96% ethanol were included in the molecular analyses. In addition, 2-4 adult specimens from localities never cited before were stored in 70% ethanol for anatomical studies. Empty shells were also collected for morphometrics analyses. Only adult shells characterised by a dull peristome slightly reflected toward the umbilicus, were

selected for morphometry studies to avoid scoring shape changes associated with the ontogenetic process. A total of 133 shells were included in the geometric morphometrics analyses (shell number per species ranged from 8 to 21 except for *P. daanidentata* for which only 5 shells were available). Information about the specimens and shells is provided in **Supplementary Table S1** and **Supplementary Table S2**, respectively.

## 2.2. Analysis of DNA sequences

### 2.2.1. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from a piece of foot using the DNAeasy Tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer's guidelines. Two mitochondrial genes, the cytochrome c oxidase subunit I (*COI*) and 16S RNA ribosomal subunit (*16S*), were selected along with the nuclear rDNA gene cluster consisting of the 3´ end of the ITS1 region, the gene 5.8S, the complete ITS2 region and 5´ end of the large subunit 28S (*ITS1-5.8S-ITS2-28S*). PCR conditions for DNA amplification were as follows for all fragments: 96 ºC for 1 min, 35x (96 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 1 min) and 72 ºC for 10 min. The primers used are listed in **Supplementary Table S3**. PCR products were purified and sequenced at Macrogen Inc using ABI3730XL or ABI3700 sequencers. Previously available sequences were downloaded from GenBank.

### 2.2.2. Phylogenetic analyses

Each data set was aligned with the Probabilistic Alignment Kit algorithm (PRANK) (Löytynoja and Goldman, 2005) which is known to outperform other alignment methods for indel-rich sequences (Löytynoja and Goldman, 2008). For each codon position in COI, substitution saturation was assessed following the entropy-based information method (Xia et al., 2003) with DAMBE v.6.1.19 (Xia, 2013).

Both maximum likelihood (ML) and Bayesian inference (BI) were used on the concatenated dataset. The dataset was partitioned by genes, further dividing *COI* into three partitions according to codon positions. The best evolutionary models fitting each partition were selected applying the Akaike Information Criterion (AIC) (Akaike, 1974) as implemented in jModelTest v.2.1.6 (Darriba et al., 2012). ML analyses were conducted with the program GARLI v.2.01 (Zwickl, 2006). The most likely tree topology was inferred from 48 independent runs setting a random starting tree. The best tree (i.e. the one with the highest likelihood score) was used as starting tree in the bootstrap analyses comprising 2,000 pseudo-replicates. Based on the bootstrap analyses trees, a majority rule consensus tree was created with the SumTrees program of the DendroPy Phylogenetic Computing Library v.4.1.0

(Sukumaran and Holder, 2010) and support values were mapped to the best tree. MrBayes v.3.2.6 (Ronquist et al., 2012) was used for BI analysis running it for $20x10^6$ generations with a 25% burn-in value. Both GARLI and MrBayes analyses were implemented at CIPRES Science Gateway (Miller et al., 2010). The species *Cryptosaccus cabrerensis*, *Portugala inchota* and *Hygromia limbata* were used as outgroups.

### 2.2.3. Molecular species delimitation

Using multilocus sequence data, multispecies coalescent species delimitation methods accommodate lineage sorting due to ancestral polymorphism to estimate the probability of different species delimitation hypotheses in a Bayesian framework. Two of these methods were used to explore species limits: i) BPP v.3.1 program (Yang, 2015; Yang and Rannala, 2010) and ii) the STACEY package v.1.1.1 (Jones, 2017) for BEAST2 v.2.3.2 (Bouckaert et al., 2014). For ease of analysis, we avoid using 5.8S and 28S genes due to their low variability (i.e. only genes *COI, 16S*, *ITS1* and *ITS2* were used).

As BPP requires *a priori* assigning of sampled specimens to candidate species, two molecular species delimitation methods that do not require specifying group membership but that rely only on mtDNA were used to define the species hypothesis: i) Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) and ii) the Bayesian implementation of the General Mixed Yule Coalescent model (bGMYC) (Reid and Carstens, 2012). ABGD statistically infers the "barcode gap" that occurs between intraspecific and interspecific divergence of a distribution of pairwise distances and uses it to group sequences. The *COI* alignment with all the specimen sequences was uploaded at the ABGD webpage (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) and run with the default settings (Pmin = 0.001, Pmax = 0.1, Steps = 10, X (relative gap width) = 1.5) and Kimura 2-parameter distances calculated with TS/TV = 4.4 as inferred by PAUP* v4.0b10 (Swofford, 2002). The GMYC model introduced by Pons et al. (2006) combines a model of stochastic lineage growth (Yules model) with a coalescence null model to determine the point of transition from species-level to population-level evolutionary processes on an ultrametric tree in a maximum likelihood framework. Its Bayesian implementation (bGMYC) incorporates phylogenetic uncertainty by sampling over a posterior distribution of sampled trees. bGMYC analysis was conducted for the concatenated mitochondrial dataset (*COI+16S*) from which identical haplotypes were eliminated because zero length branches have been shown to over-split the results (Monaghan et al., 2009). The ultrametric trees were constructed with BEAST v.1.8.4 (Drummond et al., 2012) under a relaxed lognormal molecular clock. From the posterior tree distribution, 100 trees were evenly sampled for bGMYC. Parameter py2 was set to 1.3 and t2 to 50 while the starting parameters for the MCMC were fixed at 1, 0.5 and 25 for Yule, coalescent and threshold priors respectively; otherwise default parameters were used. bGMYC was run for 50,000

generations with a burn-in of 40,000. Comparing the results of these two analyses, a consensus delimitation scheme was inferred to guide multilocus delimitation analysis.

Because of computing limitations, for BPP we used a reduced sequence matrix of 46 specimens and the number of individuals was equally distributed throughout the candidate species. BPP has two options for species delimitation: one requires a guide species tree to scan different delimitation schemes while the other explores both different species delimitation models and different species trees. Species limits were explored using the same parameters for both options because the first is very dependent on the guide tree and often datasets contain more information about species delimitation than about their relationships (Yang, 2015). The guide species tree was estimated with the *BEAST option (Heled and Drummond, 2010) of the BEAST v.1.8.4 package. Analyses were carried out setting the gamma priors $\theta \sim G(2, 33)$ for ancestral population sizes and $\tau \sim G(2, 200)$ for the root age, after testing that the prior means were reasonable for the data by checking the posterior distribution created for the parameters under the coalescent model when the species phylogeny is fixed. The jrMCMC was run for 300,000 generations (sampling intervals of three) with a burn-in period of 30,000. To ensure that convergence was achieved, two independent runs for each of the rjMCMC algorithms (0 and 1) implemented in the program were performed for each analysis.

For STACEY analysis all the specimens except those with missing *loci* were included. One of the advantages of STACEY is that there is no need to *a priori* assign individuals to species. Accordingly, this analysis was conducted on the specimens clustered by population. Exceptionally, because some of the individuals from the same locality were not grouped in the same clusters in mtDNA clustering analyses, they were introduced separately as suggested by these methods. A single tree and a single relaxed lognormal molecular clock were specified for mitochondrial genes. Individual strict clocks and trees were defined for each nuclear marker. A Yule speciation tree prior with 0.008 *collapseheight* was set and two independent runs of $50x10^6$ generations with 20% burn-in were carried out. To assess the statistical support of species delimitation, the posterior tree distribution was analysed using SpeciesDelimitationAnalyser v.1.8.0 (Jones et al., 2015) raising *collapseheight* to 0.01. A similarity matrix was then constructed in R v.3.3.2.

## 2.3. Morphological analysis

### 2.3.1. Data acquisition

3D images of shells were captured with Nikon XT H 225 Computed Tomography (CT) equipment at Metrología Sariki S.A. For a target resolution of 0.038 mm, the scanning

space was restricted to 75x75x75 mm. This means that in each scan we could include all shells that fitted in this cube. To position the shells in the cube they were glued to sticks with their front view upwards and the sticks fixed to a polystyrene sheet. The applied X-ray power depends on target density so shells were separated accordingly. From the STL file returned by the CT, each shell was individualized using Meshlab v.1.3.3 (Cignoni et al., 2008; available at http://meshlab.sourceforge.net/).

The lack of discrete morphological characters on the shell only allowed us to define three homologous landmarks. Thus, to capture the whole shape, we placed equally spaced semilandmarks throughout two homologous curves; these were the suture (80 semilandmarks) and peristome outline (22 semilandmarks) (**Fig. 1, Supplementary Table S4**). Landmark digitalization was performed with the R package geomorph v.3.0.1 (Adams and Otárola-Castillo, 2013).

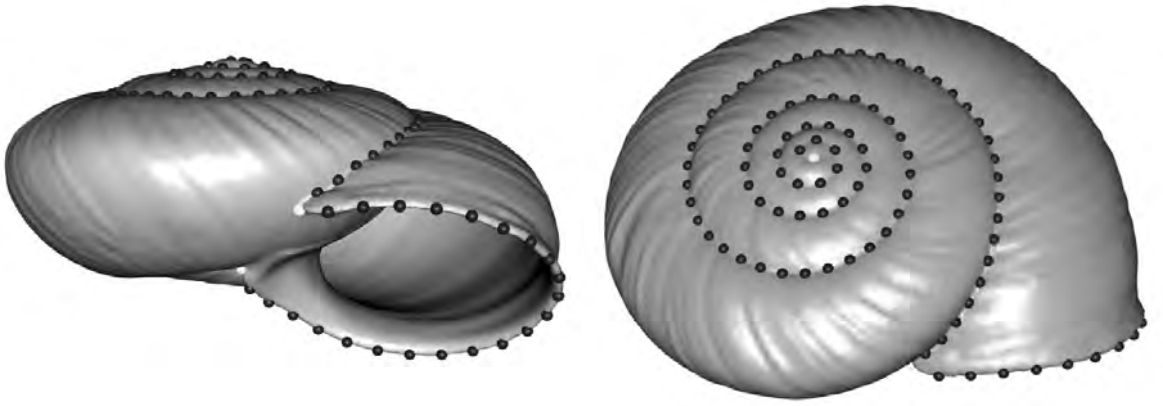
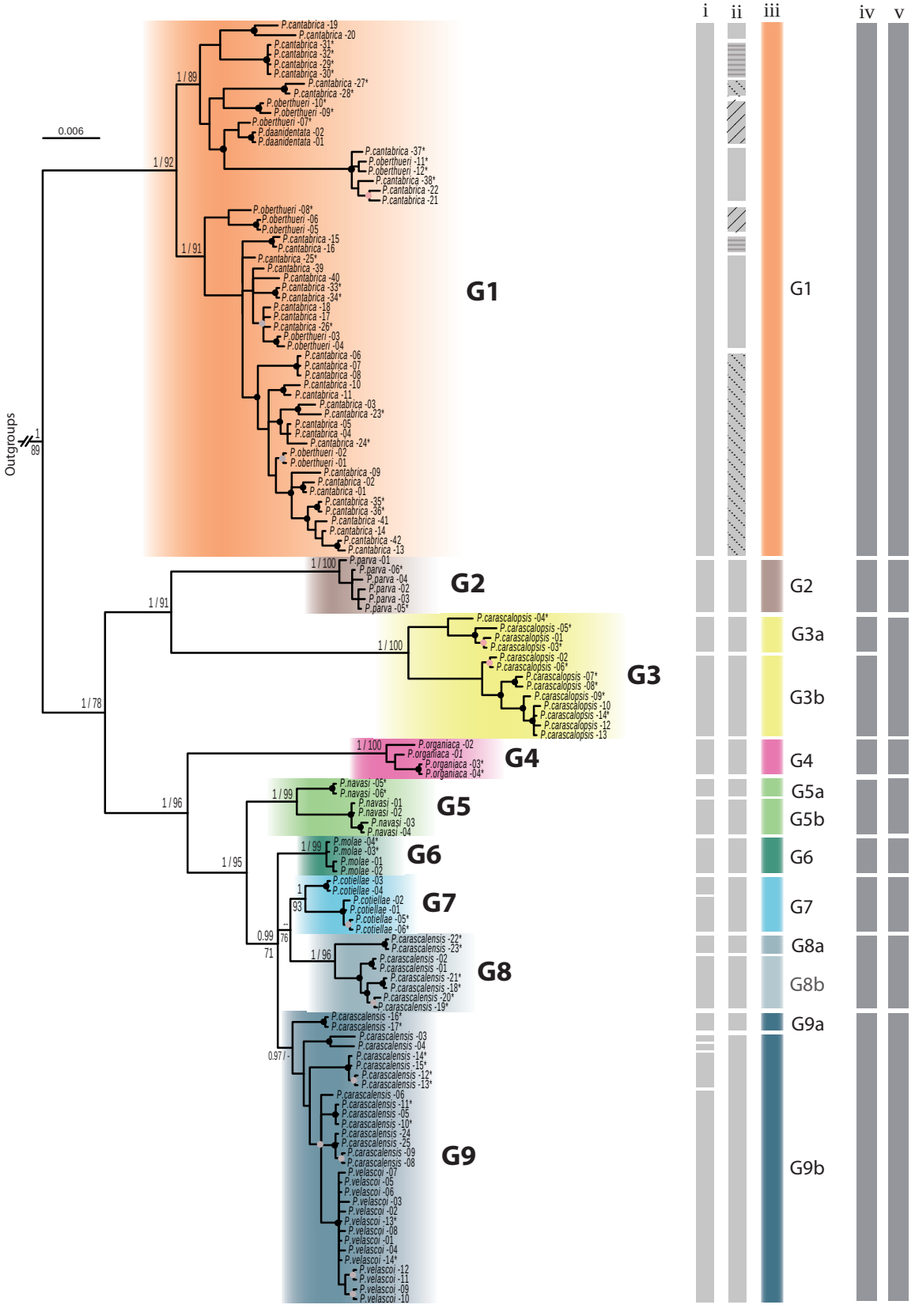


**Figure 1.** Placement of landmarks (white) and semilandmarks (dark grey) along the peristome and the suture on frontal (left) and superior (right) views.

### 2.3.2. Shape variation and morphological distinctiveness of groups

The resulting coordinate dataset was subjected to a full Procrustes fit and projection into tangent space as implemented in the R package Morpho v.2.4.1.1 (Schlager, 2016) to eliminate variation due to scaling (size), position and orientation and only shape variation was retained. Semilandmarks were allowed to slide along their tangent directions minimizing bending energy during superimposition as suggested by Gunz et al. (2005).

Principal component analysis (PCA) was used to reduce the dimensionality of shape space and to record the main patterns of variation in shape across the genus, as well as to *a priori* identify morphologically distinct groups. The shape change direction of the first principal components (PCs) was also visualized by warping the scanned surface of a shell to the extremes of each PC axis.


i
ii
iii
iv
v
0.006
Outgroups
G1
G2
G3
G4
G5
G6
G7
G8
G9
G1
G2
G3a
G3b
G4
G5a
G5b
G6
G7
G8a
G8b
G9a
G9b

Despite PCA provides some insight about morphologically differentiated groups, when variation within a group is anisotropic around the group mean, the distinctness of groups also depends on the directionality of variation within groups which is overlooked in PCA (Klingenberg and Monteiro, 2005). Hence, to test for shape differences between groups, canonical variate analysis (CVA) was used. CVA assumes that within-group covariance matrices are alike to maximize differences between groups relative to the variation within them. This is thus the best way to detect the morphological distinctiveness of groups (Klingenberg and Monteiro, 2005). We tested the groups that became identifiable in PCA along with the groups returned by the molecular species delimitation methods. Statistical significance of differences between groups was assessed via permutation test (1000 permutations) for Mahalanobis distances. All analyses were performed with Morpho.

### 2.3.3. Disparity comparisons

Variation in morphological disparity (i.e. the extent of morphological variation within a sample) was inspected using Procrustes variance as the disparity measure, which is the mean squared Procrustes distance of each specimen from the mean shape of the group (Zelditch et al., 2003). Differences among groups were statistically assessed through permutation test (1000 permutations). Both Procrustes variances and permutation test calculations were accomplished with geomorph software.

### 2.3.4. Allometry

To examine how size influences shape, multivariate regression of shape on centroid size was used and statistical significance was determined by permutation testing (1000 permutations). Preliminary results showed better linear relationship when centroid size was log-transformed so we used the transformed measure. Allometry was analysed for the whole genus, but to address if there was any evolutionary allometry, mean shapes of each of the groups delimited in previous analyses were also computed and regressed on their mean log-transformed centroid size. Shape changes associated with size were illustrated by warping a scanned surface to both size extremes.

We then calculated the angles formed between the different shape change vectors obtained from allometry regressions and PCA to determine whether there was correspondence

**Figure 2.** Phylogenetic tree of *Pyrenaearia* obtained by Bayesian inference from the concatenated dataset including *COI*, *16S* and the nuclear gene cluster *ITS1-5.8S-ITS2-28S*. Specimens' codes with asterisks indicate the new individuals added in this work comparing to Elejalde et al. (2009). Numbers on the nodes correspond to BI posterior probabilities and ML bootstrap values respectively. Dots on the nodes also indicate support information: black dots for nodes supported by BI > 0.95 and ML >70%, grey dots for BI > 0.95 and ML < 70% and pink dots for BI < 0.95 and ML > 70%. The tree is coloured to distinguish the nine main phylogroups. Bars on the right summarized species delimitation results: i) ABGD, ii) bGMYC (boxes with the same line pattern denoted clusters recovered in the bGMYC analysis not recovered together in the phylogeny), iii) consensus mtDNA delimitation with the candidate species to be tested by BPP, iv) BPP and v) Stacey.

between shape changes associated to them. As the direction of PC vectors is arbitrary (Klingenberg and Marugán-Lobón, 2013), angle calculation was limited to range from 0º to 90º. Statistical significance of the angles was estimated following Li (2011), who suggested that the probability of a random vector to form an angle with one of our vectors equal or less the angle formed between our two vectors can be estimated as the area of the cap defined by our angle divided by the area of the complete hypersphere.

# 3. Results

## 3.1. Gene sequences

The final concatenated data set comprised 2429 aligned characters for 132 individuals (GenBank accession numbers for each specimen are provided in **Supplementary Table S1**). No saturation was detected at any codon position within *COI* (index of substitution saturation was significantly lower than the critical substitution saturation index). Substitution models selected for each gene and each codon position in COI are provided in **Supplementary Table S5**.

## 3.2. Phylogenetic inference

The phylogeny recovered by phylogenetic analyses on the concatenated mitochondrial and nuclear dataset is shown in **Figure 2.** The ML analyses results mirrored those obtained through BI such that the topology of the phylogeny is based only on BI. However, both BI posterior probabilities and ML bootstrap values are indicated at the nodes clades when BI ≥ 0.95 and ML ≥ 70%. Our results recovered nine well supported clades (groups 1-9) and provided good support for most relationships among them. **Figure 3** shows sampled localities coloured according to the nine main phylogroups. A tree based on mitochondrial information and another one based on nuclear markers have been included separately as supplementary information (**Supplementary Fig. S1** and **Fig. S2** respectively).

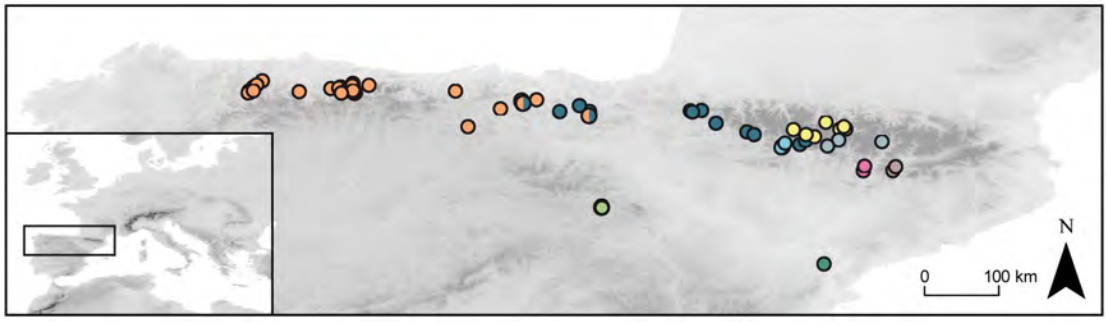



**Figure 3.** Location of the sampled populations. Colours correspond to the 9 main phylogroups obtained in the phylogeny (**Fig. 2**).

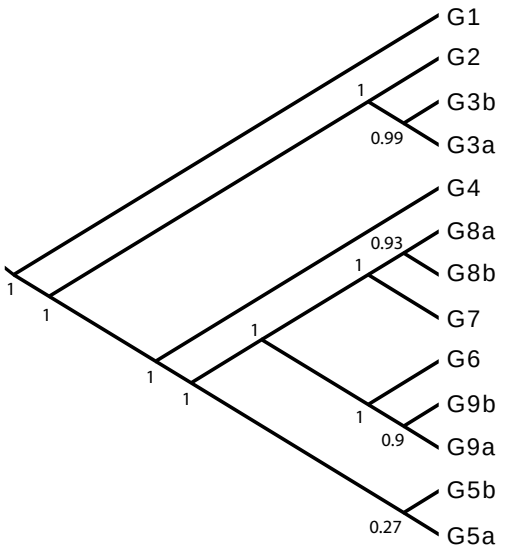



**Figure 4.** BPP results obtained using the guide species tree obtained with *BEAST for the candidate species recovered in mtDNA delimitation analyses. Species split probabilities are provided in each node.

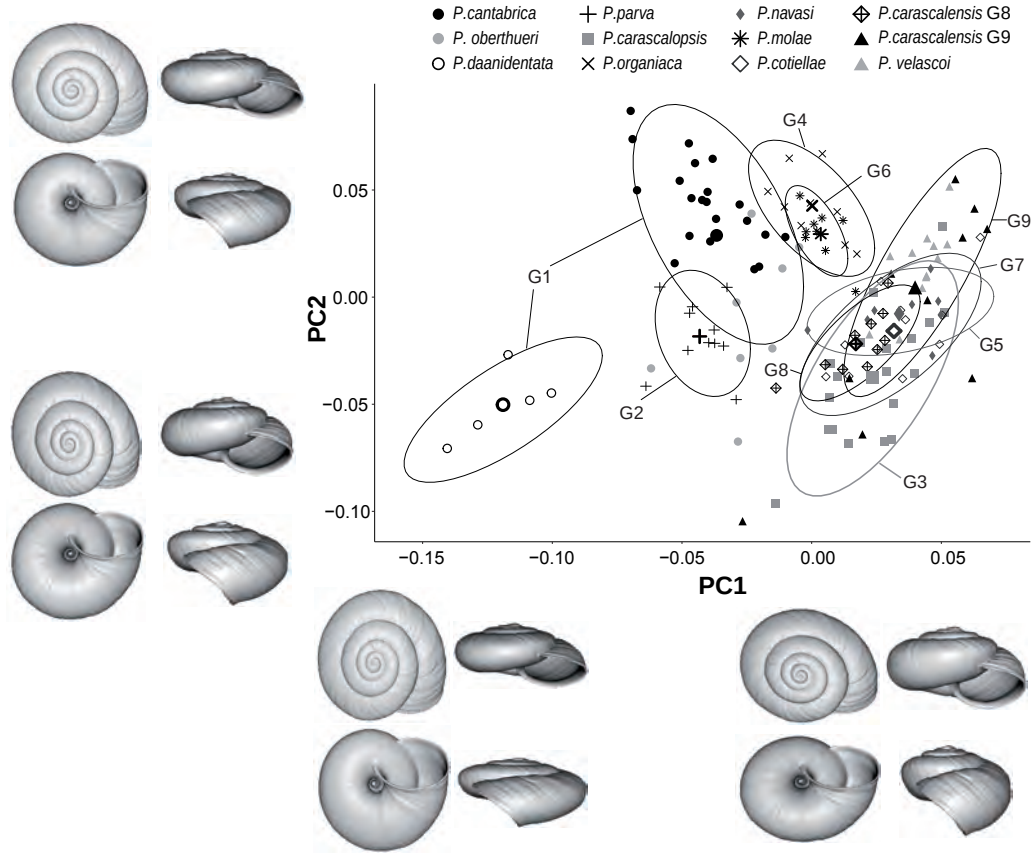



**Figure 5.** First two dimensions of the PC scores and shape change representations associated to each of them. Each symbol represents the different nominal species according to Elejalde et al. (2009), although to point out the two groups molecularly found for *P. carascalensis*, they are provided with different symbols. Confidence ellipses given a 0.9 probability are indicate for the species delimited molecularly but G1 is further splitted into two to highlight the morphological distinctiveness of *P. daanidentata*. For each of these groups, the centroid is also denoted with bold bigger symbols.

Group 1 was strongly supported by both analyses (BI = 1; ML = 92), and turned out to be the sister group of the rest. This group included only samples from the Cantabrian Mountains identified as *P. cantabrica*, *P. oberthueri* and *P. daanidentata*. Hereafter the group is referred to as the Cantabrian clade. The group was further divided into two supported sister clades. However, *P. cantabrica* and *P. oberthueri* appeared spread over both clades and also mixed within each of them and hence these species appear as paraphyletic. The specimens of *P. daanidentata* formed a well supported monophyletic group in one of the two clades contributing to the paraphyly of both *P. oberthueri* and *P. cantabrica*.

Groups 2 and 3 were fully supported (BI = 1; ML = 100) and recovered as sister groups. Group 2 comprised only *P. parva* while Group 3 was formed by *P. carascalopsis*. The clade joining G2 and G3 was sister to a highly supported clade grouping Groups 4 to 9 (BI = 1; ML = 96). Group 4 contained *P. organiaca* and was sister to a clade joining G5 to G9. Group 5 gathered *P. navasi* and was sister to Groups 6, 7, 8 and 9 which formed a polytomy. Group 6 enclosed only *P. molae*. Groups 7 and 8 were recovered as sister groups, but only ML analyses supported their relationship (ML = 76). Group 7 included *P. cotiellae* and Group 8 *P. carascalensis* from the east of its distribution range. Both BI and ML analyses recovered Group 9, but it was supported only by the former (BI = 0.97). This last group comprised both *P. velascoi* and western and central *P. carascalensis* populations.

**Table 1.** Stacey delimitation results for the 9 best schemes obtained.

| Count | Posterior probability | n clusters | Clusters | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1004 | 0.06 | 9 | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | | |
| 544 | 0.03 | 8 | G1 | G2 | G3 | G4 | G5 | G6+G9 | G7 | G8 | | | |
| 527 | 0.03 | 10 | G1 | G2 | G3 | G4 | G5a | G5b | G6 | G7 | G8 | G9 | |
| 392 | 0.02 | 10 | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9a | G9b | |
| 374 | 0.02 | 9 | G1 | G2 | G3 | G4 | G5 | G6+G9a | G7 | G8 | G9b | | |
| 311 | 0.02 | 10 | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8a | G8b | G9 | |
| 269 | 0.01 | 10 | G1.1 | G1.2 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | |
| 246 | 0.01 | 11 | G1 | G2 | G3 | G4 | G5a | G5b | G6 | G7 | G8 | G9a | G9b |
| 192 | 0.01 | 11 | G1 | G2 | G3 | G4 | G5a | G5b | G6 | G7 | G8a | G8b | G9 |
| … | | | | | | | | | | | | | |

## 3.3. Molecular species delimitation

### 3.3.1. BPP

The results of the two methods used to set the candidate species to be tested by BPP as well as the consensus achieved by comparing them are summarised in **Figure 2**. For ABGD, different prior maximal distances (*P*) yielded different numbers of groups: 31 (*P* = 0.001000), 31 (*P* = 0.001668), 17 (*P* = 0.002783), 17 (*P* = 0.004642), 6 (*P* = 0.007743), 6 (*P* = 0.012915), 5 (*P* = 0.021544) and 1 (*P* = 0.035938). Following

Puillandre et al. (2012), geographical distribution and phylogenetic relationships were used as independent data to choose among the different partitions recovered by the method, and we considered the result with 17 groups ($P = 0.004642$) as the most plausible. Instead, bGMYC recovered 18 groups whose members showed a posterior probability ≥ 0.95 of belonging to the same species. Next, both sets of results were compared to build a congruent consensus candidate species scheme. Consensus candidate species matched the main phylogenetic groups except for groups G3, G5, G8 and G9 which were each split into two.

BPP analyses with and without a guide species tree returned the same delimitation scheme: of 13 candidate species tested, G5a and G5b were not supported and grouped together as also observed for candidate species G8a and G8b, and G9a and G9b; the rest were strongly supported and 10 species were delimited. In both analyses, convergence was achieved as the two independent runs for each of the algorithms (0 and 1) yielded the same results. **Figure 4** shows the results obtained with guide tree. Species posterior probabilities from analyses without guide tree are provided in **Supplementary Table S6**.

### 3.3.2. Stacey

**Table 1** lists the nine best clusterings obtained with the Stacey software. The clustering with the highest posterior probability (PP = 0.06) delimited nine species that perfectly matched the nine main phylogroups recovered. Posterior probabilities values for the two subsequent clusterings dropped to half, and for the remaining clusterings, probabilities were further reduced. When inspecting the similarity matrix (**Supplementary Fig. S3**), the assignment of individuals (grouped by population) to the nine groups was supported with high posterior probabilities (PP ≥ 0.90) in most cases.

**Table 2.** Mahalanobis distances between the 10 groups suggested by molecular and morphological evidence. Statistically significant differences are indicated by asterisks: '*' $p < 0.05$ and '**' $p < 0.01$.

| Tested groups | **G1a** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **G1b** | 22.744** | **G1b** | | | | | | | |
| **G2** | 12.739** | 26.446** | **G2** | | | | | | |
| **G3** | 15.689** | 27.088** | 17.835** | **G3** | | | | | |
| **G4** | 11.873** | 26.180** | 15.711** | 15.036** | **G4** | | | | |
| **G5** | 17.292** | 29.115** | 15.644** | 11.368** | 18.009** | **G5** | | | |
| **G6** | 11.403** | 26.972** | 15.695** | 11.599** | 12.303** | 13.958** | **G6** | | |
| **G7** | 16.594** | 29.980** | 18.839** | 9.738** | 15.098** | 15.435** | 14.419** | **G7** | |
| **G8** | 13.983** | 29.860** | 14.689** | 11.098** | 16.251** | 11.677** | 11.374** | 12.982** | **G8** |
| **G9** | 14.595** | 28.731** | 17.523** | 7.090** | 14.003** | 12.382** | 10.499** | 7.549* | 9.744** |

## 3.4. Shape variation and morphological distinctiveness of groups

The first three PCs accounted for 71.6% of the total shape variation in *Pyrenaearia*. PC1 and PC2 described the highest amount of variation, 33.7% and 28.0% respectively, while PC3 explained 9.9% of the variation. Each of the remaining PCs (PC4 to PC132) contributed negligible amounts to the shape variation, less than 6.5% each of the PCs from PC4 to PC12 and less than 0.5% each of the PCs from PC13 to PC132.

**Figure 5** shows the scatter plot of the first two principal components scores and illustrates the shape changes associated with them. Shape change in the first PC was related to the shell tube section increment through coiling and shell contour (i.e. shell perimeter in superior and inferior views). Negative change from the mean shifted

**Table 3.** Procrustes variances for the 10 groups suggested by molecular and morphological evidence and pairwise differences between them. Statistically significant differences are indicated by asterisks: '*' $p < 0.05$ and '**' $p < 0.01$.

| **Within group variances** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Procrustes variance | | Pairwise differences | | | | | | | | | |
| **G1a** | 0.0037 | | **G1a** | | | | | | | | |
| **G1b** | 0.0025 | **G1b** | 0.00111 | **G1b** | | | | | | | |
| **G2** | 0.0029 | **G2** | 0.00074 | 0.00037 | **G2** | | | | | | |
| **G3** | 0.0027 | **G3** | 0.00099 | 0.00012 | 0.00025 | **G3** | | | | | |
| **G4** | 0.0016 | **G4** | 0.00202* | 0.00091 | 0.00128 | 0.00103 | **G4** | | | | |
| **G5** | 0.0012 | **G5** | 0.00250* | 0.00139 | 0.00176 | 0.00151 | 0.00048 | **G5** | | | |
| **G6** | 0.0010 | **G6** | 0.00269** | 0.00158 | 0.00195 | 0.00170 | 0.00067 | 0.00019 | **G6** | | |
| **G7** | 0.0017 | **G7** | 0.00200* | 0.00089 | 0.00126 | 0.00101 | 0.00002 | 0.00050 | 0.00069 | **G7** | |
| **G8** | 0.0011 | **G8** | 0.00256** | 0.00145 | 0.00182 | 0.00157 | 0.00054 | 0.00006 | 0.00013 | 0.00056 | **G8** |
| **G9** | 0.0032 | **G9** | 0.00049 | 0.00062 | 0.00025 | 0.00050 | 0.00153 | 0.00201* | 0.00220* | 0.00151 | 0.002 |
| **Group variances with respect to total mean** | | | | | | | | | | | |
| Procrustes variance | | Pairwise differences | | | | | | | | | |
| **G1a** | 0.0059 | | **G1a** | | | | | | | | |
| **G1b** | 0.0206 | **G1b** | 0.01471** | **G1b** | | | | | | | |
| **G2** | 0.0060 | **G2** | 0.00008 | 0.01463** | **G2** | | | | | | |
| **G3** | 0.0048 | **G3** | 0.00115 | 0.01585** | 0.00123 | **G3** | | | | | |
| **G4** | 0.0049 | **G4** | 0.00102 | 0.01573** | 0.00110 | 0.00013 | **G4** | | | | |
| **G5** | 0.0029 | **G5** | 0.00299 | 0.01769** | 0.00306 | 0.00184 | 0.00197 | **G5** | | | |
| **G6** | 0.0026 | **G6** | 0.00330* | 0.01801** | 0.00338 | 0.00215 | 0.00228 | 0.00031 | **G6** | | |
| **G7** | 0.0032 | **G7** | 0.00271 | 0.01742** | 0.00279 | 0.00156 | 0.00169 | 0.00028 | 0.00059 | **G7** | |
| **G8** | 0.0026 | **G8** | 0.00330** | 0.01800** | 0.00338 | 0.00215 | 0.00228 | 0.00031 | 0.00000 | 0.00059 | **G8** |
| **G9** | 0.0049 | **G9** | 0.00107 | 0.01577** | 0.00115 | 0.00008 | 0.00005 | 0.00192 | 0.00223 | 0.00164 | 0.002 |

towards constant increment of the shell tube section and circular contour, which translated into a sharp angle for the aperture with respect to coiling (visible in lateral view) and flattened shells. In the positive direction, shells showed a logarithmic increment of the shell tube section through coiling and an elliptic perimeter, being more globose and with a less sharply angular aperture. PC2 described changes relative to aperture shape. In the negative direction, apertures were rounded and oblique and shells displayed a wide and globose last whorl. Towards the positive direction, the shells featured apertures elongated perpendicularly to the coiling axis, with a narrow, slightly angular last whorl.

Taking into account 0.9 confidence interval around groups' centroids, PCA recovered five different groups. Individuals belonging to *P. daanidentata* formed morphologically identifiable and well separated group. *P. cantabrica* and *P. oberthueri* formed a continuous group that overlapped with *P. parva* slightly. *P. molae* and *P. organiaca* specimens overlapped largely, yet together they formed a conspicuous group that marginally overlapped with *P. cantabrica*. Specimens identified as *P. cotiellae*, *P. carascalensis* (from both G8 and G9), *P. velascoi*, *P. carascalopsis* and *P. navasi* were indiscernible and gathered together in a group separated from the rest.

CVA was used to test the morphological distinctiveness of the 9 main phylogroups recovered as they were proposed as different species by molecular delimitation methods. Additionally, G1 was split to test *P. daanidentata* as a separate group as

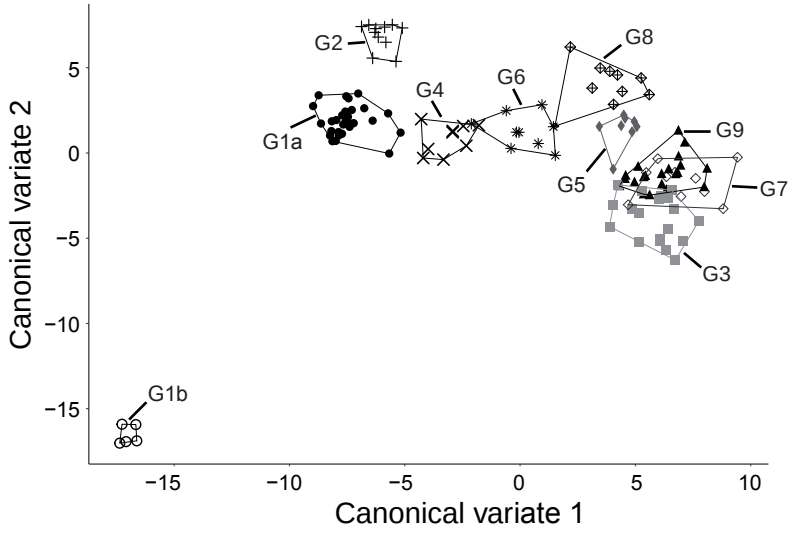



**Figure 6.** Canonical variate analysis for the 10 groups suggested by molecular and morphological evidence. Each group is represented by unique symbol and independent convex hull.

clearly suggested by PCA (G1a contained *P. cantabrica* and *P. oberthueri* specimens; G1b *P. daanidentata* individuals). The analysis showed significant Mahalanobis distances among these 10 groups, indicating they were different from each other (**Table 2**, graphically displayed in **Fig. 6**).

## 3.5. Disparity

Intra-group disparity and disparity of groups in relation to genus mean was inspected for the 10 groups obtained combining molecular and morphological information. Procrustes variances for each of the 10 delimited groups and the pairwise differences between them are provided in **Table 3**. For within group disparity, G1a and G9 showed the highest variation; G1b, G2 and G3 displayed medium variability while the amount of shape variation of G4, G5, G6, G7 and G8 was clearly low. Results showed that G1b was the group that deviated most from the genus mean and the only one that did it significantly.

## 3.6. Allometry

Multivariate regression of shape on log centroid size revealed a clear allometry within the genus and also a strong evolutionary allometry: for the entire genus 16.8% of shape variation was explained by size (**Fig. 7a**) and this value increased to 37.7% when evolutionary allometry was considered (**Fig. 7b**), both being statistically significant ($p < 0.001$ and $p < 0.01$ respectively). It was also observed that G1b formed a group that deviated from the general trend.

Shape change related to general allometry seemed to resemble the change associated with evolutionary allometry (**Fig. 7c**). This similarity was confirmed by the angular comparison as the angle between the two allometric regression vectors was of 16.1º ($p = 0$). Moreover, the representations of the shape change associated to allometry shown in **Figure 7c** were similar to the PC1 illustrations (**Fig. 4**). Also, when calculating the angle they formed between the allometry vectors and PC1 vector, their similarity proved significant (angle between general allometry vector and PC1 vector 19.7º, $p = 0$; angle between evolutionary allometry vector and PC1 vector 22.4º, $p = 0$). However, while PC1 explained 33.7% of the total shape variation, allometric regression only accounted for 16.8%. This indicates that although allometry seemed to be an important factor for shape change, other processes must have also played a role in its diversification.

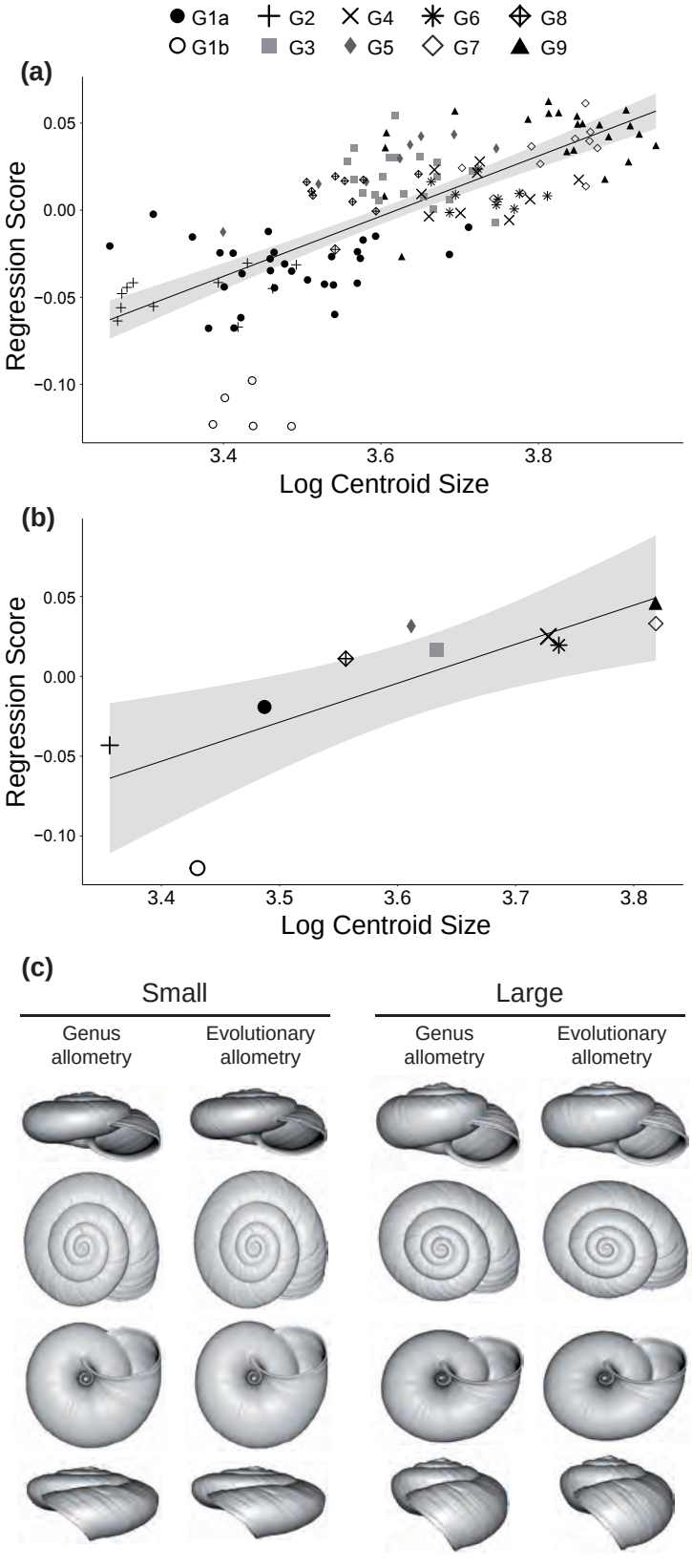



**Figure 7.** Allometry as characterized by the regression of shape on log centroid size.

(a) Allometry within the genus.

(b) Evolutionary allometry.

(c) Shape changes associated to allometry.

# 4. Discussion

## 4.1. Species delimitation

In this study, we have addressed for the first time species delimitation in the land snail genus *Pyrenaearia* using an integrative approach combining molecular information with 3D morphological data.

The two molecular delimitation methods yielded the same delimitation scheme in which the nine main phylogroups obtained were recovered as species. The only exception was G3 which was supported as a single species by Stacey and split into two by BPP. Owing to computation limitations, we used a reduced data matrix for BPP and, specifically, five G3 individuals out of 13 were excluded. The drawback of this approach is that, in the selection process, the most divergent individuals may be retained and this can generate spurious genetic discontinuity. Indeed, sampling gaps are known to hinder genealogical interpretations (Lohse, 2009; Niemiller et al., 2012). The fact that Stacey, which includes more specimens, did not detect differentiation between the two groups may be suggesting that the genetic discontinuity detected by BPP may have been an artefact arising from the exclusion of some individuals. Indeed, when we re-run Stacey using the same reduced matrix as in BPP, we obtained the same results as in BPP with G3 splitted into two (**Supplementary Table S7**) confirming that the differentiation was a sampling artefact.

Shell shape variation within the genus occurred mainly along two directions. Major shell variation, as depicted by PC1, was primarily related to shell tube section increment through coiling. The shells of *Pyrenaearia* also differed considerably in aperture shape which entails differences in spire height (PC2 accounts for 28% of the total variation) and along this change we found a gradient from rounded apertures and globose shells to elongated apertures and fairly flattened shells. These shell shape characters, with no further information, served to delineate five groups that did not fit the main phylogroups except for G2. However, some morphological patterns could be inferred from PC1. All the PC1 scores of clade G1 were below -0.005 so that they occupied the shape space towards constant increment in the shell tube. G2 also displayed negative scores from -0.064 to -0.029. The rest of the phylogroups (G3-9) exhibited PC scores above -0.017 (except for one clear outlier of G9 whose value was of -0.027) and, therefore, these groups show a morphological gradient from slightly constant increment of the shell tube to a more logarithmic increment with the majority of the individuals occupying the shape space towards logarithmic increment. This means that despite there is an overlapping zone between -0.017 to -0.005, the increment through coiling of the shell tube section is able to discern the two main *Pyrenaearia* clades with the exception of G2. Otherwise, aperture shape

and spire height variation did not define obvious groupings. Shells seemed highly polymorphic for this character and phylogroups were distributed across all the shape space, although some displayed less variation than others. A high variability in spire height has been reported in other land snails (Cain, 1977; Fiorentino et al., 2008; Razkin et al., 2017; Stankowski, 2011).

Surprisingly, within phylogroup G1, for which the molecular data found no genetic discontinuities, individuals belonging to the nominal species *P. daanidentata* formed a clearly distinguishable group. Further, this morphological entity appeared the most different within the whole genus with a Procrustes variance in relation to the genus mean of 0.0206, which is 3.5 times larger compared to the next most divergent group. This great deviation in shape may be reflecting that *P. daanidentata* is indeed a valid species and, instead, our failure to detect the species using molecular data could be the result of stochastic sorting of ancestral polymorphisms or hybridization which is common in recent speciation events (Edwards and Knowles, 2014) or because of the use of neutral loci in a case of selective speciation (Solís-Lemus et al., 2015). Further, according to raw morphology data, G3, G5, G7, G8 and G9 were indistinguishable from each other as well as G4 from G6. However, when we integrated molecular and morphological data taking into account the shape change direction according to the groups obtained molecularly, morphological differences were found among all of them, providing further support to the differentiation between these phylogroups. Hence, intra-specific shape variation in some of *Pyrenaearia* phylogroups gives rise to overlapping species shell morphology, but the way in which this variation occurs within each group in terms of its directionality and extension differs thus making them distinguishable.

## 4.2. Proposed taxonomic revision

Using our integrative approach to species delimitation, 10 species were identified in *Pyrenaearia* and we propose a taxonomic revision of the genus.

The nominal species *P. cantabrica*, *P. oberthueri* and *P. daanidentata*, endemic to the Cantabrian Mountains, were recovered by molecular delimitation methods as a single species (G1). However, morphological evidence undoubtedly supports the specific validity of *P. daanidentata*. The distinction between *P. cantabrica* and *P. oberthueri* has been repeatedly questioned and rather suggested that morphological variation could be reflecting an adaptive shape change following an altitudinal cline (Elejalde et al., 2009; Ortiz de Zárate, 1956; Raven, 1988). Our results support this interpretation. Morphologically the species form a continuous group showing the same range of values for PC1 scores. However, along PC2, the *P. oberthueri* specimens display lower scores and those of *P. cantabrica* show higher scores but with no break between them. This means their rate of shell tube section increment

through coiling is the same but there is a continuous gradient from globose to flat shells. Moreover, this gradient partially matches altitude: individuals from high altitudes are more globose and those of lower-lands are flattened. In view of our molecular and morphological results, we propose these two species should no longer be considered as separate lineages but as a single species distributed across the Cantabrian Mountains under the name of *P. cantabrica*, which has priority over *P. oberthueri*. In contrast, the morphological distinction of *P. daanidentata* does not fit within this adaptive clinal shape variation following altitude. *P. daanidentata* is only known from its type locality "*Collado del Perro – Canal del Burro*" and besides a differentiated shell shape, it features a strong tooth in the peristome which is unique in the whole genus (Raven, 1988). These morphological differences could be the result of either intrinsic or extrinsic barriers to reproduction and fine-scale niche partitioning, or allopatric speciation, resulting from geographical isolation. The reduced genome fraction analysed here may not be enough to support the divergence of *P. daanidentata* that morphology seems to be indicating (Edwards and Knowles, 2014), being in addition the responsible for *P. cantabrica* appearing as paraphyletic. We advocate that this species should maintain its species status thought the prove of its molecular differentiation awaits a much extended molecular dataset.

*P. parva* (G2), *P. organiaca* (G4) and *P. molae* (G6) emerge here as valid species. *P. parva* was separately supported by molecular and morphological analyses. *P. organiaca* and *P. molae* were recovered as species by molecular methods and morphologically they were different from the rest of *Pyrenaearia* species but from each other they were only distinguishable when the direction of shape change was considered. Besides, these three species can be additionally separated according to their characteristic shell striation patterns and well defined geographical distributions (Fagot, 1905; Haas, 1924; Ortiz de Zárate, 1956; Puente, 1994; Vilella, 1963).

The remaining five species that make up the *Pyrenaearia* genus were clearly delimited by molecular methods and further supported by morphological information. However, morphological differentiation between these species was only detected when the direction of shape variation within the species was considered because, due to intra-specific shape variation, their morphology overlaps. Three of these species matched the nominal species *P. cotiellae* (G7), *P. carascalopsis* (G3) and *P. navasi* (G5) and, thus, should retain their species status. *P. cotiellae* is restricted to Cotiella Massif while *P. carascalopsis* is distributed solely in the north watershed of the central Pyrenees. The Pyrenean watershed has been also identified as a factor determining the distribution range of other land snail species (Gittenberger, 1973; Kokshoorn et al., 2010). Within *P. navasi*, which apart from the characters here investigated can also be differentiated according to its brown and fragile shell, Prieto (1991) considered two subspecies: the smaller *P. navasi navasi* thriving on rock outcrops

above 1600 m, and *P. navasi sylvatica* which is larger and more globose and whose single population inhabits a beech/oak forest a completely atypical habitat for the genus. Molecular methods found no evidence of any split between these subspecies as *P. n. sylvatica* specimens (*P. navasi*-03 and *P. navasi*-04) where nested within *P. n. navasi* individuals in clade G5. Unfortunately, despite our intense sampling of the exact type locality and its surroundings, we found no shells of *P. n. sylvatica* for morphometrics studies and its shape distinctiveness could not be assessed. Prieto (1991) suggested that the subspecies evolved in response to a Quaternary glacial retreat. However, its lack of molecular differentiation, the fact that we found no additional specimens in the type locality, and its atypical habitat, make us suspect that the population may have been founded by some individuals falling from the population living at higher altitudes and that it may have become extinct.

Lineages G8 and G9 were also recovered as species. While G8 comprised *P. carascalensis* individuals living at the east of Noguera Ribagorçana Valley, G9 included the *P. carascalensis* populations distributed in the Pyrenees at the west of Noguera Ribagorçana Valley along with *P. velascoi* inhabiting the Basque Mountains. The exact location of the type locality of *P. carascalensis*, "*Forêt de Carascal en Aragon*", is unknown (Prieto, 1986; Puente, 1994). Nevertheless, all populations of phylogroup G8 were located outside Aragón province. Thus, the name *P. carascalensis* should be restricted to the G9 phylogroup. *P. velascoi* specimens also grouped together in G9, and therefore the name *P. velascoi* should be incorporated to the synonymy of *P. carascanlesis* as the name *P. velascoi* is subsequent to it. There is no available name for the so far overlooked lineage G8, because the specimens belonging to this phylogroup have always been included within *P. carascalensis* or *P. carascalopsis* (Altimira, 1965, 1994; Puente, 1994). Therefore, we have tentatively called this new cryptic species *Pyrenaearia* sp. G8, pending its formal description (in prep.).

## 4.3. Shell characters in *Pyrenaearia*

In this study, we have assessed morphological variation in snail shells through 3D geometric morphometrics instead of performing a 2D analysis of a predominantly three-dimensional structure. In geometric morphometrics, 3D structures have been often examined with 2D images. Indeed, most geometric morphometrics studies of mollusc shells have been based on 2D images and rarely have 3D images been used (Márquez and Averbuj, 2017; Márquez et al., 2011; Scalici et al., 2016). Addressing a 3D structure using 2D images will inevitably involve a loss of information and some inaccuracy in estimating size and shape (Cardini, 2014). Comparisons between results obtained for 2D and 3D data on skulls show that the former involves some extent of inaccuracy, which makes the approach unsuitable for intra-specific data (or also for cryptic species complexes) while for inter-specific data, caution

is required (Cardini, 2014). Moreover, even for the relatively flat mouse mandible, a 3D approach performs better than 2D (Navarro and Maga, 2016). In molluscs, Scalici et al. (2016) found that a 2D approximation was insufficient to capture shape differences between two mussel populations from two environmentally different locations, whereas their 3D dataset was capable of doing so. In our case, we have not carried out a comparison between 3D and 2D approaches and we cannot conclude if there are significant differences on performance for species discrimination between them. However, there is no doubt that the shape information captured by our 3D landmarks and semilandmarks approximates more to the real shape of shells than 2D data will ever do.

Morphological variation may arise from genetic differences product of population history or from selection in different environments. For gastropod shells, the action of both population history (Dillon and Jacquemin, 2015; Dowle et al., 2015) and adaptation to local environment (Stankowski, 2011; Welter-Schultes, 2000) have been reported as drivers of morphological variation. Indeed, in most cases, a combination of both is thought to determine final shape, the contribution of each factor depending on the species (Noshita et al., 2016). In *Pyrenaearia*, the presence of the morphologically clearly distinct *P. cantabrica* and *P. carascalensis* living in sympatry at least in two locations of the Basque Mountains, strongly suggests a genetic component to shell shape. In the same way, the capacity of the pattern of shell tube section increment through coiling to distinguish between the two main clades, also suggests a role of genetic factor on that characteristic. Further, we found that disparity within species was related to the breadth of their distributions, such that those with a wider distribution range showed greater variability than those with a more restricted distribution. In addition, we determined that *P. cantabrica* shows a clinal shape variation following altitude. These evidences may be suggesting that shell shape is also the result of some degree of local adaptation to the environment.

In this study, we also examined whether size affects shell shape in the genus and we found that allometry accounts for 16.8% of the shape variation and evolutionary allometry for 37.7%. These values are within the top of the range of proportions of shape variation affected by allometry reported for other groups (Cardini et al., 2015; Figueirido et al., 2010; Gonzalez et al., 2011; Klingenberg and Marugán-Lobón, 2013). Our high percentages are consistent with the results of Urdy et al. (2010) who modelled shell morphogenesis using a free-form vector model and concluded that shell shape variation in snails is highly affected by size. We observed that the change attributed to allometry resembled the change associated with PC1. Allometry is thus one of the factors affecting shape differences within the genus. However, the shape variation explained by PC1 was larger than the proportion explained by allometry, and therefore, other processes must also have contributed to shell diversification.

The deviation shown by *P. daanidentata* from the general allometric trend detected provides further support for this interpretation.

## 4.4. Phylogeographic patterns

A good delimitation scheme and a solved phylogeny are prerequisites to infer the evolutionary processes that organisms go through. Here, throughout the use of an integrative species delimitation approach and extending the sampling effort of Elejalde et al. (2009) as well as adding new nuclear DNA information, we have proposed a new species delimitation scheme for *Pyrenaearia* and gained resolution in its phylogeny, especially for basal nodes.

The genus *Pyrenaearia* has been traditionally considered cold-adapted because most of its species show a sub-Alpine distribution (Ortiz de Zárate, 1956; Prieto, 1986; Puente, 1994). Surprisingly, we have observed here that *P. cantabrica* and *P. carascalensis* thrive across wide altitude ranges (from 50 m to 2200 m and from 460 m to 2640 m respectively). Further, *P. molae,* which lives at 900 m but in places where summer temperatures easily reach 27 ºC (inferred from WorldClim database of Hijmans et al., 2005) and *P. organiaca,* which appears at 500 m, did not form a monophyletic group. Then, the low temperatures associated to high altitudes are not mandatory for some *Pyrenaearia* species, as long as shady rock outcrops are available. Therefore, despite being a cold-adapted taxon, this genus displays certain heat-tolerance and, instead, the availability of shady rock outcrops may be a more important factor shaping the genus' distribution.

According to the divergence time estimates calculated by Elejalde et al. (2009) using general diversification rates for molluscs, the split between the four main clades that they recovered, which coincide with our G1, G2, G3 and G4-9, would have predated the Pliocene, while they suggest that speciation within these clades was shaped by glacial cycles of the Pleistocene. An important role of glaciations in the speciation within the genus has also been proposed by Prieto (1991). Indeed, it has been shown that Pleistocene climate changes have contributed to the speciation of many mountain-dwelling organisms (Bidegaray-Batista et al., 2014; Dépraz et al., 2008; Gittenberger et al., 2004; Harl et al., 2014a ; Mouret et al., 2011; Pauls et al., 2006). Nevertheless, the close relationship we detected between the Pyrenean *P. cotiellae*, *P.* sp. G8 and *P. carascalensis* with the geographically distant *P. molae* of Tarragona may be suggesting that the establishment of *P. molae* could be the result of a passive dispersal event, as has been described for other terrestrial gastropods (Gittenberger et al., 2006).

Surprisingly, just two species are restricted to the Cantabrian Mountains while there are six in the Pyrenees, though the area of both mountain ranges is similar

and both have been extensively glaciated during Quaternary (Hughes et al., 2006). Calcareous rocks occupy vast areas of the Cantabrian Mountains and, arising from the erosion of glaciers and torrents, there are numerous narrow, steep valleys and many rocky walls spread across these calcareous areas (Gutiérrez-Elorza, 1994). This means that there are plenty of suitable habitats for *Pyrenaearia* in this mountain range and populations occupied extensive areas close to each other, which may be making gene flow easier. The Pyrenees on the contrary, have a more complex rock substrate (Dendaletche, 1991; Gutiérrez-Elorza, 1994). The Axial Pyrenees, where the highest summits are found, are formed by a complex mixture of sedimentary (limestone and sandstone), plutonic (granite) and metamorphic (marble, quartzite and slate) rocks, while the Inner Mountain Ranges and Pre-Pyrenees are mainly characterized by carbonate rocks. Although *P. navasi* is able to live on a siliceous substrate, the remaining species depend on calcareous rocks for their survival. Thus, the siliceous extensions of the Axial Pyrenees suppose greater distances between populations and may be hindering gene flow and promoting speciation in this mountain system. Further, this richness pattern is similar to that reported for the also obligate limestone-dweller land snail genus *Abida*, which has a single species endemic to the Cantabrian Mountains while it shows a high diversity in the Pyrenees (Gittenberger, 1973). A phylogeographic study has also identified the isolation of suitable calcareous habitats in the Alps as an important factor for diversification of the calciphilous snail genus *Orcula* (Harl et al., 2014b).

## 4.5. Conclusions

Based on the results of a species delimitation approach combining multilocus genetic data and morphological information, we propose a taxonomic revision of the land snail genus *Pyrenaearia*. Besides identifying a hitherto unrecognised cryptic species and the synonymization of two species, our data also provide further support for most of the nominal species of this genus. The results indicate the integration of multiple characters as an indispensable tool for delimiting species. In effect, some of the genus' species were only recognized once molecular methods had revealed their existence, and one species (*P. daanidentata*) could not be discerned through the molecular markers used in this work. Shell morphology in the genus seems to be the product of both population history and adaptation to local environment and allometry emerged as an important factor affecting shell shape. We suggest that *Pyrenaearia* is a cold-adapted taxon with certain heat-tolerance so that the availability of shady rock outcrops emerges as a determining factor shaping the genus' distribution. The greater complexity of the rock substrate in the Pyrenees could be promoting the genus diversification and thus be responsible for its greater richness in this mountain range.

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# References


Adams, D.C., Otárola-Castillo, E., 2013. Geomorph: an r package for the collection and analysis of geometric morphometric shape data. Methods Ecol. Evol. 4, 393–399.

Adams, D.C., Rohlf, F.J., Slice, D.E., 2004. Geometric morphometrics: Ten years of progress following the "revolution". Ital. J. Zool. 71, 5–16.

Adams, D.C., Rohlf, F.J., Slice, D.E., 2013. A field comes of age: geometric morphometrics in the 21st century. Hystrix 24, 7–14.

Agapow, P.M., Bininda-Emonds, O.R.P., Crandall, K.A., Gittleman, J.L., Mace, G.M., Marshall, J.C., Purvis, A., 2004. The impact of species concept on biodiversity studies. Q. Rev. Biol. 79, 161–179.

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Automat. Contr. 19, 716–723.

Altimira, C., 1965. Notas malacológicas. 1. Moluscos interesantes del Parque Nacional de Aigues Tortes. Misc. Zool. 2, 19–22.

Altimira, C., 1994. Moluscos del Parque Nacional de Aigüestortes. Butll. Inst. Cat. Hist. Nat. 62, 53–64.

Bidegaray-Batista, L., Ferrández, M.Á., Arnedo, M.A., 2014. Winter is coming: Miocene and Quaternary climatic shifts shaped the diversification of Western-Mediterranean *Harpactocrates* (Araneae, Dysderidae) spiders. Cladistics 30, 428–446.

Blair, C., Méndez de la Cruz, F.R., Law, C., Murphy, R.W., 2015. Molecular phylogenetics and species delimitation of leaf-toed geckos (Phyllodactylidae: *Phyllodactylus*) throughout the Mexican tropical dry forest. Mol. Phylogenet. Evol. 84, 254–265.

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.H., Xie, D., Suchard, M.A., Rambaut, A., Drummond, A.J., 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. PLoS Comput. Biol. 10, e1003537.

Cain, A.J., 1977. Variation in the spire index of some coiled gastropod shells, and its evolutionary significance. Phil. Trans. R. Soc. Lond. B 277, 377–428.

Cardini, A., 2014. Missing the third dimension in geometric morphometrics: how to assess if 2D images really are a good proxy for 3D structures? Hystrix 25, 73–81.

Cardini, A., Polly, D., Dawson, R., Milne, N., 2015. Why the long face? Kangaroos and wallabies follow the same "rule" of cranial evolutionary allometry (CREA) as placentals. Evol. Biol. 42, 169–176.

Cignoni, P., Callieri, M., Corsini, M., Dellepiane, M., Ganovelli, F., Ranzuglia, G., 2008. MeshLab: an open-source mesh processing tool. In: Scarano, V., De Chiara, R., Erra, U. (Eds.), Sixth Eurographics Italian Chapter Conference. The Eurographics Association, Salerno, Italy, pp. 129–136.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9, 772.

de Queiroz, K., 2007. Species Concepts and Species Delimitation. Syst. Biol. 56, 879–886.

Dendaletche, C., 1991. Guía de los Pirineos: elementos de geología, ecología y biología, Translation, Omega, Barcelona, Spain.

Dépraz, A., Cordellier, M., Hausser, J., Pfenninger, M., 2008. Postglacial recolonization at a snail's pace (*Trochulus villosus*): confronting competing refugia hypotheses using model selection. Mol. Ecol. 17, 2449–2462.

Dillon, R.T., Jacquemin, S.J., 2015. The heritability of shell morphometrics in the freshwater pulmonate gastropod *Physa*. PLoS ONE 10, e0121962.

Dowle, E.J., Morgan-Richards, M., Brescia, F., Trewick, S.A., 2015. Correlation between shell phenotype and local environment suggests a role for natural selection in the evolution of *Placostylus* snails. Mol. Ecol. 24, 4205–4221.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973.

Edwards, D.L., Knowles, L.L., 2014. Species detection and individual assignment in species delimitation: can integrative data increase efficacy? Proc. R. Soc. B 281, 20132765.

Elejalde, M.A., Madeira, M.J., Prieto, C.E., Backeljau, T., Gómez-Moliner, B.J., 2009. Molecular phylogeny, taxonomy, and evolution of the land snail genus Pyrenaearia (Gastropoda, Helicoidea). Am. Malac. Bull. 27, 69–81.

Ence, D.D., Carstens, B.C., 2011. SpedeSTEM : a rapid and accurate method for species delimitation. Mol. Ecol. Resour. 11, 473–480.

Evin, A., Baylac, M., Ruedi, M., Mucedda, M., Pons, J.M., 2008. Taxonomy, skull diversity and evolution in a species complex of Myotis (Chiroptera: Vespertilionidae): a geometric morphometric appraisal. Biol. J. Linn. Soc. 95, 529–538.

Fagot, P., 1905. Contributions á la faune malacologique de la Catalogne. Butll. Inst. Cat. Hist. Nat. 5, 122–124.

Figueirido, B., Serrano-Alarcón, F.J., Slater, G.J., Palmqvist, P., 2010. Shape at the cross-roads: homoplasy and history in the evolution of the carnivoran skull towards herbivory. J. Evol. Biol. 23, 2579–2594.

Fiorentino, V., Salomone, N., Manganelli, G., Giusti, F., 2008. Phylogeography and morphological variability in land snails: the Sicilian *Marmorana* (Pulmonata, Helicidae). Biol. J. Linn. Soc. 94, 809–823.

Gittenberger, E., 1973. Beiträge zur Kenntnis der Pupillacea III. Chondrininae. Zool. Verh. 127, 1–267.

Gittenberger, E., Groenenberg, D.S.J., Kokshoorn, B., Preece, R.C., 2006. Molecular trails from hitch-hiking snails. Nature 439, 409.

Gittenberger, E., Piel, W.H., Groenenberg, D.S.J., 2004. The Pleistocene glaciations and the evolutionary history of the polytypic snail species *Arianta arbustorum* (Gastropoda, Pulmonata, Helicidae). Mol. Phylogenet. Evol. 30, 64–73.

Gonzalez, P.N., Perez, S.I., Bernal, V., 2011. Ontogenetic allometry and cranial shape diversification among human populations from South America. Anat. Rec. 294, 1864–1874.

Gunz, P., Mitteroecker, P., Bookstein, F., 2005. Semilandmarks in three dimensions, in: Slice, D.E. (Ed.), Modern morphometrics in physical anthropology. Kluwer Academic/Plenum Publishers, New York, USA, pp. 73-98.

Gutiérrez-Elorza, M., 1994. Geomorfología de España. Rueda, Madrid, Spain.

Haas, F., 1924. Contribució a la malacofauna de la conca inferior del Ebre. Butll. Inst. Cat. Hist. Nat. 4, 48–63.

Harl, J., Duda, M., Kruckenhauser, L., Sattmann, H., Haring, E., 2014a. In search of glacial refuges of the land snail *Orcula dolium* (Pulmonata, Orculidae) - An integrative approach using DNA sequence and fossil data. PLoS ONE 9, e96012.

Harl, J., Páll-Gergely, B., Kirchner, S., Sattmann, H., Duda, M., Kruckenhauser, L., Haring, E., 2014b. Phylogeography of the land snail genus *Orcula* (Orculidae, Stylommatophora) with emphasis on the Eastern Alpine taxa: speciation, hybridization and morphological variation. BMC Evol. Biol. 14, 223.

Hausdorf, B., Hennig, C., 2010. Species delimitation using dominant and codominant multilocus markers. Syst. Biol. 59, 491–503.

Heled, J., Drummond, A.J., 2010. Bayesian inference of species trees from multilocus data. Mol. Biol. Evol. 27, 570–580.

Hijmans, R.J., Cameron, S.E., Parra, J.L., Jones, P.G., Jarvis, A., 2005. Very high resolution interpolated climate surfaces for global land areas. Int. J. Climatol. 25, 1965–1978.

Hughes, P.D., Woodward, J.C., Gibbard, P.L., 2006. Quaternary glacial history of the Mediterranean mountains. Prog. Phys. Geogr. 30, 334–364.

Jones, G., 2017. Algorithmic improvements to species delimitation and phylogeny estimation under the multispecies coalescent. J. Math. Biol. 74, 447–467.

Jones, G., Aydin, Z., Oxelman, B., 2015. DISSECT: An assignment-free Bayesian discovery method for species delimitation under the multispecies coalescent. Bioinformatics 31, 991–998.

Klingenberg, C.P., Marugán-Lobón, J., 2013. Evolutionary covariation in geometric morphometric data: analyzing integration, modularity, and allometry in a phylogenetic context. Syst. Biol. 62, 591–610.

Klingenberg, C.P., Monteiro, L.R., 2005. Distances and directions in multidimensional shape spaces: implications for morphometric applications. Syst. Biol. 54, 678–688.

Knowles, L.L., Carstens, B.C., 2007. Delimiting species without monophyletic gene trees. Syst. Biol. 56, 887–895.

Kokshoorn, B., van Schoor, M., Erkelens, I., Gittenberger, E., 2010. Waves of dispersal in island-hopping *Chondrina* species (Gastropoda, Pulmonata, Chondrinidae). Zool. Anz. 249, 71–79.

Leaché, A.D., Fujita, M.K., 2010. Bayesian species delimitation in West African forest geckos (*Hemidactylus fasciatus*). Proc. R. Soc. B 277, 3071–3077.

Li, S., 2011. Concise formulas for the area and volume of a hyperspherical cap. Asian J. Math. Stat. 4, 66–70.

Lohse, K., 2009. Can mtDNA barcodes be used to delimit species? A response to Pons et al. (2006). Syst. Biol. 58, 439–442.

Löytynoja, A., Goldman, N., 2005. An algorithm for progressive multiple alignment of sequences with insertions. Proc. Natl. Acad. Sci. USA 102, 10557–10562.

Löytynoja, A., Goldman, N., 2008. Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. Science 320, 1632–1635.

Márquez, F., Averbuj, A., 2017. Sexual dimorphism in the shell of a nassariid gastropod. A 3D geometric morphometrics approach. J. Mar. Biol. Assoc. UK 97, 249–255.

Márquez, F., González-José, R., Bigatti, G., 2011. Combined methods to detect pollution effects on shell shape and structure in Neogastropods. Ecol. Indic. 11, 248–254.

Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010*, New Orleans, LA, pp. 1–8.

Mitteroecker, P., Gunz, P., 2009. Advances in Geometric Morphometrics. Evol. Biol. 36, 235–247.

Monaghan, M.T., Wild, R., Elliot, M., Fujisawa, T., Balke, M., Inward, D.J.G., Lees, D.C., Ranaivosolo, R., Eggleton, P., Barraclough, T.G., Vogler, A.P., 2009. Accelerated species inventory on Madagascar using coalescent-based models of species delineation. Syst. Biol. 58, 298–311.

Mouret, V., Guillaumet, A., Cheylan, M., Pottier, G., Ferchaud, A.L., Crochet, P.A., 2011. The legacy of ice ages in mountain species: post-glacial colonization of mountain tops rather than current range fragmentation determines mitochondrial genetic diversity in an endemic Pyrenean rock lizard. J. Biogeogr. 38, 1717–1731.

Navarro, N., Maga, A.M., 2016. Does 3D phenotyping yield substantial insights in the genetics of the mouse mandible shape? G3 (Bethesda) 6, 1153–1163.

Niemiller, M.L., Near, T.J., Fitzpatrick, B.M., 2012. Delimiting species using multilocus data: diagnosing cryptic diversity in the southern cavefish, *Typhlichthys subterraneus* (Teleostei: Amblyopsidae). Evolution 66, 846–866.

Noshita, K., Shimizu, K., Sasaki, T., 2016. Geometric analysis and estimation of the growth rate gradient on gastropod shells. J. Theor. Biol. 389, 11–19.

O'Meara, B.C., 2010. New heuristic methods for joint species delimitation and species tree inference. Syst. Biol. 59, 59–73.

Ortiz de Zárate, A., 1956. Observaciones anatómicas y posición sistemática de varios Helícidos españoles. IV. Género *Pyrenaearia* Hesse, 1921. Bol. R. Soc. Esp. Hist. Nat. 54, 35–61.

Padial, J.M., Miralles, A., De la Riva, I., Vences, M., 2010. The integrative future of taxonomy. Front. Zool. 7, 16.

Pauls, S.U., Lumbsch, H.T., Haase, P., 2006. Phylogeography of the montane caddisfly *Drusus discolor*: evidence for multiple refugia and periglacial survival. Mol. Ecol. 15, 2153–2169.

Pons, J., Barraclough, T.G., Gomez-zurita, J., Cardoso, A., Duran, D.P., Hazell, S., Kamoun, S., Sumlin, W.D., Vogler, A.P., 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Syst. Biol. 55, 595–609.

Prieto, C.E., 1986. Estudio sistemático y biogeográfico de los Helicidae *sensu* Zilch, 1959-60 (Gastropoda: Pulmonata: Stylommatophora) del País Vasco y regiones adyacentes. Leioa, PhD thesis, University of the Basque Country (EHU/UPV), Spain.

Prieto, C.E., 1991. *Pyrenaearia navasi sylvatica ssp.nov.*, una subespecie originada por una regresión glaciar cuaternaria en el macizo del Moncayo. Kobie 20, 69–75.

Puente, A.I., 1994. Estudio taxonómico y biogeográfico de la superfamilia Helicoidea Rafinesque, 1815 (Gastropoda: Pulmonata: Stylommatophora) de la Península Ibérica e Islas Baleares. Leioa, PhD thesis, University of the Basque Country (EHU/UPV), Spain.

Puillandre, N., Lambert, a, Brouillet, S., Achaz, G., 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Mol. Ecol. 21, 1864–77.

Rato, C., Harris, D.J., Carranza, S., Machado, L., Perera, A., 2015. The taxonomy of the *Tarentola mauritanica* species complex (Gekkota: Phyllodactylidae): Bayesian species delimitation supports six candidate species. Mol. Phylogenet. Evol. 94, 271–278.

Raven, J.G., 1988. *Pyrenaearia daanidentata* spec. nov. (Helicidae), a toothed species from the Cantabrian mountains, Spain. Basteria 52, 121–123.

Razkin, O., Gómez-Moliner, B.J., Vardinoyannis, K., Martínez-Ortí, A., Madeira, M.J., 2017. Species delimitation for cryptic species complexes: case study of *Pyramidula* (Gastropoda, Pulmonata). Zool. Scr. 46, 55–72.

Reid, N.M., Carstens, B.C., 2012. Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. BMC Evol. Biol. 12, 196.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.

Satler, J.D., Carstens, B.C., Hedin, M., 2013. Multilocus species delimitation in a complex of morphologically conserved trapdoor spiders (Mygalomorphae, Antrodiaetidae, *Aliantypus*). Syst. Biol. 62, 805–823.

Scalici, M., Traversetti, L., Spani, F., Bravi, R., Malafoglia, V., Persichini, T., Colasanti, M., 2016. Using 3D virtual surfaces to investigate molluscan shell shape. Aquat. Living Resour. 29, 207.

Schlager, S., 2016. Package 'Morpho: calculations and visualisations related to geometric morphometric' version 2.4.1.1. Available from https://cran.r-project.org/web/packages/Morpho/index.html

Sites, J.W., Marshall, J.C., 2003. Delimiting species: a Renaissance issue in systematic biology. Trends Ecol. Evol. 18, 462–470.

Solís-Lemus, C., Knowles, L.L., Ané, C., 2015. Bayesian species delimitation combining multiple genes and traits in a unified framework. Evolution 69, 492–507.

Stankowski, S., 2011. Extreme, continuous variation in an island snail: local diversification and association of shell form with the current environment. Biol. J. Linn. Soc. 104, 756–769.

Stankowski, S., 2013. Ecological speciation in an island snail: evidence for the parallel evolution of a novel ecotype and maintenance by ecologically dependent postzygotic isolation. Mol. Ecol. 22, 2726–2741.

Sukumaran, J., Holder, M.T., 2010. DendroPy: a Python library for phylogenetic computing. Bioinformatics 26, 1569–1571.

Sukumaran, J., Knowles, L.L., 2017. Multispecies coalescent delimits structure, not species. Proc. Natl. Acad. Sci. 114, 1607–1612.

Swofford, D.L., 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sunderland, MA, Sinauer Associates.

Toussaint, E.F.A., Morinière, J., Müller, C.J., Kunte, K., Turlin, B., Hausmann, A., Balke, M., 2015. Comparative molecular species delimitation in the charismatic Nawab butterflies (Nymphalidae, Charaxinae, *Polyura*). Mol. Phylogenet. Evol. 91, 194–209.

Urdy, S., Goudemand, N., Bucher, H., Chirat, R., 2010. Allometries and the morphogenesis of the molluscan shell: a quantitative and theoretical model. J. Exp. Zool. (Mol. Dev. Evol) 314B, 280–302.

Vilella, M., 1963. Notas malacológicas I. Contribución al conocimiento de *Pyrenaearia molae* Hass. Misc. Zool. 1, 27–32.

Welter-Schultes, F.W., 2000. The pattern of geographical and altitudinal variation in the land snail *Albinaria idaea* from Crete (Gastropoda: Clausiliidae). Biol. J. Linn. Soc. 71, 237–250.

Xia, X., 2013. DAMBE5: a comprehensive software package for data analysis in molecular biology and evolution. Mol. Biol. Evol. 30, 1720–1728.

Xia, X., Xie, Z., Salemi, M., Chen, L., Wang, Y., 2003. An index of substitution saturation and its application. Mol. Phylogenet. Evol. 26, 1–7.

Yang, Z., 2015. A tutorial of BPP for species tree estimation and species delimitation. Curr. Zool. 61, 854–865.

Yang, Z., Rannala, B., 2010. Bayesian species delimitation using multilocus sequence data. Proc. Natl. Acad. Sci. USA 107, 9264–9269.

Zelditch, M.L., Sheets, H.D., Fink, W.L., 2003. The ontogenetic dynamics of shape disparity. Paleobiology 29, 139–156.

Zwickl, D.J., 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD thesis, The University of Texas at Austin, USA.

# Supplementary material

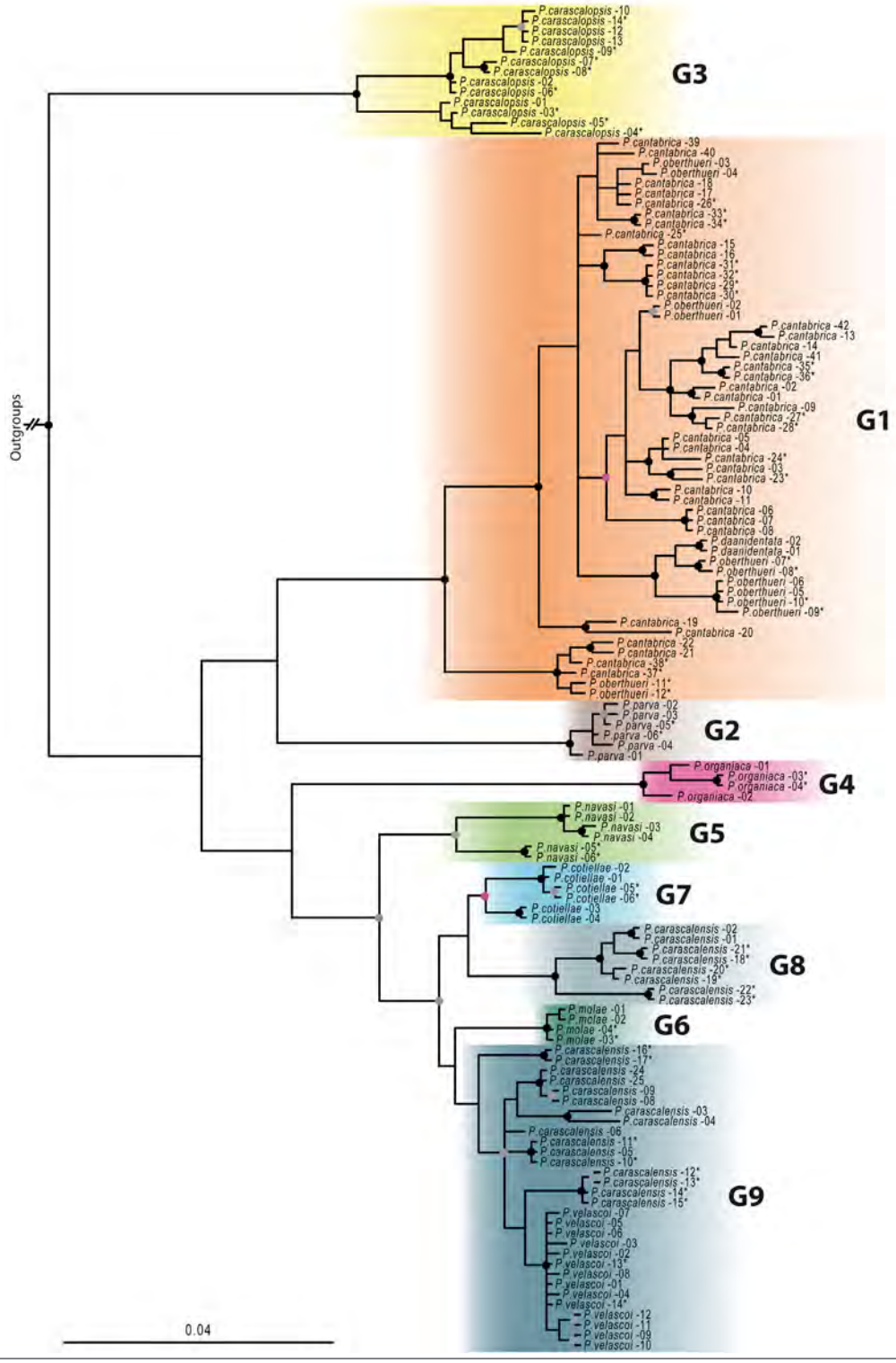


**Figure S1.** Phylogenetic tree of *Pyrenaearia* obtained by Bayesian inference based on mitochondrial information (including *COI* and *16S*). Specimens' codes with asterisks correspond to the new individuals added in this work comparing to Elejalde et al. (2009). Dots on the nodes indicate support information: black dots for nodes supported by BI > 0.95 and ML > 70%, grey dots for BI > 0.95 and ML < 70% and pink dots for BI < 0.95 and ML > 70%. The tree is coloured to distinguish the nine main phylogroups as defined in the text.


**Figure S2.** Phylogenetic tree of *Pyrenaearia* obtained by Bayesian inference based on the nuclear gene cluster *ITS1-5.8S-ITS2-28*. Specimens' codes with asterisks correspond to the new individuals added in this work comparing to Elejalde et al. (2009). Dots on the nodes indicate support information: black dots for nodes supported by BI > 0.95 and ML > 70% and grey dots for BI > 0.95 and ML < 70%. The tree is coloured to distinguish the nine main phylogroups as defined in the text.

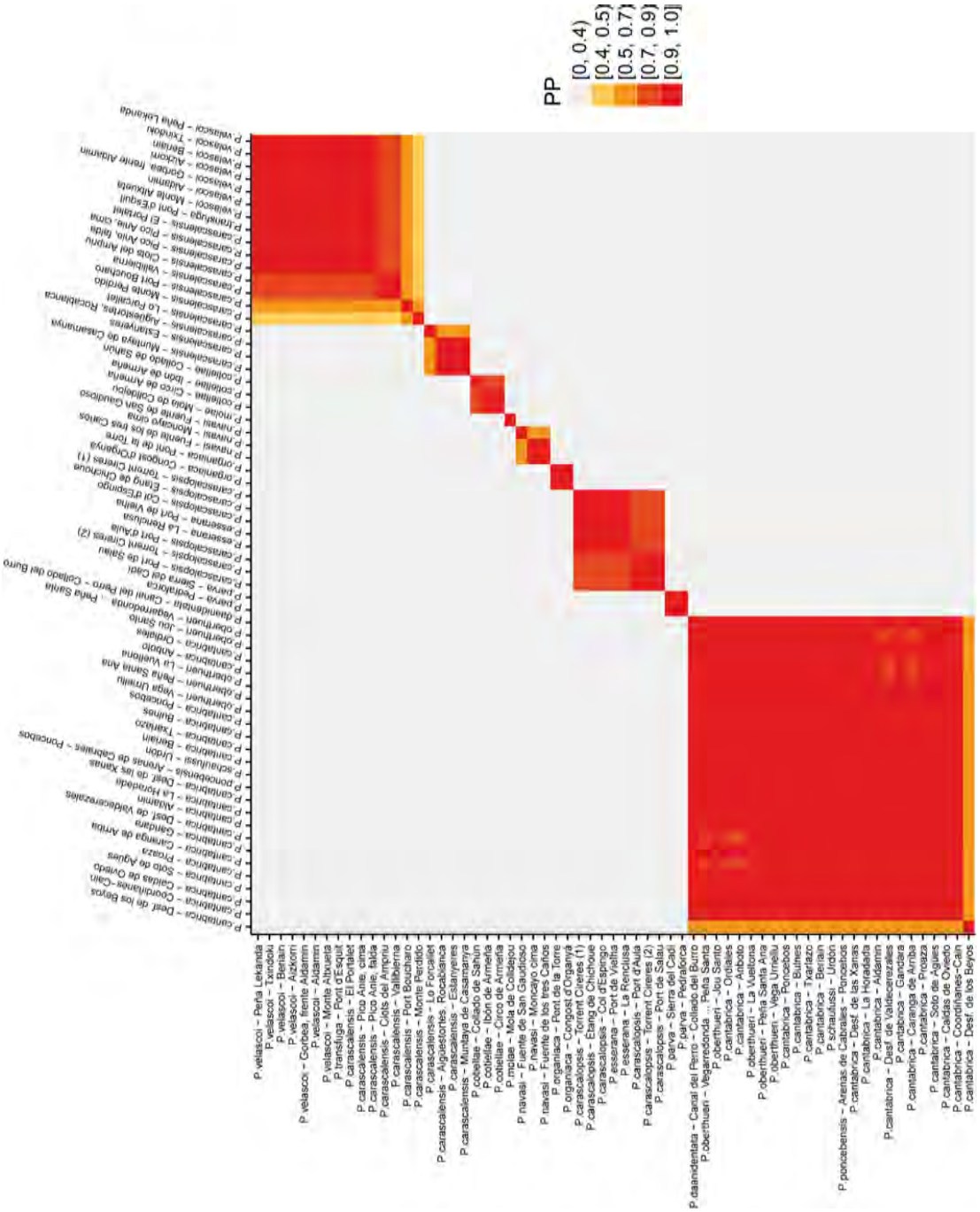


**Figure S3.** Similarity matrix constructed for Stacey results with specimens clustered by population (PP: posterior probabilities).

**Table S1.** List of specimens studied molecularly in this work with voucher number, relation to Elejalde et al. (2009) codes, locality information and GenBank accession numbers. Grey shades indicate individuals whose accession numbers and localities were erroneous in Elejalde et al. (2009).

| Paper Code | Elejalde et al. (2009) Code | Voucher | Locality | Province | Country | HUSO | X | Y | GenBank accession number | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | COI | 16s | ITS1-5.8s-ITS2-28s |
| *P.cantabrica* -01 | *P.cantabrica* -01 | EHUMC-980 | Caldas de Oviedo, Fuso de la Reina, Peña Avis | Asturias | Spain | 30T | 263189 | 4800445 | EU310048 | EU310127 | MG013736 |
| *P.cantabrica* -02 | *P.cantabrica* -02 | EHUMC-979 | Caldas de Oviedo, Fuso de la Reina, Peña Avis | Asturias | Spain | 30T | 263189 | 4800445 | EU310049 | EU310128 | MG013735 |
| *P.cantabrica* -03 | *P.cantabrica* -03 | EHUMC-998 | Gorbea, Peña Lekanda | Bizkaia | Spain | 30T | 516698 | 4768325 | EU310050 | EU310129 | EU310208 |
| *P.cantabrica* -04 | *P.cantabrica* -04 | EHUMC-967 | Txarlazo | Bizkaia | Spain | 30T | 496668 | 4758912 | EU310051 | EU310130 | MG013725 |
| *P.cantabrica* -05 | *P.cantabrica* -05 | EHUMC-966 | Txarlazo | Bizkaia | Spain | 30T | 496668 | 4758912 | EU310052 | EU310131 | MG013724 |
| *P.cantabrica* -06 | *P.cantabrica* -06 | EHUMC-961 | Beriain | Nafarroa | Spain | 30T | 583154 | 4748921 | EU310053 | EU310132 | MG013719 |
| *P.cantabrica* -07 | *P.cantabrica* -07 | EHUMC-962 | Beriain | Nafarroa | Spain | 30T | 583154 | 4748921 | EU310054 | EU310133 | MG013720 |
| *P.cantabrica* -08 | *P.cantabrica* -08 | EHUMC-963 | Beriain | Nafarroa | Spain | 30T | 583154 | 4748921 | EU310055 | EU310134 | MG013721 |
| *P.cantabrica* -09 | *P.cantabrica* -09 | EHUMC-983 | Proaza, "senda del oso" | Asturias | Spain | 29T | 741591 | 4792340 | EU310056 | EU310135 | MG013739 |
| *P.cantabrica* -10 | *P.cantabrica* -10 | EHUMC-981 | Soto de Agües, ruta del Alba | Asturias | Spain | 30T | 298978 | 4784855 | EU310057 | EU310136 | MG013737 |
| *P.cantabrica* -11 | *P.cantabrica* -11 | EHUMC-982 | Soto de Agües, ruta del Alba | Asturias | Spain | 30T | 298978 | 4784855 | EU310058 | EU310137 | MG013738 |
| *P.cantabrica* -13 | *P.cantabrica* -13 | EHUMC-997 | Arenas de Cabrales - Poncebos: tuneles | Asturias | Spain | 30T | 351913 | 4794620 | EU310060 | EU310139 | MG013752 |
| *P.cantabrica* -14 | *P.cantabrica* -14 | EHUMC-996 | Arenas de Cabrales - Poncebos: tuneles | Asturias | Spain | 30T | 351913 | 4794620 | EU310061 | EU310140 | MG013751 |
| *P.cantabrica* -15 | *P.cantabrica* -15 | EHUMC-984 | Caranga de Arriba - Las Agüeras | Asturias | Spain | 29T | 741616 | 4787453 | EU310062 | EU310141 | MG013740 |
| *P.cantabrica* -16 | *P.cantabrica* -16 | EHUMC-985 | Caranga de Arriba - Las Agüeras | Asturias | Spain | 29T | 741616 | 4787453 | EU310063 | EU310142 | MG013741 |
| *P.cantabrica* -17 | *P.cantabrica* -17 | EHUMC-986 | Cordiñanes-Caín | León | Spain | 30T | 345432 | 4785243 | EU310064 | EU310143 | MG013742 |
| *P.cantabrica* -18 | *P.cantabrica* -18 | EHUMC-952 | Cordiñanes-Caín | León | Spain | 30T | 345432 | 4785243 | EU310065 | EU310144 | MG013710 |
| *P.cantabrica* -19 | *P.cantabrica* -19 | EHUMC-995 | Desfiladero de los Beyos | Asturias | Spain | 30T | 330152 | 4788131 | EU310066 | EU310145 | MG013750 |
| *P.cantabrica* -20 | *P.cantabrica* -20 | EHUMC-931 | Desfiladero de los Beyos | Asturias | Spain | 30T | 330152 | 4788131 | EU310067 | EU310146 | MG013693 |
| *P.cantabrica* -21 | *P.cantabrica* -21 | EHUMC-1000 | Anboto | Bizkaia | Spain | 30T | 532173 | 4770990 | EU310068 | EU310147 | MG013754 |
| *P.cantabrica* -22 | *P.cantabrica* -22 | EHUMC-999 | Anboto | Bizkaia | Spain | 30T | 532173 | 4770990 | EU310069 | EU310148 | MG013753 |
| *P.cantabrica* -23* | | EHUMC-1922 | Aldamin | Bizkaia | Spain | 30T | 518240 | 4765250 | MG013576 | MG013632 | MG013760 |
| *P.cantabrica* -24* | | EHUMC-1923 | Alto de Gándara | Cantabria | Spain | 30T | 452508 | 4782917 | MG013577 | MG013633 | MG013761 |

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| *P.cantabrica* -25* | | EHUMC-1924 | Bulnes | Asturias | Spain | 30T | 352242 | 4788528 | MG013578 | MG013634 | MG013762 |
| *P.cantabrica* -26* | | EHUMC-1925 | Bulnes | Asturias | Spain | 30T | 352242 | 4788528 | MG013579 | MG013635 | MG013763 |
| *P.cantabrica* -27* | | EHUMC-1926 | Desfiladero de las Xanas | Asturias | Spain | 30T | 257582 | 4795207 | MG013580 | MG013636 | MG013764 |
| *P.cantabrica* -28* | | EHUMC-1927 | Desfiladero de las Xanas | Asturias | Spain | 30T | 257582 | 4795207 | MG013581 | MG013637 | MG013765 |
| *P.cantabrica* -29* | | EHUMC-1928 | Desfiladero de Valdecerezales | Asturias | Spain | 29T | 737104 | 4785008 | MG013582 | MG013638 | MG013766 |
| *P.cantabrica* -30* | | EHUMC-1929 | Desfiladero de Valdecerezales | Asturias | Spain | 29T | 737104 | 4785008 | MG013583 | MG013639 | MG013767 |
| *P.cantabrica* -31* | | EHUMC-1930 | Entrago | Asturias | Spain | 29T | 736540 | 4784331 | MG013584 | MG013640 | MG013768 |
| *P.cantabrica* -32* | | EHUMC-1931 | Entrago | Asturias | Spain | 29T | 736540 | 4784331 | MG013585 | MG013641 | MG013769 |
| *P.cantabrica* -33* | | EHUMC-1932 | La Horadada | Burgos | Spain | 30T | 464603 | 4735039 | MG013586 | MG013642 | MG013770 |
| *P.cantabrica* -34* | | EHUMC-1933 | La Horadada | Burgos | Spain | 30T | 464603 | 4735039 | MG013587 | MG013643 | MG013771 |
| *P.cantabrica* -35* | | EHUMC-1934 | Poncebos | Asturias | Spain | 30T | 351396 | 4791460 | MG013588 | MG013644 | MG013772 |
| *P.cantabrica* -36* | | EHUMC-1935 | Poncebos | Asturias | Spain | 30T | 351396 | 4791460 | MG013589 | MG013645 | MG013773 |
| *P.cantabrica* -37* | | EHUMC-1936 | Vegarredonda, mirador Ordiales | Asturias | Spain | 30T | 337940 | 4788656 | MG013590 | MG013646 | MG013774 |
| *P.cantabrica* -38* | | EHUMC-1937 | Vegarredonda, mirador Ordiales | Asturias | Spain | 30T | 337940 | 4788656 | MG013591 | MG013647 | MG013775 |
| *P.cantabrica* -39 | *P.schaufussi*-01 | EHUMC-926 | Urdón | Cantabria | Spain | 30T | 367571 | 4791784 | EU310080 | EU310159 | MG013689 |
| *P.cantabrica* -40 | *P.schaufussi*-02 | EHUMC-930 | Urdón | Cantabria | Spain | 30T | 367571 | 4791784 | EU310081 | EU310160 | MG013692 |
| *P.cantabrica* -41 | *P.poncebensis* -01 | EHUMC-992 | Arenas de Cabrales - Poncebos: tuneles | Asturias | Spain | 30T | 351913 | 4794620 | EU310078 | EU310157 | MG013748 |
| *P.cantabrica* -42 | *P.poncebensis* -02 | EHUMC-958 | Arenas de Cabrales - Poncebos: tuneles | Asturias | Spain | 30T | 351913 | 4794620 | EU310079 | EU310158 | MG013716 |
| *P.carascalensis* -01 | *P.carascalensis* -01 | EHUMC-975 | Muntaya de Casamanya | Ordino | Andorra | 31T | 382653 | 4715934 | EU310025 | EU310104 | MG013731 |
| *P.carascalensis* -02 | *P.carascalensis* -02 | EHUMC-974 | Muntaya de Casamanya | Ordino | Andorra | 31T | 382653 | 4715934 | EU310026 | EU310105 | MG013730 |
| *P.carascalensis* -03 | *P.carascalensis* -03 | EHUMC-987 | Port Boucharo, Gavarnie | Hautes Pyrénées | France | 30T | 740548 | 4732068 | EU310027 | EU310106 | MG013743 |
| *P.carascalensis* -04 | *P.carascalensis* -04 | EHUMC-988 | Port Boucharo, Gavarnie | Hautes Pyrénées | France | 30T | 740548 | 4732068 | EU310028 | EU310107 | MG013744 |
| *P.carascalensis* -05 | *P.carascalensis* -05 | EHUMC-968 | Pico Anie, cima | Pyrénées-Atlantiques | France | 30T | 685951 | 4757089 | EU310029 | EU310108 | MG013726 |
| *P.carascalensis* -06 | *P.carascalensis* -06 | EHUMC-969 | Pico Anie, cima | Pyrénées-Atlantiques | France | 30T | 685951 | 4757089 | EU310030 | EU310109 | EU310188 |
| *P.carascalensis* -08 | *P.carascalensis* -08 | EHUMC-989 | El Portalet | Pyrénées-Atlantiques | France | 30T | 709695 | 4742751 | EU310032 | EU310111 | MG013745 |
| *P.carascalensis* -09 | *P.carascalensis* -09 | EHUMC-970 | Pico Anie, cima | Pyrénées-Atlantiques | France | 30T | 685951 | 4757089 | EU310033 | EU310112 | MG013727 |

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| *P.carascalensis* -10* | | EHUMC-1938 | El Portalet | Pyrénées-Atlantiques | France | 30T | 709695 | 4742751 | MG013592 | MG013648 | MG013776 |
| *P.carascalensis* -11* | | EHUMC-1939 | Pico Anie, falda (casa metal) | Pyrénées-Atlantiques | France | 30T | 683895 | 4758724 | MG013593 | MG013649 | MG013777 |
| *P.carascalensis* -12* | | EHUMC-1940 | Vallibierna, hacia Cuello de Culebres | Huesca | Spain | 31T | 306855 | 4718868 | MG013594 | MG013650 | MG013778 |
| *P.carascalensis* -13* | | EHUMC-1941 | Vallibierna, hacia Cuello de Culebres | Huesca | Spain | 31T | 306855 | 4718868 | MG013595 | MG013651 | MG013779 |
| *P.carascalensis* -14* | | EHUMC-1942 | Clots del Ampriu (Cerler) | Huesca | Spain | 31T | 301210 | 4714023 | MG013596 | MG013652 | MG013780 |
| *P.carascalensis* -15* | | EHUMC-1943 | Clots del Ampriu (Cerler) | Huesca | Spain | 31T | 301210 | 4714023 | MG013597 | MG013653 | MG013781 |
| *P.carascalensis* -16* | | EHUMC-1944 | Monte Perdido | Huesca | Spain | 31T | 256128 | 4728518 | MG013598 | MG013654 | MG013782 |
| *P.carascalensis* -17* | | EHUMC-1945 | Monte Perdido | Huesca | Spain | 31T | 256128 | 4728518 | MG013599 | MG013655 | MG013783 |
| *P.carascalensis* -18* | | EHUMC-1946 | Aigüestortes, Canal des Estanyeres | Lleida | Spain | 31T | 339763 | 4718950 | MG013600 | MG013656 | MG013784 |
| *P.carascalensis* -19* | | EHUMC-1947 | Aigüestortes, Canal des Estanyeres | Lleida | Spain | 31T | 339763 | 4718950 | MG013601 | MG013657 | MG013785 |
| *P.carascalensis* -20* | | EHUMC-1880 | Aigüestortes, Rocablanca | Lleida | Spain | 31T | 339326 | 4718268 | KY850422 | KY850514 | MG013759 |
| *P.carascalensis* -21* | | EHUMC-1948 | Aigüestortes, Rocablanca | Lleida | Spain | 31T | 339326 | 4718268 | MG013602 | MG013658 | MG013786 |
| *P.carascalensis* -22* | | EHUMC-1949 | Lo Forcallet, Montanyo de Llacs | Lleida | Spain | 31T | 328536 | 4711093 | MG013603 | MG013659 | MG013787 |
| *P.carascalensis* -23* | | EHUMC-1950 | Lo Forcallet, Montanyo de Llacs | Lleida | Spain | 31T | 328536 | 4711093 | MG013604 | MG013660 | MG013788 |
| *P.carascalensis* -24 | *P.transfuga* -01 | EHUMC-945 | Pont d´Esquit | Pyrénées-Atlantiques | France | 30T | 694914 | 4759475 | EU310034 | EU310113 | MG013705 |
| *P.carascalensis* -25 | *P.transfuga* -02 | EHUMC-947 | Pont d´Esquit | Pyrénées-Atlantiques | France | 30T | 694914 | 4759475 | EU310035 | EU310114 | MG013706 |
| *P.carascalopsis* -01 | *P.carascalopsis* -01 | EHUMC-953 | Port de Salau | Ariège | France | 31T | 347150 | 4733559 | EU310004 | EU310083 | MG013711 |
| *P.carascalopsis* -02 | *P.carascalopsis* -02 | EHUMC-1002 | Rocablanca, Torrent Cireres | Lleida | Spain | 31T | 341452 | 4733445 | EU310005 | EU310084 | MG013756 |
| *P.carascalopsis* -03* | | EHUMC-1951 | Port de Salau | Ariège | France | 31T | 347150 | 4733559 | MG013605 | MG013661 | MG013789 |
| *P.carascalopsis* -04* | | EHUMC-1952 | Rocablanca, Torrent Cireres | Lleida | Spain | 31T | 341452 | 4733445 | MG013606 | MG013662 | MG013790 |
| *P.carascalopsis* -05* | | EHUMC-1953 | Port d'Aula | Ariège | France | 31T | 345342 | 4736735 | MG013607 | MG013663 | MG013791 |
| *P.carascalopsis* -06* | | EHUMC-1954 | Port d'Aula | Ariège | France | 31T | 345342 | 4736735 | MG013608 | MG013664 | MG013792 |
| *P.carascalopsis* -07* | | EHUMC-1955 | Sentein, Étang de Chichoué | Ariège | France | 31T | 327773 | 4743118 | MG013609 | MG013665 | MG013793 |
| *P.carascalopsis* -08* | | EHUMC-1956 | Sentein, Étang de Chichoué | Ariège | France | 31T | 327773 | 4743118 | MG013610 | MG013666 | MG013794 |
| *P.carascalopsis* -09* | | EHUMC-1957 | Col d'Espingo (subiendo desde Lac d'Oô) | Haute-Garonne | France | 31T | 295411 | 4734026 | MG013611 | MG013667 | MG013795 |
| *P.carascalopsis* -10 | *P.esserana* -01 | EHUMC-932 | La Renclusa | Huesca | Spain | 31T | 307643 | 4726898 | EU310006 | EU310085 | MG013694 |

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| *P.carascalopsis* -12 | *P.esserana* -02 | EHUMC-990 | Port de Vielha | Lleida | Spain | 31T | 316007 | 4723973 | EU310007 | EU310086 | MG013746 |
| *P.carascalopsis* -13 | *P.esserana* -03 | EHUMC-991 | Port de Vielha | Lleida | Spain | 31T | 316007 | 4723973 | EU310008 | EU310087 | MG013747 |
| *P.carascalopsis* -14* | | EHUMC-1960 | La Renclusa | Huesca | Spain | 31T | 307643 | 4726898 | MG013614 | MG013670 | MG013798 |
| *P.cotiellae* -01 | *P.cotiellae* -01 | EHUMC-949 | Circo de Armeña | Huesca | Spain | 31T | 282372 | 4709986 | EU310021 | EU310100 | MG013708 |
| *P.cotiellae* -02 | *P.cotiellae* -02 | EHUMC-939 | Circo de Armeña | Huesca | Spain | 31T | 282372 | 4709986 | EU310022 | EU310101 | MG013700 |
| *P.cotiellae* -03 | *P.cotiellae* -03 | EHUMC-971 | Collado de Sahún | Huesca | Spain | 31T | 286403 | 4716141 | EU310023 | EU310102 | MG013728 |
| *P.cotiellae* -04 | *P.cotiellae* -04 | EHUMC-972 | Collado de Sahún | Huesca | Spain | 31T | 286403 | 4716141 | EU310024 | EU310103 | MG013729 |
| *P.cotiellae* -05* | | EHUMC-1958 | Ibón de Armeña | Huesca | Spain | 31T | 282718 | 4710237 | MG013612 | MG013668 | MG013796 |
| *P.cotiellae* -06* | | EHUMC-1959 | Ibón de Armeña | Huesca | Spain | 31T | 282718 | 4710237 | MG013613 | MG013669 | MG013797 |
| *P.daanidentata* -01 | *P.daanidentata* -01 | EHUMC-1001 | Canal del Perro - Collado del Burro | León | Spain | 30T | 340235 | 4781917 | EU310070 | EU310149 | MG013755 |
| *P.daanidentata* -02 | *P.daanidentata* -02 | EHUMC-929 | Canal del Perro - Collado del Burro | León | Spain | 30T | 340235 | 4781917 | EU310071 | EU310150 | MG013691 |
| *P.molae* -01 | *P.molae* -01 | EHUMC-936 | Mola de Colldejou | Tarragona | Spain | 31T | 321089 | 4553127 | EU310019 | EU310098 | EU310177 |
| *P.molae* -02 | *P.molae* -02 | EHUMC-940 | Mola de Colldejou | Tarragona | Spain | 31T | 321089 | 4553127 | EU310020 | EU310099 | EU310178 |
| *P.molae* -03* | | EHUMC-1961 | Mola de Colldejou | Tarragona | Spain | 31T | 321089 | 4553127 | MG013615 | MG013671 | MG013799 |
| *P.molae* -04* | | EHUMC-1962 | Mola de Colldejou | Tarragona | Spain | 31T | 321089 | 4553127 | MG013616 | MG013672 | MG013800 |
| *P.navasi* -01 | *P.navasi* -01 | EHUMC-927 | Moncayo, cima | Zaragoza | Spain | 30T | 597192 | 4626319 | EU310015 | EU310094 | EU310173 |
| *P.navasi* -02 | *P.navasi* -02 | EHUMC-941 | Moncayo, cima | Zaragoza | Spain | 30T | 597192 | 4626319 | EU310016 | EU310095 | MG013701 |
| *P.navasi* -03 | *P.navasi* -03 | EHUMC-937 | Moncayo, fuente de los Tres Caños | Zaragoza | Spain | 30T | 598198 | 4629240 | EU310017 | EU310096 | MG013698 |
| *P.navasi* -04 | *P.navasi* -04 | EHUMC-950 | Moncayo, fuente de los Tres Caños | Zaragoza | Spain | 30T | 598198 | 4629240 | EU310018 | EU310097 | MG013709 |
| *P.navasi* -05* | | EHUMC-1963 | Moncayo, fuente de San Gaudioso | Zaragoza | Spain | 30T | 598446 | 4626990 | MG013617 | MG013673 | MG013801 |
| *P.navasi* -06* | | EHUMC-1964 | Moncayo, fuente de San Gaudioso | Zaragoza | Spain | 30T | 598446 | 4626990 | MG013618 | MG013674 | MG013802 |
| *P.oberthueri* -01 | *P.oberthueri* -01 | EHUMC-976 | Vega Urriellu | Asturias | Spain | 30T | 352000 | 4785073 | EU310072 | EU310151 | MG013732 |
| *P.oberthueri* -02 | *P.oberthueri* -02 | EHUMC-956 | Vega Urriellu | Asturias | Spain | 30T | 352000 | 4785073 | EU310073 | EU310152 | MG013714 |
| *P.oberthueri* -03 | *P.oberthueri* -03 | EHUMC-928 | Vega Urriellu | Asturias | Spain | 30T | 352000 | 4785073 | EU310074 | EU310153 | MG013690 |
| *P.oberthueri* -04 | *P.oberthueri* -04 | EHUMC-943 | Vega Urriellu | Asturias | Spain | 30T | 352000 | 4785073 | EU310075 | EU310154 | MG013703 |
| *P.oberthueri* -05 | *P.oberthueri* -05 | EHUMC-955 | Peña Santa Ana | Cantabria | Spain | 30T | 352509 | 4782295 | EU310076 | EU310155 | MG013713 |

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| *P.oberthueri* -06 | *P.oberthueri* -06 | EHUMC-954 | Peña Santa Ana | Cantabria | Spain | 30T | 352509 | 4782295 | EU310077 | EU310156 | MG013712 |
| *P.oberthueri* -07* | | EHUMC-1965 | Jou Santo - Peña Santa | Asturias | Spain | 30T | 340121 | 4786370 | MG013619 | MG013675 | MG013803 |
| *P.oberthueri* -08* | | EHUMC-1966 | Jou Santo - Peña Santa | Asturias | Spain | 30T | 340121 | 4786370 | MG013620 | MG013676 | MG013804 |
| *P.oberthueri* -09* | | EHUMC-1967 | La Vueltona, cabaña Veronica | Cantabria | Spain | 30T | 352431 | 4781138 | MG013621 | MG013677 | MG013805 |
| *P.oberthueri* -10* | | EHUMC-1968 | La Vueltona, cabaña Veronica | Cantabria | Spain | 30T | 352431 | 4781138 | MG013622 | MG013678 | MG013806 |
| *P.oberthueri* -11* | | EHUMC-1969 | Vegarredonda - Peña Santa, antes del zig-zag | Asturias | Spain | 30T | 338904 | 4787609 | MG013623 | MG013679 | MG013807 |
| *P.oberthueri* -12* | | EHUMC-1970 | Vegarredonda - Peña Santa, antes del zig-zag | Asturias | Spain | 30T | 338904 | 4787609 | MG013624 | MG013680 | MG013808 |
| *P.organiaca* -01 | *P.organiaca* -01 | EHUMC-935 | Congost d´Organyà | Lleida | Spain | 31T | 363442 | 4677083 | EU310013 | EU310092 | MG013697 |
| *P.organiaca* -02 | *P.organiaca* -02 | EHUMC-942 | Congost d´Organyà | Lleida | Spain | 31T | 363442 | 4677083 | EU310014 | EU310093 | MG013702 |
| *P.organiaca* -03* | | EHUMC-1971 | Congost del Pont de la Torre | Lleida | Spain | 31T | 363704 | 4679023 | MG013625 | MG013681 | MG013809 |
| *P.organiaca* -04* | | EHUMC-1972 | Congost del Pont de la Torre | Lleida | Spain | 31T | 363704 | 4679023 | MG013626 | MG013682 | MG013810 |
| *P.parva* -01 | *P.parva* -01 | EHUMC-973 | Pedraforca, subida por el canal | Barcelona | Spain | 31T | 393016 | 4676882 | EU310009 | EU310088 | EU310167 |
| *P.parva* -02 | *P.parva* -02 | EHUMC-934 | Pedraforca, subida por el canal | Barcelona | Spain | 31T | 393016 | 4676882 | EU310010 | EU310089 | MG013696 |
| *P.parva* -03 | *P.parva* -03 | EHUMC-977 | Sierra de Cadí | Barcelona | Spain | 31T | 395680 | 4682110 | EU310011 | EU310090 | MG013733 |
| *P.parva* -04 | *P.parva* -04 | EHUMC-978 | Sierra de Cadí | Barcelona | Spain | 31T | 395680 | 4682110 | EU310012 | EU310091 | MG013734 |
| *P.parva* -05* | | EHUMC-1973 | Sierra de Cadí | Barcelona | Spain | 31T | 395680 | 4682110 | MG013627 | MG013683 | MG013811 |
| *P.parva* -06* | | EHUMC-1974 | Pedraforca, subida por el canal | Barcelona | Spain | 31T | 393016 | 4676882 | MG013628 | MG013684 | MG013812 |
| *P.velascoi* -01 | *P.velascoi* -01 | EHUMC-944 | Gorbea, frente Aldamin | Bizkaia | Spain | 30T | 517739 | 4765043 | EU310036 | EU310115 | MG013704 |
| *P.velascoi* -02 | *P.velascoi* -02 | EHUMC-965 | Txindoki | Gipuzkoa | Spain | 30T | 574294 | 4763755 | EU310037 | EU310116 | MG013723 |
| *P.velascoi* -03 | *P.velascoi* -03 | EHUMC-964 | Txindoki | Gipuzkoa | Spain | 30T | 574294 | 4763755 | EU310038 | EU310117 | MG013722 |
| *P.velascoi* -04 | *P.velascoi* -04 | EHUMC-957 | Aitzgorri, cima orientación norte | Gipuzkoa | Spain | 30T | 555116 | 4755504 | EU310039 | EU310118 | MG013715 |
| *P.velascoi* -05 | *P.velascoi* -05 | EHUMC-933 | Aldamin | Bizkaia | Spain | 30T | 518240 | 4765250 | EU310040 | EU310119 | MG013695 |
| *P.velascoi* -06 | *P.velascoi* -06 | EHUMC-938 | Aldamin | Bizkaia | Spain | 30T | 518240 | 4765250 | EU310041 | EU310120 | MG013699 |
| *P.velascoi* -07 | *P.velascoi* -07 | EHUMC-925 | Monte Altxueta, Macizo de Aralar | Nafarroa | Spain | 30T | 584523 | 4756061 | EU310042 | EU310121 | MG013688 |
| *P.velascoi* -08 | *P.velascoi* -08 | EHUMC-948 | Aitzgorri, cima orientación norte | Gipuzkoa | Spain | 30T | 555116 | 4755504 | EU310043 | EU310122 | MG013707 |
| *P.velascoi* -09 | *P.velascoi* -09 | EHUMC-993 | Gorbea, Peña Lekanda | Bizkaia | Spain | 30T | 516698 | 4768325 | EU310044 | EU310123 | MG013749 |

| Paper Code | Elejalde et al. (2009) Code | Voucher | Locality | Province | Country | HUSO | X | Y | GenBank accession number | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | COI | 16s | ITS1-5.8s-ITS2-28s |
| *P.velascoi* -10 | *P.velascoi* -10 | EHUMC-994 | Gorbea, Peña Lekanda | Bizkaia | Spain | 30T | 516698 | 4768325 | EU310045 | EU310124 | EU310203 |
| *P.velascoi* -11 | *P.velascoi* -11 | EHUMC-960 | Beriain | Nafarroa | Spain | 30T | 583154 | 4748921 | EU310046 | EU310125 | MG013718 |
| *P.velascoi* -12 | *P.velascoi* -12 | EHUMC-959 | Beriain | Nafarroa | Spain | 30T | 583154 | 4748921 | EU310047 | EU310126 | MG013717 |
| *P.velascoi* -13* | | EHUMC-1975 | Gorbea, frente Aldamin | Bizkaia | Spain | 30T | 517739 | 4765043 | MG013629 | MG013685 | MG013813 |
| *P.velascoi* -14* | | EHUMC-1976 | Monte Altxueta, Macizo de Aralar | Nafarroa | Spain | 30T | 584523 | 4756061 | MG013630 | MG013686 | MG013814 |
| *C. cabrerensis* | | EHUMC-1977 | Alto del Peñon | León | Spain | 29T | 701788 | 4675552 | MG013631 | MG013687 | MG013815 |
| *H. limbata* | | EHUMC-1027 | Queralbs: Daió | Girona | Spain | --- | --- | --- | KY818322 | KJ458529 | MG013757 |
| *P. inchoata* | | EHUMC-1035 | Ruinas de Conimbriga (Sierra de Coinbra) | --- | Portugal | --- | --- | --- | KY818340 | KJ458549 | MG013758 |

**Table S2.** List of shells studied in this work.

| Shell Code | Species | Locality | Country | HUSO | X | Y |
|---|---|---|---|---|---|---|
| C-Pcan-028 | *P. cantabrica* | Beriain | Spain | 30T | 583154 | 4748921 |
| C-Pcan-029 | *P. cantabrica* | Beriain | Spain | 30T | 583154 | 4748921 |
| C-Pcan-033 | *P. cantabrica* | Beriain | Spain | 30T | 583154 | 4748921 |
| C-Pcan-121 | *P. cantabrica* | Desfiladero de Valdecerezales | Spain | 29T | 737104 | 4785008 |
| C-Pcan-122 | *P. cantabrica* | Desfiladero de Valdecerezales | Spain | 29T | 737104 | 4785008 |
| C-Pcan-172 | *P. cantabrica* | La Horadada | Spain | 30T | 464603 | 4735039 |
| C-Pcan-174 | *P. cantabrica* | La Horadada | Spain | 30T | 464603 | 4735039 |
| C-Pcan-231 | *P. cantabrica* | Arenas de Cabrales – Poncebos: tuneles | Spain | 30T | 351913 | 4794620 |
| C-Pcan-233 | *P. cantabrica* | Bulnes | Spain | 30T | 352242 | 4788528 |
| C-Pcan-234 | *P. cantabrica* | Bulnes | Spain | 30T | 352242 | 4788528 |
| C-Pcan-238 | *P. cantabrica* | Soto de Agües, ruta del Alba | Spain | 30T | 298978 | 4784855 |
| C-Pcan-239 | *P. cantabrica* | Soto de Agües, ruta del Alba | Spain | 30T | 298978 | 4784855 |
| C-Pcan-240 | *P. cantabrica* | Soto de Agües, ruta del Alba | Spain | 30T | 298978 | 4784855 |
| C-Pcan-245 | *P. cantabrica* | Urdón | Spain | 30T | 367571 | 4791784 |
| C-Pcan-246 | *P. cantabrica* | Urdón | Spain | 30T | 367571 | 4791784 |
| C-Pcan-260 | *P. cantabrica* | Desfiladero de los Beyos | Spain | 30T | 330152 | 4788131 |
| C-Pcan-261 | *P. cantabrica* | Desfiladero de los Beyos | Spain | 30T | 330152 | 4788131 |
| C-Pcan-262 | *P. cantabrica* | Desfiladero de los Beyos | Spain | 30T | 330152 | 4788131 |
| C-Pcan-265 | *P. cantabrica* | Desfiladero de las Xanas | Spain | 30T | 257582 | 4795207 |
| C-Pcan-266 | *P. cantabrica* | Desfiladero de las Xanas | Spain | 30T | 257582 | 4795207 |
| C-Pcan-267 | *P. cantabrica* | Desfiladero de las Xanas | Spain | 30T | 257582 | 4795207 |
| C-Pcot-269 | *P. cotiellae* | Collado de Sahun | Spain | 31T | 286403 | 4716141 |
| C-Pcot-270 | *P. cotiellae* | Collado de Sahun | Spain | 31T | 286403 | 4716141 |
| C-Pcot-271 | *P. cotiellae* | Collado de Sahun | Spain | 31T | 286403 | 4716141 |
| C-Pcot-465 | *P. cotiellae* | Circo de Armeña | Spain | 31T | 282372 | 4709986 |
| C-Pcot-466 | *P. cotiellae* | Circo de Armeña | Spain | 31T | 282372 | 4709986 |
| C-Pcot-467 | *P. cotiellae* | Circo de Armeña | Spain | 31T | 282372 | 4709986 |
| C-Pcot-468 | *P. cotiellae* | Circo de Armeña | Spain | 31T | 282372 | 4709986 |
| C-Pcot-470 | *P. cotiellae* | Circo de Armeña | Spain | 31T | 282372 | 4709986 |
| C-Pcot-477 | *P. cotiellae* | Collado de Sahun | Spain | 31T | 286403 | 4716141 |
| C-Pcot-478 | *P. cotiellae* | Collado de Sahun | Spain | 31T | 286403 | 4716141 |
| C-Pcot-479 | *P. cotiellae* | Collado de Sahun | Spain | 31T | 286403 | 4716141 |
| C-Pcrl-110 | *P. carascalensis* | Pico Anie, cima | France | 30T | 685951 | 4757089 |
| C-Pcrl-111 | *P. carascalensis* | Pico Anie, cima | France | 30T | 685951 | 4757089 |
| C-Pcrl-226 | *P. carascalensis* | Monte Perdido | Spain | 31T | 256128 | 4728518 |
| C-Pcrl-227 | *P. carascalensis* | Monte Perdido | Spain | 31T | 256128 | 4728518 |
| C-Pcrl-228 | *P. carascalensis* | Monte Perdido | Spain | 31T | 256128 | 4728518 |
| C-Pcrl-386 | *P. carascalensis* | El Portalet | France | 30T | 709695 | 4742751 |
| C-Pcrl-388 | *P. carascalensis* | El Portalet | France | 30T | 709695 | 4742751 |
| C-Pcrl-417 | *P. carascalensis* | Port Boucharo, Gavarnie | France | 30T | 740548 | 4732068 |
| C-Pcrl-418 | *P. carascalensis* | Port Boucharo, Gavarnie | France | 30T | 740548 | 4732068 |
| C-Pcrp-142 | *P. carascalopsis* | Port de Salau | France | 31T | 347150 | 4733559 |
| C-Pcrp-143 | *P. carascalopsis* | Port de Salau | France | 31T | 347150 | 4733559 |

| Shell Code | Species | Locality | Country | HUSO | X | Y |
|---|---|---|---|---|---|---|
| C-Pcrp-144 | *P. carascalopsis* | Port de Salau | France | 31T | 347150 | 4733559 |
| C-Pcrp-145 | *P. carascalopsis* | Port de Salau | France | 31T | 347150 | 4733559 |
| C-Pcrp-146 | *P. carascalopsis* | Port de Salau | France | 31T | 347150 | 4733559 |
| C-Pcrp-148 | *P. carascalopsis* | Port de Salau | France | 31T | 347150 | 4733559 |
| C-Pcrp-149 | *P. carascalopsis* | Port de Salau | France | 31T | 347150 | 4733559 |
| C-Pcrp-150 | *P. carascalopsis* | Port de Salau | France | 31T | 347150 | 4733559 |
| C-Pcrp-151 | *P. carascalopsis* | Port de Salau | France | 31T | 347150 | 4733559 |
| C-Pdaa-063 | *P. daanidentata* | Canal del Perro - Collado del Burro | Spain | 30T | 340235 | 4781917 |
| C-Pdaa-064 | *P. daanidentata* | Canal del Perro - Collado del Burro | Spain | 30T | 340235 | 4781917 |
| C-Pdaa-065 | *P. daanidentata* | Canal del Perro - Collado del Burro | Spain | 30T | 340235 | 4781917 |
| C-Pdaa-066 | *P. daanidentata* | Canal del Perro - Collado del Burro | Spain | 30T | 340235 | 4781917 |
| C-Pdaa-067 | *P. daanidentata* | Canal del Perro - Collado del Burro | Spain | 30T | 340235 | 4781917 |
| C-Pess-187 | *P. carascalopsis* | Port de Vielha | Spain | 31T | 316007 | 4723973 |
| C-Pess-188 | *P. carascalopsis* | Port de Vielha | Spain | 31T | 316007 | 4723973 |
| C-Pess-189 | *P. carascalopsis* | Port de Vielha | Spain | 31T | 316007 | 4723973 |
| C-Pess-192 | *P. carascalopsis* | Port de Vielha | Spain | 31T | 316007 | 4723973 |
| C-Pess-458 | *P. carascalopsis* | Port de Vielha | Spain | 31T | 316007 | 4723973 |
| C-Pess-491 | *P. carascalopsis* | La Renclusa | Spain | 31T | 307643 | 4726898 |
| C-Pess-492 | *P. carascalopsis* | La Renclusa | Spain | 31T | 307643 | 4726898 |
| C-Pess-493 | *P. carascalopsis* | La Renclusa | Spain | 31T | 307643 | 4726898 |
| C-Pess-495 | *P. carascalopsis* | La Renclusa | Spain | 31T | 307643 | 4726898 |
| C-Pess-496 | *P. carascalopsis* | La Renclusa | Spain | 31T | 307643 | 4726898 |
| C-Pmol-093 | *P. molae* | Mola de Colldejou | Spain | 31T | 321089 | 4553127 |
| C-Pmol-094 | *P. molae* | Mola de Colldejou | Spain | 31T | 321089 | 4553127 |
| C-Pmol-096 | *P. molae* | Mola de Colldejou | Spain | 31T | 321089 | 4553127 |
| C-Pmol-097 | *P. molae* | Mola de Colldejou | Spain | 31T | 321089 | 4553127 |
| C-Pmol-098 | *P. molae* | Mola de Colldejou | Spain | 31T | 321089 | 4553127 |
| C-Pmol-099 | *P. molae* | Mola de Colldejou | Spain | 31T | 321089 | 4553127 |
| C-Pmol-100 | *P. molae* | Mola de Colldejou | Spain | 31T | 321089 | 4553127 |
| C-Pmol-101 | *P. molae* | Mola de Colldejou | Spain | 31T | 321089 | 4553127 |
| C-Pmol-102 | *P. molae* | Mola de Colldejou | Spain | 31T | 321089 | 4553127 |
| C-Pnav-272 | *P. navasi* | Moncayo, cima | Spain | 30T | 597192 | 4626319 |
| C-Pnav-273 | *P. navasi* | Moncayo, fuente de San Gaudioso | Spain | 30T | 598446 | 4626990 |
| C-Pnav-274 | *P. navasi* | Moncayo, fuente de San Gaudioso | Spain | 30T | 598446 | 4626990 |
| C-Pnav-275 | *P. navasi* | Moncayo, fuente de San Gaudioso | Spain | 30T | 598446 | 4626990 |
| C-Pnav-346 | *P. navasi* | Moncayo, fuente de San Gaudioso | Spain | 30T | 598446 | 4626990 |
| C-Pnav-347 | *P. navasi* | Moncayo, fuente de San Gaudioso | Spain | 30T | 598446 | 4626990 |
| C-Pnav-348 | *P. navasi* | Moncayo, fuente de San Gaudioso | Spain | 30T | 598446 | 4626990 |
| C-Pnav-447 | *P. navasi* | Moncayo, cima | Spain | 30T | 597192 | 4626319 |
| C-Pobr-196 | *P. cantabrica* | Vega Urriello | Spain | 30T | 352000 | 4785073 |
| C-Pobr-197 | *P. cantabrica* | Vega Urriello | Spain | 30T | 352000 | 4785073 |
| C-Pobr-198 | *P. cantabrica* | Vega Urriello | Spain | 30T | 352000 | 4785073 |
| C-Pobr-199 | *P. cantabrica* | Vega Urriello | Spain | 30T | 352000 | 4785073 |
| C-Pobr-428 | *P. cantabrica* | La Vueltona, cabaña Veronica | Spain | 30T | 352431 | 4781138 |
| C-Pobr-429 | *P. cantabrica* | La Vueltona, cabaña Veronica | Spain | 30T | 352431 | 4781138 |

| Shell Code | Species | Locality | Country | HUSO | X | Y |
|---|---|---|---|---|---|---|
| C-Pobr-430 | *P. cantabrica* | La Vueltona, cabaña Veronica | Spain | 30T | 352431 | 4781138 |
| C-Pobr-441 | *P. cantabrica* | Jou Santu – Peña Santa | Spain | 30T | 340121 | 4786370 |
| C-Porg-078 | *P. organiaca* | Congost del Pont de la Torre | Spain | 31T | 363704 | 4679023 |
| C-Porg-079 | *P. organiaca* | Congost del Pont de la Torre | Spain | 31T | 363704 | 4679023 |
| C-Porg-080 | *P. organiaca* | Congost del Pont de la Torre | Spain | 31T | 363704 | 4679023 |
| C-Porg-081 | *P. organiaca* | Congost del Pont de la Torre | Spain | 31T | 363704 | 4679023 |
| C-Porg-082 | *P. organiaca* | Congost del Pont de la Torre | Spain | 31T | 363704 | 4679023 |
| C-Porg-083 | *P. organiaca* | Congost del Pont de la Torre | Spain | 31T | 363704 | 4679023 |
| C-Porg-085 | *P. organiaca* | Congost del Pont de la Torre | Spain | 31T | 363704 | 4679023 |
| C-Porg-086 | *P. organiaca* | Congost del Pont de la Torre | Spain | 31T | 363704 | 4679023 |
| C-Porg-087 | *P. organiaca* | Congost del Pont de la Torre | Spain | 31T | 363704 | 4679023 |
| C-Ppar-128 | *P. parva* | Pedraforca, subida por el canal | Spain | 31T | 393016 | 4676882 |
| C-Ppar-130 | *P. parva* | Pedraforca, subida por el canal | Spain | 31T | 393016 | 4676882 |
| C-Ppar-131 | *P. parva* | Pedraforca, subida por el canal | Spain | 31T | 393016 | 4676882 |
| C-Ppar-132 | *P. parva* | Pedraforca, subida por el canal | Spain | 31T | 393016 | 4676882 |
| C-Ppar-133 | *P. parva* | Pedraforca, subida por el canal | Spain | 31T | 393016 | 4676882 |
| C-Ppar-157 | *P. parva* | Sierra de Cadí | Spain | 31T | 395680 | 4682110 |
| C-Ppar-158 | *P. parva* | Sierra de Cadí | Spain | 31T | 395680 | 4682110 |
| C-Ppar-159 | *P. parva* | Sierra de Cadí | Spain | 31T | 395680 | 4682110 |
| C-Ppar-160 | *P. parva* | Sierra de Cadí | Spain | 31T | 395680 | 4682110 |
| C-Ppar-161 | *P. parva* | Sierra de Cadí | Spain | 31T | 395680 | 4682110 |
| C-Ppar-162 | *P. parva* | Sierra de Cadí | Spain | 31T | 395680 | 4682110 |
| C-Psp-001 | *P. sp* | Aigüestortes, Rocablanca | Spain | 31T | 339326 | 4718268 |
| C-Psp-193 | *P. sp* | Aigüestortes, Rocablanca | Spain | 31T | 339326 | 4718268 |
| C-Psp-194 | *P. sp* | Aigüestortes, Rocablanca | Spain | 31T | 339326 | 4718268 |
| C-Psp-195 | *P. sp* | Aigüestortes, Rocablanca | Spain | 31T | 339326 | 4718268 |
| C-Psp-362 | *P. sp* | Aigüestortes, Rocablanca | Spain | 31T | 339326 | 4718268 |
| C-Psp-402 | *P. sp* | Muntaya de Casamanya | Andorra | 31T | 382653 | 4715934 |
| C-Psp-403 | *P. sp* | Muntaya de Casamanya | Andorra | 31T | 382653 | 4715934 |
| C-Psp-405 | *P. sp* | Muntaya de Casamanya | Andorra | 31T | 382653 | 4715934 |
| C-Psp-489 | *P. sp* | Lo Forcallet, Montanyo de Llacs | Spain | 31T | 328536 | 4711093 |
| C-Psp-490 | *P. sp* | Lo Forcallet, Montanyo de Llacs | Spain | 31T | 328536 | 4711093 |
| C-Ptrans-109 | *P. carascalensis* | Pont d´Esquit | France | 30T | 694914 | 4759475 |
| C-Ptrans-115 | *P. carascalensis* | Pont d´Esquit | France | 30T | 694914 | 4759475 |
| C-Pvel-002 | *P. carascalensis* | Aizkorri | Spain | 30T | 555116 | 4755504 |
| C-Pvel-003 | *P. carascalensis* | Aizkorri | Spain | 30T | 555116 | 4755504 |
| C-Pvel-004 | *P. carascalensis* | Aizkorri | Spain | 30T | 555116 | 4755504 |
| C-Pvel-017 | *P. carascalensis* | Aldamin | Spain | 30T | 518240 | 4765250 |
| C-Pvel-020 | *P. carascalensis* | Aldamin | Spain | 30T | 518240 | 4765250 |
| C-Pvel-037 | *P. carascalensis* | Beriain | Spain | 30T | 583154 | 4748921 |
| C-Pvel-038 | *P. carascalensis* | Beriain | Spain | 30T | 583154 | 4748921 |
| C-Pvel-039 | *P. carascalensis* | Beriain | Spain | 30T | 583154 | 4748921 |
| C-Pvel-048 | *P. carascalensis* | Gorbea frente Aldamin | Spain | 30T | 517739 | 4765043 |
| C-Pvel-049 | *P. carascalensis* | Gorbea frente Aldamin | Spain | 30T | 517739 | 4765043 |
| C-Pvel-050 | *P. carascalensis* | Gorbea frente Aldamin | Spain | 30T | 517739 | 4765043 |

**Table S3.** Primers used for amplification and sequencing.

| Gene | Primer | Sequence | Reference |
|---|---|---|---|
| COI | LCO1490 (Fw) | 5' GGTCAACAAATCATAAAGATATTGG 3' | Folmer *et al*. (1994) |
| | HCO2198 (Rv) | 5' TAAACTTCAGGGTGACCAAAAAATCA 3' | Folmer *et al*. (1994) |
| 16S | 16SarI (Fw) | 5' CGCCTGTTTATCAAAAACAT 3' | Palumbi *et al*. (1991) |
| | 16SbrH (Rv) | 5' CCGGTCTGAACTCAGATCACGT 3' | Palumbi *et al*. (1991) |
| ITS1-5.8S | ITS1L (Fw) | 5' TCCGTAGGTGAACCTGCGGAAGGAT 3' | Hillis and Dixon (1991) |
| | 58C (Rv) | 5' TGCGTTCAAGATATCGATGTTCAA 3' | Hillis and Dixon (1991) |
| 5.8S-ITS2-28S | LSU-1 (Fw) | 5' CTAGCTGCGAGAATTAATGTGA 3' | Wade *et al*. (2006) |
| | LSU-3 (Rv) | 5' ACTTTCCCTCACGGTACTTG 3' | Wade *et al*. (2006) |

**References:**

Hillis, D.M., Dixon, M.T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q. Rev. Biol. 66, 411–453.

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G. 1991. The Simple Fool's Guide to PCR, Version 2.0. Department of Zoology, University of Hawaii, Honolulu, HI.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Wade, C.M., Mordan, P.B., Naggs, F. 2006. Evolutionary relationships among the Pulmonate land snails and slugs (Pulmonata, Stylommatophora). Biol. J. Linn. Soc. 87, 593–610.

**Table S4.** Landmark and semilandmark description.

| **Landmarks** | |
|---|---|
| ID | Description |
| L1 | Intersection of the peristome with parietal area |
| L2 | Intersection of the peristome with columellar area |
| L3 | Starting point of the suture |
| **Semilandmarks** | |
| ID | Description |
| SL1-SL22 | Peristome outline, from L1 to L2 (22 semilandmarks) |
| SL23-SL80 | Suture, from L3 to L1 (80 semilandmarks) |

**Table S5.** Evolutionary models selected using Akaike Information Criteria implemented in jModeltest for the different partitions of the ingroup.

| **Gene** | **Best model** | **Proportion of invariant sites** | **Gamma** |
|---|---|---|---|
| COI-1st | TrN+G | -- | 0.12 |
| COI-2nd | F81 | -- | -- |
| COI-3rd | GTR+G | -- | 1.33 |
| 16s | TIM1+I+G | 0.47 | 0.42 |
| ITS1 | HKY | -- | -- |
| 8.5s | JC | -- | -- |
| ITS2 | K80 | -- | -- |
| 28s | HKY | -- | -- |

**Table S6.** BPP results obtained without guide species tree for the candidate species recovered in mtDNA delimitation analyses before and after colapsing unsupported candidate species. Grey shades indicate not supported candidate species (PP < 0.95).

**13 candidate species**

| Candidate species | Posterior probabilities | | | |
|---|---|---|---|---|
| | Algorithm 0 | | Algorithm 1 | |
| G1 | 1.000 | 1.000 | 1.000 | 1.000 |
| G2 | 1.000 | 1.000 | 1.000 | 1.000 |
| G3a | 0.989 | 0.988 | 0.990 | 0.991 |
| G3b | 0.989 | 0.988 | 0.990 | 0.991 |
| G4 | 1.000 | 1.000 | 1.000 | 1.000 |
| G5a | 0.222 | 0.238 | 0.214 | 0.223 |
| G5b | 0.222 | 0.238 | 0.214 | 0.223 |
| G6 | 0.933 | 0.949 | 0.943 | 0.952 |
| G7 | 0.999 | 0.999 | 0.999 | 0.998 |
| G8a | 0.926 | 0.932 | 0.930 | 0.927 |
| G8b | 0.932 | 0.928 | 0.927 | 0.932 |
| G9a | 0.871 | 0.883 | 0.877 | 0.884 |
| G9b | 0.956 | 0.959 | 0.959 | 0.960 |

**10 candidate species**

| Candidate species | Posterior probabilities | | | |
|---|---|---|---|---|
| | Algorithm 0 | | Algorithm 1 | |
| G1 | 1.000 | 1.000 | 1.000 | 1.000 |
| G2 | 1.000 | 1.000 | 1.000 | 1.000 |
| G3a | 0.990 | 0.989 | 0.990 | 0.989 |
| G3b | 0.990 | 0.989 | 0.990 | 0.989 |
| G4 | 1.000 | 1.000 | 1.000 | 1.000 |
| G5 | 1.000 | 1.000 | 1.000 | 1.000 |
| G6 | 0.998 | 0.998 | 0.999 | 0.998 |
| G7 | 1.000 | 1.000 | 1.000 | 1.000 |
| G8 | 0.999 | 0.999 | 1.000 | 0.999 |
| G9 | 0.999 | 0.999 | 0.999 | 0.999 |

**Table S7.** Stacey delimitation results for the 9 best schemes obtained when using a reduced matrix of 46 individuals (the same as in BPP).

| **Count** | **Posterior probability** | **n clusters** | **Clusters** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8392 | 0.10 | 10 | G1 | G2 | G3a | G3b | G4 | G5 | G6 | G7 | G8 | G9 | |
| 6332 | 0.08 | 9 | G1 | G2 | G3a | G3b | G4 | G5 | G6+G9 | G7 | G8 | | |
| 5210 | 0.07 | 9 | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | | |
| 4991 | 0.06 | 8 | G1 | G2 | G3 | G4 | G5 | G6+G9 | G7 | G8 | | | |
| 3638 | 0.05 | 11 | G1 | G2 | G3a | G3b | G4 | G5 | G6 | G7 | G8a | G8b | G9 |
| 3293 | 0.04 | 11 | G1 | G2 | G3a | G3b | G4 | G5a | G5b | G6 | G7 | G8 | G9 |
| 3265 | 0.04 | 11 | G1 | G2 | G3a | G3b | G4 | G5 | G6 | G7 | G8 | G9a | G9b |
| 1909 | 0.02 | 10 | G1 | G2 | G3a | G3b | G4 | G5 | G6+G9b | G7 | G8 | G9a | |
| 1862 | 0.02 | 10 | G1 | G2 | G3a | G3b | G4 | G5 | G6+G9 | G7 | G8a | G8b | |
| … | | | | | | | | | | | | | |

# I. ARTIKULUA

## Multilocus DNA datuak eta 3D morfometria geometrikoa integratzea espezieen arteko mugak argitzeko *Pyrenaearia*-ren (Pulmonata: Hygromiidae) kasuan

- **Laburpena**
- **Eztabaida**
- **Ondorioak**

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# Multilocus DNA datuak eta 3D morfometria geometrikoa integratzea espezieen arteko mugak argitzeko *Pyrenaearia*-ren (Pulmonata: Hygromiidae) kasuan

## Laburpena

Espezieak modu zorrotzean zedarritzeko funtsezkoa da karaktere ezberdinak erabiltzea, datu guztiek (esaterako ezaugarri morfologikoek, molekularrek edo ekologikoek) ez baitituzte berdin islatzen espeziazio prozesuak. Morfometria geometrikoari eta 3D teknologiaren garapenari esker, egingarri bihurtu da datu morfologiko eta molekularrak modu esanguratsuan elkartzea orain arte forma aldaketak modu zehatzean kuantifikatzea eta neurtzea ezinezko zen taldeetan, gasteropodoen oskolean esaterako. Ikerketa honetan, espezie zedarritze metodo koaleszente *multilocus*-ak eta oskolari aplikatutako 3D morfometria geometrikoa konbinatu ditugu, *Pyrenaearia* lurreko barraskilo generoaren baitan espezieak zedarritzeko. Generoarentzat eskema taxonomiko berri bat eraiki da ondorioz, hamar espezie identifikatuz bere baitan. Bi espezie nominal sinonimizatu egin dira eta oraino ezezaguna zen espezie kriptiko bat identifikatu da. Gure aurkikuntzek berretsi egiten dute ebidentzia iturri ugariren konbinazioa garrantzitsua dela, datu molekularrek eta morfologikoek ez baitzuten, bereizita, emaitza bera eman. Are gehiago, datu morfologiko eta molekularren integrazioak oskolen forman alometria zein garrantzitsua den erakutsi du eta iradokitzen du oskolen bariazio morfologikoan populazio historiak eta ingurune ezberdinetarako hautespenak eragin konbinatua izan dutela. Gure taxonomia berriak eta berreraikuntza filogenetikoak proposatzen dute, Pleistozenoko glaziazioez gain, dispertsio pasiboak eta arroka substratuaren konplexutasunak ere eragina eduki ahal izan dutela genero honetako espeziazioan.

## Gako-hitzak

*Pyrenaearia*; espezie zedarritzea; 3D; morfometria geometrikoa; filogenia molekularra; taxonomia integratzailea

# 4. Eztabaida

## 4.1. Espezie zedarritzea

Ikerketa honetan, lehen aldiz, *Pyrenaearia* lur barraskilo generoko espezieen zedarritzeari ekin diogu, datu molekularrak eta 3D morfologia datuak elkartzen dituen hurbiltze integratzaile bat erabiliz.

Erabilitako bi zedarritze metodo molekularrek zedarritze eskema bera eman zuten, lorturiko bederatzi filotalde nagusiak espezietzat hartuz. Salbuespen bakarra G3 izan zen, Stacey-k espezie bakartzat hartu baitzuen eta BPP-k bi espezietan bereizita. Konputazio mugak direla eta, BPP-rentzat datu matrize murriztua erabili genuen eta G3ren kasuan, zehazki, 13 aletatik bost baztertuak izan ziren. Hurbiltze honen eragozpena da hautaketa prozesuan alerik dibergenteenak atxiki daitezkeela eta horrek etendura genetiko faltsua sor dezakeela. Izan ere, jakina da laginketa hutsuneek interpretazio genealogikoa eragotzi dezaketela (Lohse, 2009; Niemiller et al., 2012). Ale gehiagorekin Stacey-k bi taldeen arteko desberdintasunik detektatu ez izanak iradoki zezakeen BPP-k detektaturiko etendura genetikoa artefaktu bat zela, ale batzuk baztertzeak eragindakoa. Horrela, BPP-rekin erabilitako matrize murriztuarekin Stacey berrabiarazi genuenean, BPP-ren emaitza bera lortu genuen, G3 bitan banaturik (**S7 taula osagarria**), desberdintze hori artefaktu bat zela berretsiz.

Oskolaren formaren bariazioa generoaren baitan nagusiki bi norabidetan gertatzen da. Oskol bariazio nagusia, PC1-ak erakusten duen bezala, kiribiltzean oskol tutuaren sekzioaren handitzearekin lotuta dago. *Pyrenaeraria* oskolak, bestalde, nabarmenki desberdinak dira irekiduraren forman, horrek espiraren altuera baldintzatzen duelarik (PC2-a bariazio totalaren %28-aren erantzule da); aldaketa honetan gradiente bat ikus dezakegu irekidura borobil eta oskol globosoetatik, irekidura luze eta oskol nahiko zapaletaraino. Oskol formaren karaktere hauek, beste informaziorik gabe, bost talde mugatzeko balio izan zuten, filotalde nagusiekin bat egiten ez zutenak, G2-ren kasuan izan ezik. Hala ere, patroi morfologiko batzuk inferitu daitezke PC1-etik. G1 kladoan PC1-aren balio guztiak -0,005 baino baxuagoak izan ziren, oskol tutuaren handipen konstanterako forma-espazioa hartzen zuelarik. G2-k ere balio negatiboak zituen -0,064 eta -0,029 artean. Gainontzeko filotaldeen (G3-9) balioak -0,017-tik gorakoak izan ziren (G9-ren *outlier* garbi bat salbu, -0,027 balioa zeukana) eta, beraz, talde hauek erakusten duten gradiente morfologikoa xumeki konstantea den oskol tutuaren handipen batetik, handipen logaritmikoago baterakoa da, norbanako

gehienek handipen logaritmikoaren forma-espazioa hartzen dutelarik. Honek esan nahi du, -0,017 eta -0,005 artean gainjartze gune bat dagoen arren, oskola kiribildu ahala tutuaren sekzioak jasaten duen handipena gai dela bi *Pyrenaearia* klado nagusien bereizketa egiteko, G2-ren salbuespenarekin. Ordea, irekiduraren formaren eta espiraren altueraren bariazioek ez zituzten talde garbiak definitu. Karaktere hauentzat oskolak oso polimorfikoak zirela ikusi zen eta filotaldeek distribuzio zabala erakutsi zuten forma-espazio osoan zehar, nahiz eta batzuek besteek baino bariazio gutxiago agertu. Beste lur barraskilo batzuen espiraren altueran ere bariazio handia deskribatu da (Cain, 1977; Fiorentino et al., 2008; Razkin et al., 2017; Stankowski, 2011).

Gure harridurarako, G1 filotaldearen baitan, datu molekularrek ez zuten ezberdintasun genetikorik jaso, baina *P. daanidentata* espezie nominaleko norbanakoek ondo bereizitako talde morfologikoa osatu zuten. Are gehiago, entitate morfologiko hau genero osoan desberdinena izan zen, bere Procrustes bariantzak generoaren bataz bestekoarekiko 0,0206 balioa zuelarik, hurrengo talde dibergenteenarekin konparatuz 3,5 aldiz handiagoa dena. Formaren desbiderapen handi honek erakusten duena izan daiteke *P. daanidentata* baliozko espezie bat dela eta datu molekularrekin espeziea detektatu ez izana, berriz, espeziazio berrietan ohikoa den arbasoen polimorfismoen banaketa estokastikoaren edo hibridazioaren ondorio izan liteke (Edwards and Knowles, 2014), edo espeziazio selektibo baten kasuan *loci* neutralak erabiltzearen ondorio (Solís-Lemus et al., 2015). Are, morfologia datu gordinen arabera, G3, G5, G7, G8 eta G9 ezin ziren elkarrengandik bereizi, G4 kladoa G6-tik bereizezina zen bezala. Hala ere, datu molekularrak eta morfologikoak integratu genituenean eta datu molekularrekin lorturiko taldeen arabera forma aldaketaren norabidea kontuan hartu zenean, guztien artean diferentzia morfologikoak aurkitu ziren, filotalde hauen arteko diferentzia berretsiz. Beraz, *Pyrenaearia* filotalde batzuetako forma bariazio intraespezifikoak oskol morfologiaren gainjartze bat eragiten du, baina bariazio hori talde bakoitzean gertatzen den modua desberdina da norabide eta hedadurari dagokionez, taldeak bereizgarri eginez.

## 4.2. Taxonomia proposamen berria

Espezieak zedarritzeko gure hurbiltze integratzailearekin, 10 espezie identifikatu ziren *Pyrenaearia* generoan eta taxonomiaren eguneratze bat proposatzen dugu.

*P. cantabrica*, *P. oberthueri* eta *P. daanidentata* espezie nominalak, Kantauriar Mendietan endemikoak, zedarritze metodo molekularren bidez espezie bakar gisa jaso ziren (G1). Hala ere, datu morfologikoek *P. daanidentata*-rentzat espezie baliotasuna babesten dute zalantzarik gabe. *P. cantabrica* eta *P. oberthueri*-ren arteko bereizketa sarritan jarri da zalantzan eta forma bariazioa altitude klina baten araberako forma adaptazioa izan daitekeela proposatu izan da (Elejalde et al., 2009; Ortiz de Zárate, 1956; Raven, 1988). Gure emaitzek interpretazio hori babesten dute. Morfologikoki espezieek talde jarrai bat osatzen dute, balio tarte berbera erakutsiz PC1-aren balioentzat. PC2-ari dagokionez, ordea, *P. oberthueri* aleek balio baxuagoak aurkeztu dituzte eta *P. cantabrica* espeziekoek balio altuagoak, baina etendurarik gabe beraien artean. Honek esan nahi du kiribildu ahala berdina dela beraien oskol tutuaren handipena, baina gradiente bat dagoela oskol borobilagoetatik zapalagoetara. Are gehiago, gradiente honek partzialki bat egiten du altitudearekin: altitude handiko norbanakoak borobilagoak dira eta eremu baxuetakoak zapalagoak. Gure emaitza molekularrak eta morfologikoak ikusita, bi espezie hauek Kantauriar Mendietan zehar hedatzen den espezie bakar gisa hartzea proposatzen dugu *P. cantabrica* izenpean, lehentasuna baitu *P. oberthueri* izenarekiko. Ostera, *P. daanidentata* espeziearen desberdintzapen morfologikoa ez da sartzen altueraren arabera aldatzen den klina adaptatibo honen barruan. *P. daanidentata* "*Collado del Perro – Canal del Burro*" inguruan besterik ez da ezagutzen eta, oskol forma ezberdinaz aparte, peristoman hortz nabarmen bat aurkezten duen bakarra da genero osoan (Raven, 1988). Ezberdintasun morfologiko hauen eragileak ugalketa oztopatzen duten langa intrintseko edo estrintsekoak zein eskala txikiko nitxo zatiketa izan daitezke, edo baita isolamendu geografikoaren ondoriozko espeziazio alopatrikoa ere. Baliteke hemen analizatutako genoma zati murriztua morfologiak adierazten duen *P. daanidentata*-ren dibergentzia babesteko gai ez izatea (Edwards and Knoweles, 2014), eta *P. cantabrica* parafiletiko agertzearen erantzule ere izan daiteke hori. Ondorioz, espezie honek espezie estatusa mantendu behar duela defendatzen dugu, bere desberdintzapen molekularraren egiaztapena datu-base askoz ere handiago baten zain gelditzen den arren.

*P. parva* (G2), *P. organiaca* (G4) eta *P. molae* (G6) baliozko espezie gisa jasotzen dira hemen. *P. parva* analisi molekularrek eta morfologikoek independenteki babestu zuten. Metodo molekularrek *P. organiaca* eta *P. molae* espezie bezala berreskuratu zituzten eta morfologikoki beste *Pyrenaearia* espezieekiko ezberdinak ziren, nahiz eta beraien artean forma aldaketaren norabidea kontuan hartzean bakarrik desberdindu zitezkeen. Gainera, hiru espezie hauek oskolaren ildaska antolamendu

bereizgarriari eta ondo zedarritutako banaketa geografikoari esker ere bereiz daitezke (Fagot, 1905; Haas, 1924; Ortiz de Zárate, 1956; Puente, 1994; Vilella, 1963).

Metodo molekularrek *Pyrenaearia* generoa osatzen duten gainerako bost espezieak zalantzarik gabe zedarritu zituzten, informazio morfologikoak ere babestu zuelarik. Hala ere, espezie hauen arteko desberdintzapen morfologikoa espezieen barruko forma aldaketaren norabidea kontuan hartzean bakarrik antzeman zen, forma bariazio intraespezifikoaren eraginez haien morfologia gainjartzen delako. Hauetako hiru espeziek, *P. cotiellae* (G7), *P. carascalopsis* (G3) eta *P. navasi* (G5), espezie nominalekin bat egiten zuten eta, beraz, haien espezie estatusa mantendu behar dute. *P. cotiellae* Cotiella Mendigunera mugatzen da, *P. carascalopsis* erdialdeko Pirinioetako ipar isurialdean bakarrik aurkitzen den bitartean. Pirinioetako ur-banalerroa ere identifikatu izan da beste lur barraskilo espezie batzuen banaketa mugatzen duten faktoreen artean (Gittenberger, 1973; Kokshoorn et al., 2010). Hemen aztertutako ezaugarriez aparte bere oskol marroi eta hauskorrari esker ere desberdindu daitekeen *P. navasi*-ren barruan, Prieto-k (1991) bi subespezie aintzat hartzen zituen: 1600 m-tatik gorako arroka azaleratzeetan bizi den eta txikiagoa den *P. navasi navasi* eta *P. navasi sylvatica*, handiagoa eta borobilagoa, bakarrik pago/haritz baso puntu batean ezagutzen dena, generoarentzako guztiz ezohikoa den habitata. Metodo molekularrek ez zuten inolako banaketarik aurkitu subespezie hauen artean. Horrela, *P. n. sylvatica*-ren aleak (*P. navasi*-03 eta *P. navasi*-04) *P. n. navasi* norbanakoen artean nahasturik agertu ziren G5 kladoaren barruan. Zoritxarrez, subespeziearen *locus typicus* zehatza eta bere ingurumaria tentuz lagindu baziren ere, ez genuen *P. n. sylvatica*-ren oskolik aurkitu analisi morfometrikoetarako eta bere desberdintzapen morfologikoa ezin izan zen ebaluatu. Prieto-k (1991) subespezie hauek Kuaternarioko glaziar atzerakada bati erantzunez eboluzionatu zutela proposatu zuen. Hala ere, *locus typicus*-ean ale berririk aurkitu ez izateagatik, desberdintasun molekular faltagatik eta bere habitat ezohikoa dela eta, litekeena da populazio hura gorago bizi den populazio batetik eroritako norbanako batzuek sortu izana eta orain desagertu izana.

G8 eta G9 leinuak ere espezie gisa jaso ziren. G8-k Noguera Ribagorçana haranaren ekialdeko *P. carascalensis* norbanakoak biltzen zituen, G9-k haran horren mendebaldeko Pirinioetako *P. carascalensis* norbanakoak eta Euskal Mendietako *P. velascoi* aleak barne hartzen zituen bitartean. *P. carascalensis*-en *locus typicus* zehatza, "*Forêt de Carascal en Aragon*", ez da ezagutzen (Prieto, 1986; Puente, 1994). Hala ere, G8 filotaldearen populazio guztiak Aragoi erkidegotik

kanpo daude. Beraz, *P. carascalensis* izena G9 filotaldera mugatu behar da. *P. velascoi* norbanakoak G9 taldearen barruan sartu ziren eta, ondorioz, *P. velascoi* izena *P. carascalensis*-en sinonimiara batu behar da, *P. velascoi* izena berriagoa baita. Orain arte kontuan hartu gabeko G8 leinuarentzat ez dago izen eskuragarririk talde honetako aleak beti *P. carascalensis* edo *P. carascalopsis* kontsideratu baitira (Altimira, 1965, 1994; Puente, 1994). Horregatik, espezie kriptiko berri honi momentuz *Pyrenaearia* sp. G8 deitu diogu, bere deskribapen formalaren zain (in prep.).

## 4.3. Oskolaren ezaugarriak *Pyrenaearia*-n

Ikerketa honetan, 2D analisiak erabili beharrean, 3D morfometria geometrikoaren bidez aztertu dugu barraskilo oskolen bariazioa, batez ere hiru-dimentsiotako egitura bat. Morfometria geometrikoan, 3D egiturak sarritan aztertu izan dira 2D irudiak erabiliz. Berez, barraskilo oskoletan eginiko morfometria geometrikoko lan gehienak 2D irudietan oinarritu dira eta oso gutxitan erabili dira 3D irudiak (Márquez and Averbuj, 2017; Márquez et al., 2011; Scalici et al., 2016). 3D egitura bat 2D irudiekin analizatzeak informazio galera bat dakar ezinbestean eta baita zehaztasun falta ere tamaina eta forma estimatzean (Cardini, 2014). Garezurretatik lortutako 2D eta 3D datuen emaitzen konparaketak erakutsi zuenez, lehenengoek zehaztasun falta aurkezten dute, hurbiltzea desegoki bihurtuz datu intraespezifikoak lantzeko (edo baita espezie kriptikoen konplexuak lantzeko ere) eta datu interespezifikoak aztertzerakoan arretaz jokatzera behartuz (Cardini, 2014). Are gehiago, nahiko zapala den sagu barailarentzat ere, 3D hurbiltzeak 2D-ak baino hobeto funtzionatzen du (Navarro and Maga, 2016). Moluskuetan, Scalici et al.-ek (2016) 2D bidez lortutako datuak bi inguru ezberdinetako bi muskuilu populazioren arteko forma aldaketak jasotzeko gauza ez zirela aurkitu zuten, 3D datuak gai ziren bitartean. Gure kasuan, ez dugu inolako konparaketarik egin 3D eta 2D hurbiltzeen artean eta ezin dugu ondorioztatu haien artean desberdintasun esanguratsurik dagoen edo ez, espezieak zedarritzerako garaian. Hala ere, ez dago zalantzarik gure 3D landmark-ek eta semilandmark-ek jasotako formaren informazioa gehiago hurbiltzen dela oskolen egiazko formara 2D datuek inoiz lortuko dutena baino.

Bariazio morfologikoa populazio historiaren ondorioz sortutako desberdintasun genetikoek edo inguru ezberdinetan suertatutako hautespenak eragin dezakete. Gastropodoen oskolentzat, bai populazio historia (Dillon and Jacquemin, 2015; Dowle et al., 2015) eta bai inguru zehatzekiko adaptazioa (Stankowski, 2011; Welter-Schultes, 2000) identifikatu izan dira bariazio morfologikoaren erantzule gisa. Berez, kasu gehienetan, azken forma bien konbinazioak determinatzen duela uste da, faktore bakoitzaren parte-hartzea espeziearen araberakoa izanik (Noshita

et al., 2016). *Pyrenaearia*-n, morfologikoki guztiz ezberdinak diren *P. cantabrica* eta *P. carascalensis* espezieak gutxienez Euskal Mendietako bi puntutan sinpatrian bizitzeak, oskolaren formaren determinazioan parte-hartze genetikoa badela adierazten du. Era berean, bi klado nagusiak kiribiltzean oskol tutuaren sekzioaren handipenaren patroiaren arabera ezberdindu daitezke eta horrek ezaugarri horretan faktore genetiko batek esku-hartzen duela adierazten du. Bestalde, espezieen barruko desberdintasun maila haien distribuzioaren zabalerarekin erlazionatuta dagoela aurkitu genuen, banaketa zabalagoa zutenek bariazio gehiago aurkezten zutelarik mugatuagoak zeuden espezieek baino. Gainera, *P. cantabrica*-k altitudearen araberako bariazio morfologiko klina bat aurkezten duela determinatu genuen. Datu hauek iradoki dezakete oskolaren forma inguruneko adaptazioaren emaitza ere badela.

Ikerketa honetan, tamainak oskolaren forman eragina daukan ere aztertu genuen eta alometriak formaren bariazioaren %16.8-a azaltzen duela aurkitu genuen, alometria ebolutiboak %37.7 azaltzen zuen bitartean. Balio hauek beste talde batzuetan alometriak eragiten duen forma bariazioaren proportzioarekin alderatuz gero, balio altuenen artean aurkitzen dira (Cardini et al., 2015; Figueirido et al., 2010; Gonzalez et al., 2011; Klingenberg and Marugán-Lobón, 2013). Gure proportzio altuak Urdy et al.-en (2010) emaitzekin bat datoz. Autore hauek oskolaren morfogenesia modelizatu zuten forma-askeko bektore modelo bat erabilita eta tamainak barraskiloen oskolaren formaren bariazioan eragin handia zeukala ondorioztatu zuten. Alometriak eragindako forma aldaketak eta PC1-ak eragindakoak antza handia zutela antzeman genuen. Beraz, alometria generoaren barruan forma ezberdintasunak eragiten dituzten faktoreetako bat da. Hala ere, PC1-ak azaldutako forma bariazioa alometriak azaldutakoaren proportzioa baino altuagoa izan zen, beraz, oskolaren dibertsifikazioan beste prozesu batzuek ere parte hartu behar izan dute. *P. daanidentata*-k alometriaren joera orokorrarekiko aurkezten duen desbideraketak are gehiago indartzen du interpretazio hau.

## 4.4. Patroi filogeografikoak

Izaki bizidunek jasandako prozesu ebolutiboak inferitzeko ezinbestekoa da espezieak ondo zedarrituta egotea eta guztiz ebatzitako filogeniak edukitzea. Hemen, espezieak zedarritzeko hurbiltze integratzaile bat erabiliz eta Elejalde et al.-en (2009) bai laginketa eta bai markatzaile molekularren kopurua handituz, taxonomia berria proposatzen dugu *Pyrenaearia*-rentzat. Gainera, bere filogeniaren ebazpena hobetzen dugu, batez ere nodo basalenetan.

*Pyrenaearia* generoa hotzera egokitutako taxon gisa hartu izan da, bere espezie gehienek banaketa subalpetarra aurkezten baitute (Ortiz de Zárate, 1956; Prieto, 1986; Puente, 1994). Harrigarriki, *P. cantabrica* eta *P. carascalensis* espezieak altitude tarte zabalean bizi daitezkeela behatu dugu (50 m-tatik 2200 m-tara eta 460 m-tatik 2640 m-tara hurrenez hurren). Are gehiago, 27 ºC-ra aise iristen den 900 m-ko inguruan bizi den *P. molae*-k (Hijmans et al.-en (2005) WorldClim datu-basetik eratorrita) eta 500 m-tan agertzen den *P. organiaca*-k ez zuten talde monofiletiko bat osatu. Beraz, altitude handiei lotutako tenperatura baxuak ez dira beharrezkoak *Pyrenaearia* espezie batzuentzat, arroka azaleratze laiotzak eskura dauden bitartean. Ondorioz, nahiz eta hotzera egokitutako taxon bat den, genero honek beroarekiko tolerantzia gradu bat aurkezten du eta, horrela, arroka azaleratze laiotzen presentzia garrantzitsuagoa izan daiteke generoaren banaketa determinatzerako orduan.

Elejalde et al.-ek (2009) moluskuen dibertsifikazio tasa orokorra erabili zuten *Pyrenaearia*-ren barneko dibergentzia denborak kalkulatzeko. Haien kalkuluen arabera, lortu zituzten lau klado nagusien arteko banaketa, gure G1, G2, G3 eta G4-9 taldeekin bat egiten dutenak, Pliozenoa baino lehen gertatu zen. Klado horien barruko espeziazioa, ordea, Pleistozenoko ziklo glaziarrek modelatu zutela proposatu zuten. Prieto-k (1991) ere generoaren espeziazioan glaziazioek garrantzi handia izan zutela proposatu zuen. Zinez, Pleistozenoko aldaketa klimatikoek mendiko organismo askoren espeziazioan parte hartu dutela erakutsi izan da (Bidegaray-Batista et al., 2014; Dépraz et al., 2008; Gittenberger et al., 2004; Harl et al., 2014a ; Mouret et al., 2011; Pauls et al., 2006). Hala ere, Pirinioetako *P. cotiellae*, *P.* sp. G8 eta *P. carascalensis* espezieen eta urruti, Tarragonan, agertzen den *P. molae*-ren artean harreman estua antzeman genuen. Honek, *P. molae* dispertsio pasibo baten ondorioz finkatu zela adieraz dezake, beste hainbat gastropodo lurtarrentzat deskribatu izan den bezala (Gittenberger et al., 2006).

Harrigarriki, nahiz eta bi mendilerroek antzeko zabalera daukaten eta biek Kuaternarioan zehar glaziazio handia jasan zuten (Hughes et al., 2006), Kantauriar Mendietan bi *Pyrenaearia* espezie baino ez dira aurkitzen, Pirinioetan sei espezie bizi diren bitartean. Kantauriar Mendietan kareharriek inguru zabalak hartzen dituzte eta, glaziarren eta erreken erosioaren eraginez, haran sakon, estu ugari eta harrizko horma asko daude inguruan zehar sakabanatuta (Gutiérrez-Elorza, 1994). Honek mendilerro honetan *Pyrenaearia*-rentzat habitat egoki asko daudela esan nahi du eta, ondorioz, populazioek haien artean gertu dauden area zabalak hartzen dituzte, gene fluxua erraztu dezakeena. Pirinioek, ordea, arroka substratu konplexuagoa aurkezten dute (Dendaletche, 1991; Gutiérrez-Elorza,

1994). Pirinio Axialak, gailur altuenak biltzen dituenak, arroka sedimentarioz (kareharriak eta hareharriak), plutonikoz (granitoa) eta metamorfikoz (marmola, kuartzita eta arbelak) osatuta daude, Aurre-Pirinioak eta Barruko Mendilerroak batez ere kareharriz osatuta dauden bitartean. Nahiz eta *P. navasi* substratu silizeotan bizitzeko gai den, gainerako *Pyrenaearia* espezieek kareharriak behar dituzte bizirauteko. Beraz, Pirinio Axialaren inguru silizeoek populazioen artean distantzia handiagoak egotea dakarte eta honek gene fluxua oztopatu dezake mendilerro honetan, espeziazioa bultzatuz. Are gehiago, dibertsitate patroi honek derrigorrez kareharria behar duen *Abida* lur barraskilo generoaren antza dauka, zeinak Kantauriar Mendietan endemikoa den espezie bakarra daukan, Pirinioetan oso dibertsoa den bitartean (Gittenberger, 1973). Ikerketa filogeografiko batek ere Alpeetako kareharrizko habitat egokien isolamendua identifikatu du *Orcula* barraskilo karefilo generoaren dibertsifikazio iturri garrantzitsu gisa (Harl et al., 2014b).

## 4.5. Ondorioak

Espezieak zedarritzeko *multilocus* datu genetikoak eta informazio morfologikoa elkartzen dituen hurbiltzetik lortutako emaitzetan oinarrituta, zuzenketa taxonomiko bat proposatzen dugu *Pyrenaearia* lur barraskilo generoarentzat. Gure datuek, orain arte aintzat hartua ez zen espezie kriptiko bat identifikatzeaz gain, bi espezieren sinonimizazioa babestu dute eta baita espezie nominal gehienen baliozkotasuna berretsi ere. Emaitzok espezieak zedarritzeko hainbat karaktereren integrazioa ezinbestekoa dela erakutsi dute. Izan ere, generoko espezie batzuk metodo molekularrek haien existentzia ezagutarazi zutenean bakarrik antzeman ziren eta espezie bat (*P. daanidentata*) ezin izan zen bereizi lan honetan erabilitako markatzaile molekularren bidez. Generoaren oskolaren forma bai populazio historiaren bai ingurunerako adaptazioaren emaitza dela dirudi eta alometria oskolaren forman eragin garrantzitsua daukan faktore bat bezala identifikatu da. *Pyrenaearia* beroarekiko tolerantzia gradu bat daukan hotzera egokitutako taxon gisa proposatzen dugu, arroka azaleratze laiotzen presentzia generoaren banaketaren faktore erabakigarri gisa aurkezten delarik. Pirinioetako substratu konplexutasun handiagoak generoaren dibertsifikazioa bultzatzen duela dirudi eta mendilerro honetako aniztasun handiagoaren erantzule izan daiteke.

## Esker onak

L.J. Chueca, J. Corbella, I. Fernández eta G. Guillén-en laguntza eskertu nahi dugu lagin bilketan. Lan hau Eusko Jaurlaritzak "*Systematics, Biogeography and Population Dynamics*" Ikerketa Taldeari emandako diru-laguntzarekin (IT575-13) finantzatua izan da partzialki. A. Caro-k Eusko Jaurlaritzaren Hezkuntza, Unibertsitate eta Ikerketa Sailaren doktoretza bekaren babesarekin burutu zuen lana (Ref. PRE_2015_2_0191).

# PAPER II

## Molecular phylogeny and biogeography of the land snail subfamily Leptaxinae (Gastropoda: Hygromiidae)

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*Molecular phylogenetics and evolution* (under review)

# Molecular phylogeny and biogeography of the land snail subfamily Leptaxinae Gastropoda: Hygromiidae)


## Abstract

The subfamily Leptaxinae is included within the highly diverse land snail family Hygromiidae. In the absence of clear diagnostic morphological differences, the subfamily status is currently based solely on molecular information and includes three disjunctly distributed tribes, Leptaxini, Cryptosaccini and Metafruticicolini. However, the phylogenetic relationships among these tribes are not fully resolved and the clustering of some of the genera to the tribes is not statistically supported. To resolve the relationships within Leptaxinae and their position within Hygromiidae, we reconstructed their phylogeny using a multi-locus approach with two mitochondrial genes and eight nuclear markers. The phylogeny was further calibrated and an analysis of ancestral area estimation was carried out to infer the biogeographic history of the group. We elevated Metafruticicolini to subfamily level (Metafruticicolinae) and we restricted Leptaxinae to Cryptosaccini and Leptaxini. The Lusitanian genus *Portugala* was moved to Leptaxini, previously containing only the Macaronesian genus *Leptaxis*. Within Cryptosaccini, a new genus strictly confined to the Sierra de la Cabrera (Spain) is described, *Fractanella* gen. nov. According to our results, Leptaxinae originated in the Early Miocene in the Iberian Peninsula, from which the Macaronesian Islands were colonized. Due to the old split recovered for the divergence between Macaronesian and Iberian lineages, we hypothesize that this colonization may have occurred via the once emerged seamounts located between the archipelagos and the European and African continents, although this could also have occurred through the oldest now emerged islands of Macaronesia. In the Iberian Peninsula, the climatic shift that began during the Middle Miocene, changing progressively from subtropical climate towards the present-day Mediterranean climate, was identified as an important factor shaping the subfamily's diversification, along with Pleistocene climatic fluctuations.



## Keywords

Hygromiidae; Leptaxinae; systematics; phylogeography; multi-locus; dart apparatus

# 1. Introduction

To establish a stable classification, but more importantly to understand the evolutionary processes promoting a high level of speciation in some lineages and not in others, an adequate knowledge about the phylogenetic relationships is needed. Moreover, when properly calibrated, well supported phylogenies allow for the estimation of the timing of the emergence of new lineages, providing crucial information for testing alternative hypotheses on the mechanisms that generated the observed diversity and to infer biogeographical patterns (Weir and Schluter, 2008). However, land snails, and especially the super-diverse superfamily Helicoidea Rafinesque, 1815, are notorious for unstable systematics, from genus level (Chueca et al., 2018) to above family level (Gómez-Moliner et al., 2013; Groenenberg et al., 2011; Hugall and Stanisic, 2011; Köhler and Criscione, 2015; Neiber et al., 2017; Perez et al., 2014; Razkin et al., 2015) which has hindered the understanding of their evolutionary history and has prevented their use to test biogeographical hypotheses.

The Leptaxinae Boettger, 1909 is included within the highly diverse land snail helicoidean family Hygromiidae Tryon, 1866. The Hygromiidae systematics above specific level has primarily been based on the morphology of the genital system, particularly on the structure of the accessory copulatory organs (or stimulatory organs) consisting on mucous glands and a dart apparatus complex constituted by dart sacs and accessory sacs (Nordsieck, 1993; Puente, 1994; Schileyko, 1991). However, the molecular phylogeny of Razkin et al. (2015) pointed out a high level of homoplasy in this anatomical character and they established a new classification dividing the former Hygromiidae delimited by genital anatomy into three new families, Hygromiidae, Geomitridae Boettger, 1909 and Canariellidae Schileyko, 1991. This division was further confirmed by Neiber et al. (2017) who performed a comprehensive molecular phylogeny of Hygromiidae *sensu* Razkin et al. (2015) covering nearly the complete genus-level diversity of the family. This work further showed a striking capacity within the newly delimited Hygromiidae for changing the number and even the basic structure of their accessory copulatory organs by parallel evolution in several lineages independently, emphasizing the importance of additional sources of information to gain adequate knowledge about their systematics. According to Neiber et al. (2017), the current Hygromiidae consists of three subfamilies, Hygromiinae, Leptaxinae and Trochulinae Lindholm, 1927. Nevertheless, without neat diagnostic morphological differences, the status of these subfamilies is based solely on molecular information and, since their phylogeny was not fully resolved, the relationships between them and even their status remain uncertain.

The Leptaxinae *sensu* Neiber et al. (2017) includes three disjunctly distributed tribes, Leptaxini, Cryptosaccini Neiber, Razkin and Hausdorf, 2017 and Metafruticicolini Schileyko, 1972. Leptaxini comprises only the Macaronesian genus *Leptaxis* Lowe, 1852 present in the Azores, Madeira and Cape Verde archipelagos. Cryptosaccini is formed by the Iberian endemisms *Cryptosaccus* Prieto and Puente, 1994, *Portugala* Gittenberger, 1980, *Mengoana* Ortiz de Zárate y López, 1949 and *Pyrenaearia* Hesse, 1921. Finally, three genera make up the Mediterranean tribe Metafruticicolini: *Hiltrudia* Nordsieck, 1993 from the Western Balkan Peninsula, *Cyrnotheba* Germain, 1929 endemic to Corsica and *Metafruticicola* Ihering, 1892 with a wide distribution in the eastern Mediterranean region. In the case of Leptaxinae, however, it is not only that its status is not statistically supported but even the phylogenetic relationships among its tribes are not fully resolved and, moreover, the clustering of some of the genera to the tribes is not statistically supported, indicating that further investigation is required to achieve an adequate classification. Furthermore, within this subfamily the high diversity of *Pyrenaearia* (10 sp.), *Leptaxis* (13 sp.) and *Metafruticicola* (16 sp.) contrasts with the low number of species in the other genera in the subfamily, but the lack of adequate knowledge about their phylogenetic relationships hinders the understanding of the evolutionary processes responsible for this richness pattern since it also averts the reconstruction of the chronological and geographical scenario of their diversification.

In this paper, we reconstruct the phylogeny of Leptaxinae *sensu* Neiber et al. (2017) on the basis of an extensive taxonomic sampling using two mitochondrial genes (*COI* and *16S*), the nuclear rDNA gene cluster and four nuclear markers recently identified for the Geomitridae genus *Candidula* Kobelt, 1871 (Chueca et al., 2018). By the inclusion of novel, independently inherited nuclear genetic markers, a deeper understanding of the phylogenetic relationships of the group is expected. The phylogeny was further calibrated and an analysis of ancestral area estimation was carried out to infer the evolutionary processes undergone by the group. More specifically, we aim to (1) solve the phylogenetic relationships within Leptaxinae, (2) determine their position within Hygromiidae, (3) update their classification and (4) reconstruct the temporal and geographical framework of their diversification.

# 2. Materials and Methods

## 2.1. Taxonomic sampling

As representatives of the ingroup taxa we included 41 specimens covering the complete genus-level diversity of Leptaxinae. A total of 16 specimens were selected to capture the diversity of the other Hygromiidae, including the Trochulinae tribes

Ciliellini Schileyko, 1970, Archaicini Schileyko, 1978, Ganulini Neiber, Razkin and Hausdorf, 2017, Trochulini, Ashfordiini Neiber, Razkin and Hausdorf, 2017 and Monachaini Wenz, 1930 (1904) and the Hygromiinae tribes Hygromiini and Perforatellini Neiber, Razkin and Hausdorf, 2017 (see Neiber et al., 2017). Additionally, five specimens of the closely related family Geomitridae representing the tribes Geomitrini, Helicellini Ihering, 1909, Trochoideini Nordsieck, 1987 and Cernuellini Schileyko, 1991 were included. Furthermore, the species *Otala lactea* (Müller, 1774), *Massylaea vermiculata* (Müller, 1774), *Corneola desmoulinsii* (Farines, 1834) and *Marmorana muralis* (Müller, 1774) of the more distantly related family Helicidae Rafinesque, 1815 (see Razkin et al., 2015) were used as outgroups. Sampling site data and voucher numbers are provided in **Supplementary Table S1**.

## 2.2. DNA extraction, PCR amplification and sequencing

Newly collected material was preserved in 96% ethanol, while some of the museum specimens were stored in 70% ethanol. Genomic DNA was extracted from a piece of foot using the DNAeasy Tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer's guidelines.

For multi-locus analyses, we selected two mitochondrial genes, the cytochrome c oxidase subunit I (*COI*) and the 16S RNA ribosomal subunit (*16S*), along with the nuclear rDNA gene cluster (including the 3′ end of the ITS1 region, the 5.8S rRNA gene, the complete ITS2 region and the 5′ end of the large subunit 28S rRNA gene (*ITS1-5.8S-ITS2-28S*)) and four additional nuclear DNA regions: *60SL17*, *60SL13*, *60SL7* and *RPL14*. Primer pairs, both previously published and newly designed internal ones, are shown in **Supplementary Table S2**. General PCR conditions for DNA amplification were as follows: an initial denaturation step at 96°C for 1 min, 35 cycles of 30 s at 96°C, 30 s at 55–64°C (depending on the annealing temperature of the primer pairs, see **Supplementary Table S2**), and 1 min at 72°C, and a final extension step at 72°C for 10 min. PCR products were purified and sequenced at Macrogen, Inc. using either an ABI3730XL or an ABI3700 sequencer. The resulting forward and reverse sequences were assembled using Geneious 8.0.2 (Kearse et al., 2012), and checked for errors/ambiguities; polymorphic sites were coded according to the IUPAC-IUB code. Sequences available from the studies of Chueca et al. (2018), Neiber et al. (2017) and Razkin et al. (2015) were downloaded from GenBank (**Supplementary Table S1**).

## 2.3. Phylogenetic analyses

Each marker data set was aligned with the Probabilistic Alignment Kit algorithm (PRANK) (Löytynoja and Goldman, 2005), which is thought to outperform other

alignment methods for indel-rich sequences (Löytynoja and Goldman, 2008). The algorithm was run with the F+ option and, for the *16S* and *ITS1-5.8S-ITS2-28S* data sets, a structure model Fast/Slow was set. Molecular character statistics, including parsimony informative sites and base frequency, were calculated with MEGA v.7.0.14 (Kumar et al., 2016). Combined alignment including outgroups is available from TREEBASE (accession number 23008).

Both Maximum Likelihood (ML) and Bayesian Inference (BI) phylogenetic analyses were performed on the combined dataset partitioned by genes and further dividing COI into three partitions according to codon positions as suggested by PARTITIONFINDER v.1.1.0 (Lanfear et al., 2012). ML analyses were conducted with RAxML v.8.2.10 (Stamatakis, 2014) under the GTRCAT model and with 1000 nonparametric bootstrapping replicates to assess node support. MrBayes v.3.2.6 (Ronquist et al., 2012) was used to conduct BI analysis. The best evolutionary models fitting each partition specified in MrBayes were selected applying the Akaike Information Criterion (AIC) (Akaike, 1974) as implemented in jModelTest v.2.1.6 (Darriba et al., 2012) and are shown in **Table 1**. MrBayes was run for 20 million generations in two parallel runs, sampling every 1000th generation with a 25% burn-in value. Convergence between runs was assessed with Traver v1.6. Both RAxML and MrBayes analyses were implemented at CIPRES Science Gateway (Miller et al., 2010).

**Table 1.** Sequence information summary of each marker including number of taxa, alignment length, parsimony informative sites, average base frequencies and evolutionary model selected by AIC as implemented in jModeltest.

| Marker | Taxa | Length (bp) | Parsimony informative sites | Average base frequency (%) | | | | Evolutionary model |
|---|---|---|---|---|---|---|---|---|
| | | | | T | C | A | G | |
| COI | 60 | 654 | 268 | 40.1 | 15.2 | 25.4 | 19.3 | TPM2uf+I+G |
| COI-1 | 60 | 218 | 47 | 30.3 | 14.4 | 28.0 | 27.3 | GTR+I+G |
| COI-2 | 60 | 218 | 8 | 44.9 | 22.7 | 12.8 | 19.6 | TPM1uf+I |
| COI-3 | 60 | 218 | 213 | 45.2 | 8.4 | 35.5 | 10.8 | GTR+I+G |
| 16S | 60 | 1549 | 572 | 35.1 | 12.2 | 36.5 | 16.1 | TIM2+I+G |
| ITS1 | 55 | 1030 | 187 | 22.2 | 28.8 | 17.8 | 31.2 | TPM2uf+G |
| 5.8S | 61 | 158 | 1 | 19.8 | 28.2 | 20.3 | 31.7 | JC |
| ITS2 | 61 | 1448 | 225 | 23.2 | 28.9 | 18.8 | 29.1 | TVM+I+G |
| 28S | 62 | 844 | 55 | 18.5 | 27.8 | 20.6 | 33.1 | TIM2+I+G |
| 60SL17 | 51 | 309 | 62 | 22.4 | 21.0 | 27.5 | 29.0 | TrN+G |
| 60SL13 | 54 | 259 | 76 | 20.3 | 24.3 | 29.4 | 26.1 | K80+G |
| 60SL7 | 47 | 427 | 119 | 23.8 | 25.2 | 26.8 | 24.3 | TIM1ef+G |
| RPL14 | 37 | 351 | 85 | 22.0 | 20.3 | 31.5 | 26.2 | TrN+I |

## 2.4. Molecular taxonomy

Nucleotide apomorphic substitutions were mapped by parsimony along the consensus tree shown in **Figure 1** based on the sequences of all genes separately using the command "describetrees / opt=deltran apolist = yes" in PAUP* (Swofford, 2002).

**Table 2.** Node age constraints used in divergence time estimates based on fossil evidence.

| Node | Crown group | Fossil genus | Fossil age | Upper limit | Source |
|---|---|---|---|---|---|
| A | Hygromiidae + Geomitridae | *Loganiopharynx* | Early Eocene | 47.8 Ma | Nordsieck (2014) |
| B | Monacha | *Monacha* | Pliocene | 5.3 Ma | Nordsieck (2014) |

## 2.5. Estimation of divergence times

Divergence times were estimated with the Bayesian algorithm implemented in BEAST v.2.4.6 (Bouckaert et al., 2014) based on the concatenated dataset and excluding the outgroup taxa. Additionally, six ingroup individuals with a high percentage of missing loci were eliminated from the analyses to facilitate the convergence between independent runs. The same data partition as in the BI analysis with MrBayes, as well as the best sequence evolution models determined by the AIC in jModelTest, were specified. Analyses were run under an uncorrelated relaxed log-normal molecular clock and the Yule model was chosen as the tree prior. Two independent runs of 80 million generations sampling every 1000th generation were performed. To assess convergence between runs, Traver v1.6 was used and 10% of the generations were discarded as burn-in. The maximum clade credibility tree and posterior probabilities as node support were obtained through TreeAnnotator v.2.4.6. Since the use of several calibration points has been shown to improve divergence times estimates (Pérez-Losada et al., 2008; Porter et al., 2005), calibration was based on two calibration points inferred from fossil evidence attributed to related Hygromiidae taxa by Nordsieck (2014) (**Table 2**). This author considered within Hygromiidae some taxa currently assigned to Geomitridae (see Neiber et al., 2017; Razkin et al., 2015) and, thus, the oldest fossil that he assigned to Hygromiidae, *Loganiopharynx rarus* (Boissy, 1840), was used to calibrate the origin of Hygromiidae and Geomitridae together. For the calibration points a log-normal distribution was assumed and the upper limit of the geological period to which the fossils were ascribed was set for divergence time estimates. Since the fossil record of the taxa under study is limited, we set diffuse priors to define the log-normal distributions (M = 2.0 and S = 0.5 for Geomitridae + Hygromiidae and M = 1.5 and S = 1.0 for *Monacha*) to minimize prior bias.

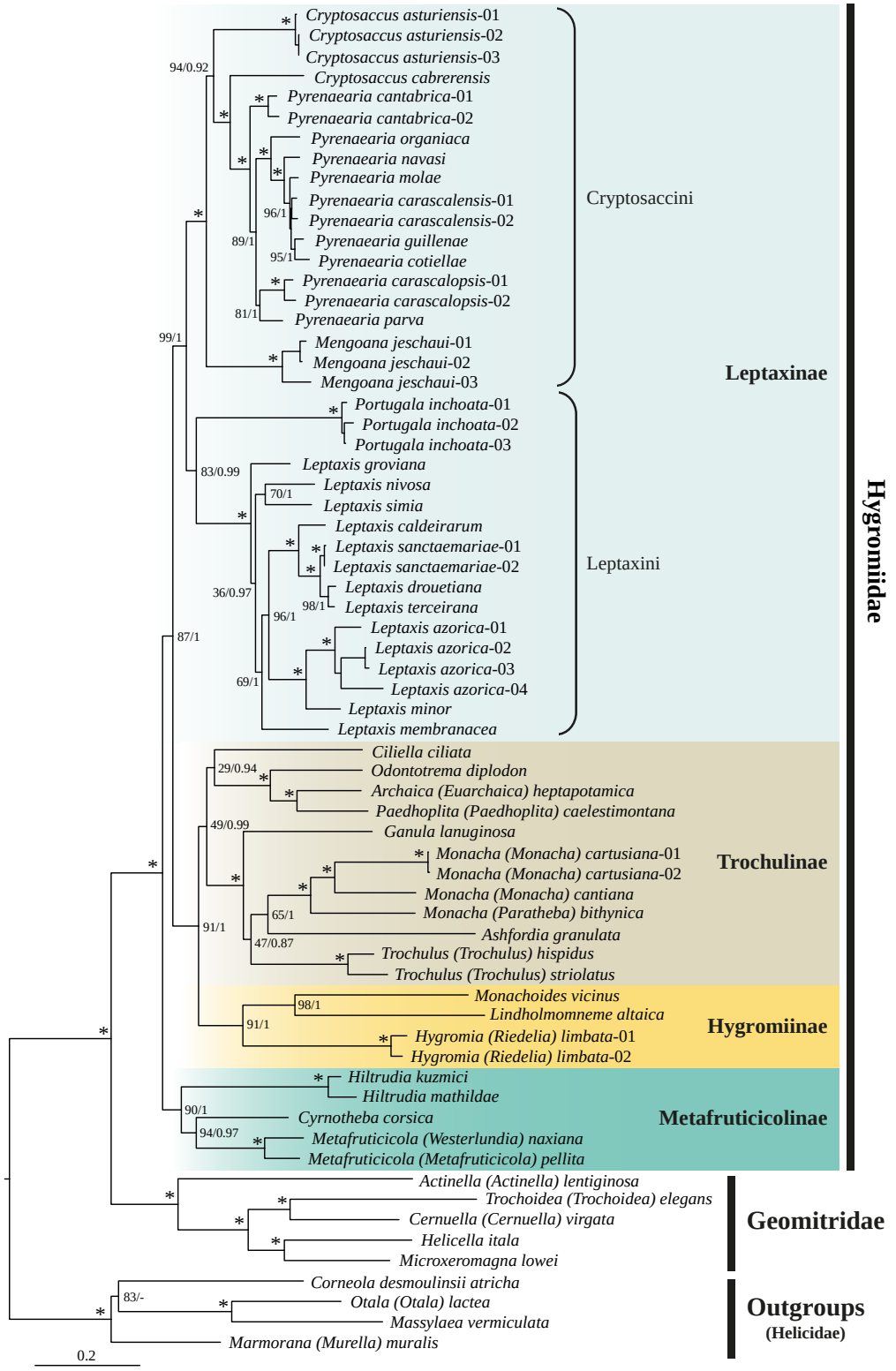



**Figure 1.** Phylogenetic tree of the Hygromiidae and Geomitridae obtained by Maximum Likelihood based on the concatenated sequences of *COI*, *16S*, *ITS1-5.8S-ITS2-28S*, *60SL17*, *60SL13*, *60SL7* and *RPL14*. Numbers on the nodes correspond to ML bootstrap values and BI posterior probabilities respectively. Asterisks (*) indicate fully supported nodes: ML bootstrap values = 100% and BI posterior probabilities = 1.00.

### 2.6. Ancestral range estimation

Based on the evidence from the above analyses, we assessed the dispersal history exclusively within the newly delimited Leptaxinae using the R package BioGeoBEARS (Matzke, 2013a). This program enables the estimation of ancestral areas under different biogeographical models (DEC (Ree et al., 2005; Ree and Smith, 2008), DIVALIKE (Ronquist, 1997) and BayAreaLIKE (Landis et al., 2013)) and further implements a parameter describing founder-event speciation (+j). Then, a comparison of the different models is made in a statistical framework to select the best-fitting model. We conducted the biogeographical analyses based on the time-calibrated tree of the BEAST analysis, estimating the ancestral ranges of all nodes. Each species was coded as belonging to one of the following seven areas: northern Iberian Peninsula, Lusitania, Madeira and the Azorean islands Santa Maria, São Miguel, Terceira/Graciosa and Faial/Pico (see **Figure 2**). We further incorporated the paleogeographical information of the formation of Macaronesian islands to the analyses so that the different islands were only available once they were emerged.

## 3. Results and discussion

### 3.1. Phylogeny and systematics

The final combined alignment included 66 individuals and comprised 7428 characters of which 1650 (22.2%) were parsimony informative sites (GenBank accession numbers are provided in **Supplementary Table S1**). Alignment length, parsimony informative sites and average base frequencies for each marker are listed in **Table 1**.

The phylogeny obtained through the analyses conducted on the combined mitochondrial and nuclear dataset is shown in **Figure 1**. The topology is based on the ML analysis results but both ML bootstrap values and BI posterior probabilities are indicated at the nodes since the resultant topologies were identical for both analysis methods. Furthermore, both methods provided support (bootstrap values (BS) > 70, posterior probabilities (PP) > 0.95) for almost all nodes, improving the resolution of the phylogenetic relationships within Hygromiidae with respect to previous molecular works, indicating the benefits of including several mitochondrial and nuclear *loci*, as has been previously documented for other groups (e.g. Barley et al., 2010; Lerner et al., 2011; McGowen, 2011). However, for some groups of organisms including snails, the availability of a wide set of primers to amplify different genome regions is limited. Here, we have successfully sequenced four nuclear markers recently

identified for the Geomitridae genus *Candidula* (Chueca et al., 2018), not only in the closely related family Hygromiidae, but also in the distantly related Helicidae. Thus, these markers may be useful in other helicoidean groups, significantly extending the available molecular markers for the family.

Our phylogenetic results recovered Hygromiidae as a maximally supported monophyletic group, with Geomitridae as sister group, also fully supported (Fig. 1). According to the phylogenies obtained by Neiber et al. (2017) and Razkin et al. (2015), a clade joining Geomitridae and Canariellidae is the sister group of Hygromiidae. Unfortunately, we were not able to include any species of the family Canariellidae and therefore we could not test its relationship to Hygromiidae and Geomitridae. On the other hand, the relationships within Geomitridae were congruent to those obtained in the preceding molecular studies of Chueca et al. (2018), Neiber et al. (2017) and Razkin et al. (2015).

Within the Hygromiidae, four main clades were recovered, which we rank as subfamilies: Metafruticicolinae, Hygromiinae, Trochulinae and Leptaxinae. Except for Trochulinae that was solely sustained by BI (BS = 49, PP = 0.99), all of these subfamilies and the relationships between them were strongly supported by both BI and ML.

**Metafruticicolinae** (BS = 90, PP = 1) as delimited here corresponds to the tribe Metafruticicolini of Neiber et al. (2017), i.e., it includes the genera *Hiltrudia*, *Cyrnotheba* and *Metafruticicola*. These authors recovered this clade as sister group of Cryptosaccini, forming a clade sister to Leptaxini, and for this reason they considered it as a Leptaxinae tribe. These relationships, however, were not statistically supported and even the affiliation of *Hiltrudia* to the group was uncertain. Here, we corroborate and provide strong support that *Hiltrudia* is indeed the sister genus of *Cyrnotheba* and *Metafruticicola*, but on the other hand, we recovered the clade as the sister group of the rest of Hygromiidae. Hence, it cannot be considered a Leptaxinae tribe and is elevated to subfamily level. It should be noted, moreover, that our consideration of Metafruticicolinae differs substantially from Metafruticicolinae *sensu* both Nordsieck (1993) and Schileyko (2006, 1978) (for an exhaustive comparison between the classifications see Neiber et al., 2017). All the taxa within Metafruticicolinae as we delimited it completely lack accessory copulatory organs and thus, it may be considered a synapomorphy of the subfamily. Notwithstanding, the loss of the accessory copulatory organs has occurred several times independently within Hygromiidae.

The subfamilies **Hygromiinae** (BS = 91, PP = 1) and **Trochulinae** (BS = 49, PP = 0.99) were recovered as sister groups forming a strongly supported clade (BS = 91, PP = 1). These two subfamilies matched, respectively, the Hygromiinae and Trochulinae as delimited by Neiber et al. (2017). Within Hygromiinae, we found

two main clades that correspond with the two tribes, Hygromiini and Perforatellini, proposed by these authors providing further support for their classification. We recovered Ashfordiini (*Ashfordia* Taylor, 1917), Ganulini (*Ganula* Gittenberger, 1970), Monachaini (*Monacha*) and Trochulini (*Trochulus* Chemnitz, 1786) *sensu* Neiber et al. (2017) joined in a clade fully supported in agreement with these authors. Moreover, despite we could not include any representatives of the tribes Urticicolini Neiber, Razkin and Hausdorf, 2017, Caucasigenini Neiber, Razkin and Hausdorf, 2017 and Halolimnohelicini Nordsieck, 1986, our results were congruent with the relationships these authors described for this clade. A clade grouping *Odontotrema diplodon* Lindholm, 1927, *Archaica heptapotamica* (Lindholm, 1927) and *Paedhoplita caelestimontana* (Tzvetkov, 1940) was fully supported, bearing out the validity of the tribe Archaicini. Albeit without support, *Ciliella ciliata* (Hartmann, 1821), the only species within Ciliellini, was recovered as sister group of Archaicini and together they were sister to the remaining tribes of Trochulinae, relationships not found by Neiber et al. (2017).

**Leptaxinae** was strongly supported (BS = 99, BI = 1) and was found to be the sister group of the clade joining Hygromiinae and Trochulinae (BS = 87, PP = 1). By excluding Metrafruticicolini from the subfamily, we recovered Leptaxinae *sensu* Razkin et al. (2015). The subfamily included two main clades that we ranked as tribes, Leptaxini and Cryptosaccini.

The Leptaxini (BS = 83, PP = 0.99) included two genera, the Macaronesian genus *Leptaxis* and the Iberian monotypic genus *Portugala*. The recovered composition of the tribe thus differs from Leptaxini as defined by Neiber et al. (2017), who only included *Leptaxis*. A relationship between *Portugala* and *Leptaxis* was previously suggested by Nordsieck (1993) since both genera possess a similar genital apparatus with a very wide dart sac without an accessory sac and two mucous glands deeply branched and located on opposite sides. However, this author also included the genera *Lindholmomneme* Haas, 1936, *Chilanodon* Westerlund, 1897, *Pseudotrichia* Schileyko, 1970, *Monachoides* Gude and Woodward, 1921, *Perforatella* Schlüter, 1838, *Urticicola* Lindholm, 1927 and *Mengoana* within Leptaxini (= *Perforatella-Leptaxis* group). Our results that placed *Portugala* as the sister group of *Leptaxis,* suggest that Macaronesia was colonized from the western Iberian Peninsula. Due to strong affinities between their taxa, Europe and especially the Iberian Peninsula have long been suggested as a source of colonization of Macaronesia, along with the Mediterranean region (Cardoso et al., 2010; Cook, 1996; Férnandez-Palacios et al., 2011). Several molecular works have recovered a sister group relationship between European and Macaronesian species including carabid beetles (Emerson et al., 2000), freshwater insects (Rutschmann et al., 2017) and land snails (Razkin et al., 2015). We included all the *Leptaxis* species endemic to the Azores archipelago (*L. minor* Backhuys, 1975, *L. azorica* (Albers,

1852), *L. caldeirarum* (Morelet and Drouët, 1857), *L. sanctaemariae* (Morelet and Drouët, 1857), *L. terceirana* (Morelet, 1860) and *L. drouetiana* (Morelet, 1860)) and recovered them as a well-supported clade (BS = 96, PP = 1) nested within the Madeiran species. The relationships recovered for the *Leptaxis* species of the Azores were strongly supported and were congruent with previous works (Jordaens et al., 2009; Van Riel et al., 2005). Unfortunately, we were not able to include *Leptaxis bollei* (Albers, 1856) from Cape Verde and, hence, we could not determine its position within the genus.

The fully supported Cryptosaccini included the Iberian genera *Mengoana*, *Cryptosaccus* and *Pyrenaearia*. *Cryptosaccus asturiensis* Prieto and Puente, 1994 and the recently described species *C. cabrerensis* Holyoak and Holyoak, 2014 did not form a monophyletic clade. Instead, *C. cabrerensis* was recovered as the sister genus of *Pyrenaearia* (BS = 100, PP = 1) while *C. asturiensis* was sister to both of them (BS = 94, PP = 0.92). Holyoak and Holyoak (2014) related *C. cabrerensis* more closely to *C. asturiensis* than to any other hygromiid because these taxa share a general shell form with a scale-like sculpture on the periphery of the body whorl and they both possess a single dart sac with a smaller accessory sac in the reproductive system. However, they also pointed out that these two species differ conspicuously both in the shell and in the genital morphology. One of the main differences concerns the accessory sac, which is completely fused to the dart sac and not visible externally in *C. asturiensis*, while in *C. cabrerensis* it is only partially fused and externally visible. Due to this characteristic of the accessory sac, these authors also hinted at the possible affinity of *C. cabrerensis* with *Pyrenaearia*, since the dart apparatus of this latter genus is formed by a dart sac and a completely separate, unfused accessory sac of similar size. A close relationship between these three taxa is corroborated by our phylogenetic analyses. Notwithstanding, in view of the phylogenetic relationships recovered here and the morphological differences stated, the establishment of a new genus, *Fractanella* gen. nov., for *C. cabrerensis* is proposed (see below for the formal description). Interestingly, we recovered the relationships within *Pyrenaearia* as described by Caro et al. (2019) and provided further support to their nodes. The genus *Mengoana* was joined as the sister group of the aforementioned Cryptosaccini genera. In *Mengoana,* the dart apparatus is formed solely by an appendix, which, according to Puente (1994), constituted an accessory sac. Therefore, within the tribe Cryptosaccini, each genus is characterised by a different morphology of the dart sac complex. Nevertheless, they all share a single dart apparatus with an accessory sac accompanied by four bifurcated mucous glands. In this way, this tribe can be distinguished from Leptaxini because its species possess two mucous glands and no accessory sac, although both tribes share a single dart apparatus complex.

When a new taxon is described, diagnostic differences from known taxa should be provided (Bauer et al., 2010). This task has traditionally been based on morphological characters; however, any heritable character including behavioural, physiological or molecular traits may be used to diagnose a taxon (Jörger and Schrödl, 2013).

Therefore, in addition to morphological characters, including molecular diagnosis is a useful practice, especially in those cases where morphological homoplasy may blur the taxonomy. The apomorphic substitutions of the subordinate family-groups and subfamily-groups of the Hygromiidae defined here are listed in **Supplementary Table S3**.

**Table 3.** Apomorphic substitutions supporting the genus *Fractanella* for each *locus*. Nucleotides before arrows indicate plesiomorphies, nucleotides after arrow indicate apomorphies and in parenthesis the position of the nucleotide in the alignment is noted. Only characters with the maximum consistency index (CI) of 1.00 are considered. nrDNA refers to ITS1-5.8S-ITS2-28S gene cluster.

| COI | 16S | nrDNA | 60SL17 | 60SL13 | 60SL7 | RPL14 |
|---|---|---|---|---|---|---|
| T → A (82) | A → T (884) | G → C (919) | None | T → A (68) | None | G → C (116) |
| G → A (121) | | G → A (1972) | | G → T (86) | | G → A (117) |
| | | C → T (2767) | | | | G → A (248) |
| | | | | | | C → T (362) |

## 3.2. Description of new taxon

***Fractanella*** Caro and Madeira gen. nov.

Type species: *Cryptosaccus cabrerensis* Holyoak and Holyoak, 2014

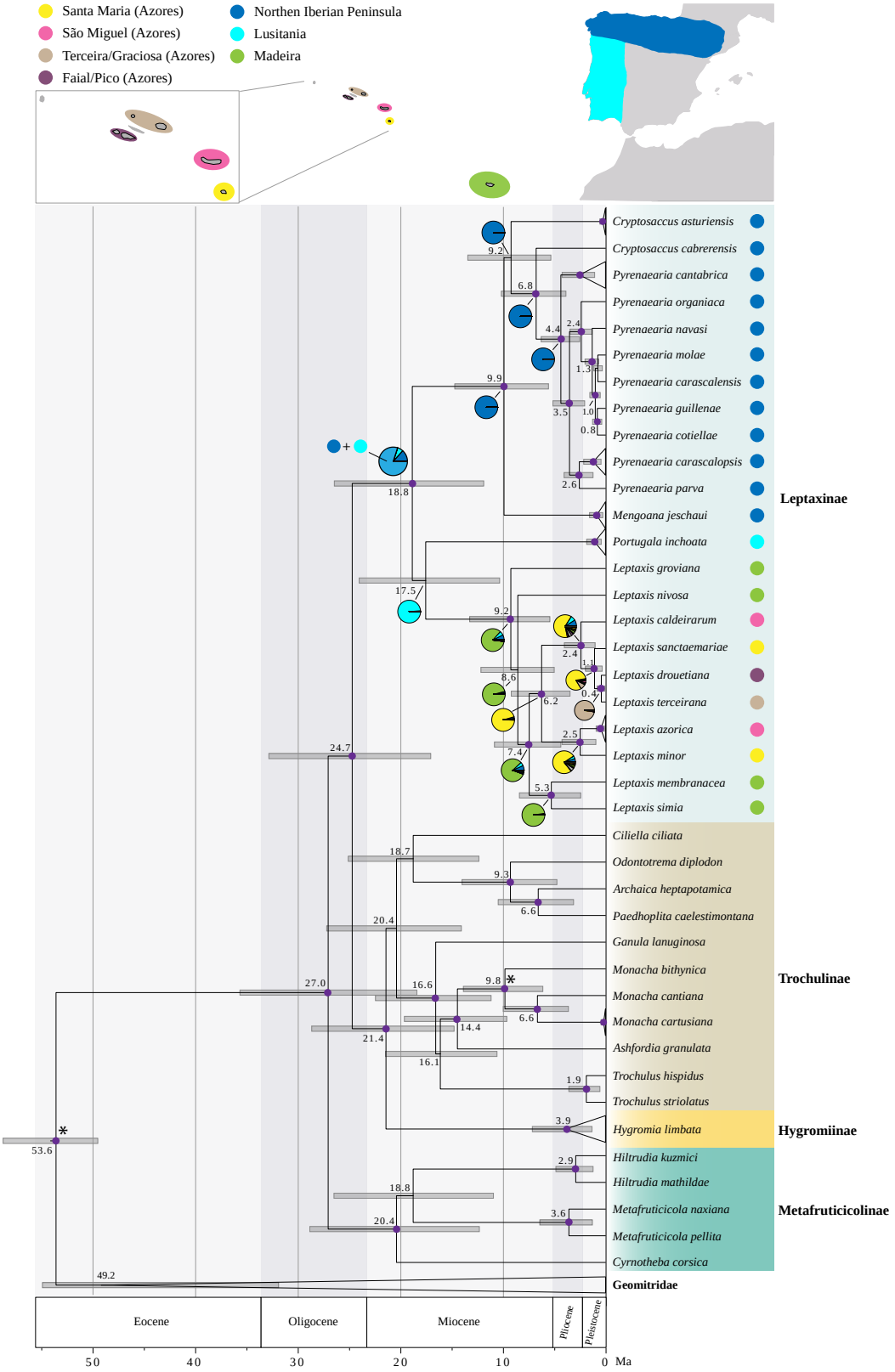
Diagnosis: The apomorphic substitutions with a maximum consistency index of 1.00 that support this Cryptosaccini genus are listed in **Table 3**. For a detailed morphological diagnosis see Holyoak and Holyoak (2014), but as mentioned above, the most conspicuous morphological characteristics of the genus are a stimulatory apparatus formed by a single dart sac partially fused with a smaller accessory sac, with four bifurcated mucous glands, and a flexible and translucent, minimally calcified shell with a microsculpture on the periphery of the body whorl that resembles small fractures.

Included taxa: *Fractanella cabrerensis* (Holyoak and Holyoak, 2014)

Etymology: Derived from the Latin fracta = fractured, it is named for the microsculpture of the shell causing it to appear fractured.

## 3.3. Timing of diversification and biogeographic patterns

Divergence time estimates inferred from the combined mitochondrial and nuclear dataset using BEAST rendered a well-resolved ultrametric tree (**Figure 2**). The only discrepancies with those topologies obtained under ML and BI phylogenetic


Santa Maria (Azores)
São Miguel (Azores)
Terceira/Graciosa (Azores)
Faial/Pico (Azores)
Northen Iberian Peninsula
Lusitania
Madeira
Cryptosaccus asturiensis
Cryptosaccus cabrerensis
Pyrenaearia cantabrica
Pyrenaearia organiaca
Pyrenaearia navasi
Pyrenaearia molae
Pyrenaearia carascalensis
Pyrenaearia guillenae
Pyrenaearia cotiellae
Pyrenaearia carascalopsis
Pyrenaearia parva
Mengoana jeschaui
Portugala inchoata
Leptaxis groviana
Leptaxis nivosa
Leptaxis caldeirarum
Leptaxis sanctaemariae
Leptaxis drouetiana
Leptaxis terceirana
Leptaxis azorica
Leptaxis minor
Leptaxis membranacea
Leptaxis simia
Ciliella ciliata
Odontotrema diplodon
Archaica heptapotamica
Paedhoplita caelestimontana
Ganula lanuginosa
Monacha bithynica
Monacha cantiana
Monacha cartusiana
Ashfordia granulata
Trochulus hispidus
Trochulus striolatus
Hygromia limbata
Hiltrudia kuzmici
Hiltrudia mathildae
Metafruticicola naxiana
Metafruticicola pellita
Cyrnotheba corsica
Geomitridae
Leptaxinae
Trochulinae
Hygromiinae
Metafruticicolinae
Eocene
Oligocene
Miocene
Pliocene
Pleistocene
50
40
30
20
10
0 Ma

analyses were related to the relationships within Metafruticicolinae and among the Madeiran *Leptaxis,* which were either weakly supported in the chronogram or in all the analyses. The BioGeoBEARS analysis to estimate ancestral areas based on this ultrametric tree, but focussing only on the Leptaxinae, recovered the DIVALIKE+j model as the one with the strongest statistical support (**Table 4**). The ancestral area estimation returned by this best biogeographical model is summarized in **Figure 2**.

Our dating analyses estimated a median age for the split between Geomitridae and Hygromiidae of *ca.* 53.6 Ma (95% highest posterior density interval (HPD): 58.7–49.5 Ma) and set the start of the diversification of the recent Hygromiidae at 27.0 Ma ago (95% HPD: 35.6–18.4 Ma). These ages are younger than the divergence time estimations of Razkin et al. (2015) and Neiber et al. (2017). Taxonomic sampling has been identified as an important factor affecting divergence time estimates (Linder et al., 2005; Poux et al., 2008; Soares and Schrago, 2015). Hence, the large differences in taxonomic sampling between our analysis and those of Razkin et al. (2015) and Neiber et al. (2017) may be the source of the variation in the age time estimates for Hygromiidae between the analyses. On the other hand, the topology obtained by Neiber et al. (2017) differs from that obtained in this work regarding the relationships of the main clades, especially in the placement of Metafruticicolinae, which may also be influencing the time inference and may be responsible for the differences in the age estimates. Molecular dating, moreover, may be beset with other problems such as, for example, the choice and placement of calibration points

**Table 4.** Results of the BioGeoBEARS model comparison for the seven areas. LnL: log-likelihood of the model; d: rate of dispersal; e: rate *o*f extinction; j: relative probability of founder-event speciation at cladogenesis; AIC: Akaike's information criterion; AICc: Akaike's information criterion corrected for small sample size. In bold the best model.

| Dispersal model | Ln*L* | No. of parameters | d | e | j | AIC | AICc | Relative probability |
|---|---|---|---|---|---|---|---|---|
| DEC | -44.26 | 2 | 0.012 | 0.018 | 0.000 | 92.51 | 93.11 | 0% |
| DEC+j | -32.54 | 3 | 0.002 | 0.008 | 0.047 | 71.08 | 72.35 | 42% |
| DIVALIKE | -39.74 | 2 | 0.010 | 0.010 | 0.000 | 83.48 | 84.08 | 0% |
| **DIVALIKE+j** | **-32.40** | **3** | **0.002** | **0.007** | **0.043** | **70.81** | **72.07** | **49%** |
| BayAreaLIKE | -55.55 | 2 | 0.014 | 0.071 | 0.000 | 115.11 | 115.71 | 0% |
| BayAreaLIKE+j | -34.09 | 3 | 0.002 | 0.008 | 0.053 | 74.18 | 75.45 | 9% |

**Figure 2.** Dated maximum clade credibility tree of the Hygromiidae and Geomitridae constructed with BEAST for the concatenated dataset of *COI, 16S, ITS1-5.8S-ITS2-28S, 60SL17, 60SL13, 60SL7* and *RPL14*. Values at the nodes represent estimated mean ages in Ma and bars indicate their 95% HPD interval. Dots indicated supported nodes (PP > 0.95). Constrained nodes are marked with asterisks (*). Pie charts represent proportion of origin derived from the results of ancestral area estimation according to the best supported BioGeoBEARS model. Colours of the pie charts correspond to the seven geographical regions as delimited in the top map. For each Leptaxinae tip, coloured dots at right indicated to which of these areas they belong.

(Hedges and Kumar, 2004; Vasconcelos et al., 2017). Therefore, without a correct understanding of the potential effects of the different factors influencing divergence inferences, caution is required when interpreting age estimates (Linder et al., 2005). Our dating estimates for Leptaxinae, which is ultimately our target, are consistent with the geological history of Macaronesia and do not contradict fossil evidence. Thus, our divergence inference seems reasonable though the possibility of some level of bias cannot be discarded.

According to our chronological and biogeographical analysis, the diversification of Leptaxinae started in the Early Miocene (18.8 Ma ago; 95% HPD: 26.4–11.9 Ma) in the Iberian Peninsula. The split between *Portugala* and *Leptaxis* was dated at 17.5 Ma ago (95% HPD: 24.0–10.3 Ma). These estimates predate the emergence of the oldest Madeira archipelago island, Porto Santo, with its oldest subaerial deposits dated at 14.2 to 13.1 Ma before present (Geldmacher et al., 2000). The diversification within Madeira *Leptaxis* species, however, was estimated to start 9.2 Ma years ago (95% HPD: 13.2–5.4 Ma), when Porto Santo was already emerged. The old split

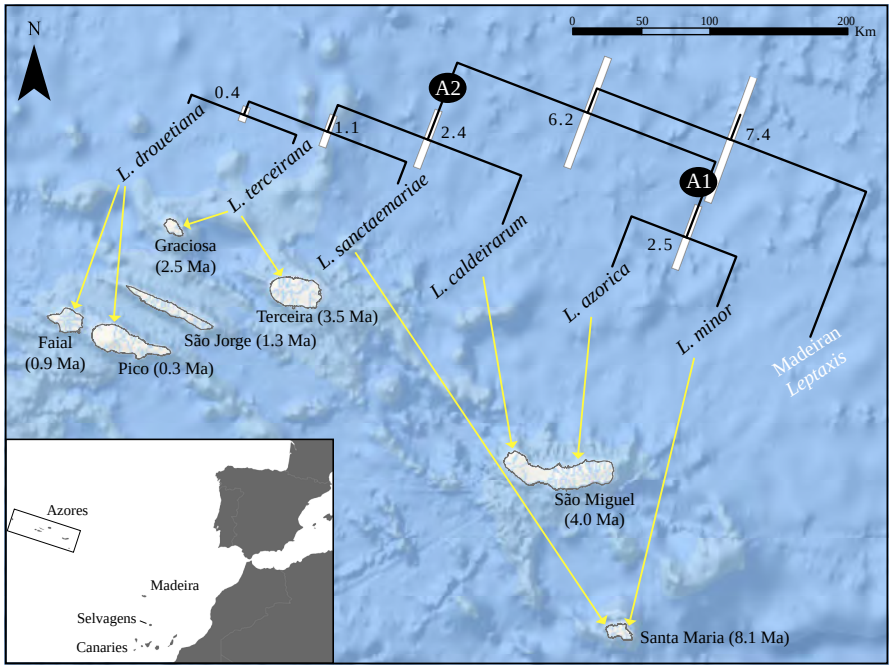



**Figure 3.** Geospatial representation of the dated maximum clade credibility tree of Azorean *Leptaxis* species. For each island the oldest geochronological ages, as gathered by Larrea et al. (2014), are reported in brackets. On the chronogram, values at the nodes represent estimated mean ages in Ma, bars indicate their 95% HPD interval and black boxes on branches denote the two main lineages defined in the text.

between *Leptaxis* and *Portugala* 17.5 Ma ago may reflect the fact that the Selvagens archipelago or the oldest islands of the Canary archipelago, emerged at that time, were colonized first and that the Madeira archipelago was reached posteriorly from there. Later, the genus would have become extinct in the Selvagens archipelago or in the Canary Islands. A *Leptaxis* fossil from the Canary Islands dated at 6–12 Ma ago (Gittenberger and Ripken, 1985) actually supports the presence of the genus in that archipelago. Nevertheless, there is increasing geological evidence, synthesized by Férnandez-Palacios et al. (2011), that suggests that the seamounts and guyots located between the Madeira and Canary archipelagos and the European and African continents may have been emerged and were thus available for colonization as early as 60 Ma ago and until 5 Ma before present. This means that when the split between the two main Leptaxini lineages took place, more islands than those of the Selvagens archipelago and the oldest Canary Islands might have been available and, moreover, closer to the Iberian Peninsula. Therefore, alternatively, Madeira and the Canaries could have been colonized via these now submerged islands. Divergence times older than the emergence of the extant islands have also been reported for the endemic Madeiran moss *Brachythecium percurrens*, as its divergence from the most recent common ancestor within its family (Helicodontioideae) was estimated *ca*. 40 Ma (Aigoin et al., 2009), which even predates the emergence of the oldest extant Macaronesian island. It can also not be ruled out that the diversification of the two Leptaxini lineages started in the mainland and that Macaronesia was colonized afterwards.

*Leptaxis* from Madeira and the Azores diverged from each other 7.4 Ma years ago (95% HPD: 10.8–4.4 Ma) according to our divergence inference. This coincides with the emergence of Santa Maria dated *ca.* 8.1 Ma (Abdel-Monem et al., 1975). Our phylogenetic and temporal reconstruction for the Azorean *Leptaxis* species did not support a clear progression rule (**Figure 3**) and, indeed, our estimation of ancestral area confirmed a lack of progression. Instead, our results support that two lineages (A1 and A2, Fig. 3) originated in Santa Maria and that they independently colonized São Miguel, the second oldest island dated ca. 4.0 Ma (Abdel-Monem et al., 1975). Posteriorly, according to the biogeographical model, Lineage A2 would have colonized Terceira (3.5 Ma) and/or Graciosa (2.5 Ma) from Santa Maria. This is also supported by the fact that when the split between *L. sanctaemariae* from Santa Maria and the clade joining *L. terceirana* (endemic to Terceira and Graciosa) and *L. drouetiana* (endemic to Faial and Pico) occurred *ca.* 1.1 Ma ago (95% HPD: 2.0–0.3 Ma), Terceira and Graciosa were the only islands inhabited by *Leptaxis* emerged (along with Santa Maria and São Miguel). Finally, Faial (0.9 Ma) would have been colonized later from Terceira or Graciosa, which is consistent with the divergence between L. terceirana and L. droutetiana estimated ca. 0.4 Ma (95% HPD: 0.8–0.1 Ma). The colonization of Pico (0.3 Ma) would then likely have occurred from Faial.

Based on our time and ancestral area estimates, the diversification of extant Cryptosaccini began *ca*. 9.9 Ma (95% HPD: 14.7–5.6 Ma), in the Late Miocene in the northern Iberian Peninsula, and the divergence events between the genera preceded the Pliocene. Since the split of Cryptosaccini from its sister group traced back to the Early Miocene, there is an apparent diversification gap of 9 Ma within the tribe, which suggests one or several major extinction events between the origin of the tribe and the diversification of extant species. During the Miocene, the western Mediterranean region underwent a major climatic shift. Starting in the Middle Miocene (*ca.* 15–7 Ma), the overall subtropical climate of the region progressively changed towards cooler and drier conditions, culminating about 3.2 Ma ago in the onset of the present-day Mediterranean climate characterized by a dual seasonality with a marked summer drought (Jiménez-Moreno et al., 2010; Suc, 1984). All the species within Cryptosaccini are associated with shady environments and do not tolerate xeric conditions. Hence, this dramatic climatic change may have had a great impact and been an important driver of the extinction events undergone by the tribe at the beginning of its evolutionary history, as suggested by our time inference. This climatic shift has been related to the extinction in the Mediterranean of numerous organisms, including plants (Férnandez-Palacios et al., 2011), vertebrates (Böhme, 2003; Casanovas-Vilar et al., 2010) and arthropods (Bidegaray-Batista et al., 2014). The surviving Cryptosaccini lineage may have persisted thanks to the moister conditions offered during the final part of the Neogene by the mountain ranges they inhabit (Jiménez-Moreno et al., 2010), as suggested for the ground-dwelling spider genus *Harpactocrates*, distributed throughout the mountain ranges surrounding the western Mediterranean and dependent on humid environments (Bidegaray-Batista et al., 2014).

Finally, *Pyrenaearia* was estimated to have begun diversifying in the Pliocene (4.4 Ma ago; 95% HPD: 6.3–2.5 Ma) such that, before the beginning of the Pleistocene glacial cycles, the three main clades of the genus had already evolved. However, most speciation events were estimated to have occurred during the Pleistocene glaciation cycles. Therefore, as for many other mountain-dwelling organisms (Bidegaray-Batista et al., 2014; Dépraz et al., 2008; Gittenberger et al., 2004; Harl et al., 2014; Mouret et al., 2011; Pauls et al., 2006), the Pleistocene climate changes seem to have been important drivers in the speciation of *Pyrenaearia*.

Interestingly, the statistical comparison between the different biogeographical models conducted with BioGeoBEARS pointed out better fitting to the data when the parameter describing the founder-event (+j) was considered, with the models where it was implemented displaying lower log-likelihood values. Moreover, the Likelihood Ratio Tests (LRT) between the nested models also rejected the null hypothesis that the models with and without founder-event confer the same likelihood on the data

(**Supplementary Table S4**). These evidences highlight that the founder-event is an explanatory process to be considered in biogeographical analysis but, in addition, in the best biogeographical model (DIVALIKE+j), the founder-event was recovered as a crucial process in the speciation of the subfamily Leptaxinae (j = 0.043 in contrast to d = 0.002 and e = 0.007, **Table 4**). This is in agreement with different biogeographical studies that have showed that founder-event speciation is a crucial process in systems with island clades, as is the case here (Matzke 2013b, 2014).

# 4. Conclusions

Through a multi-locus molecular approach, the phylogeny of the land snail subfamily Leptaxinae has been resolved with strong support and further insight has been gained on the relationships within Hygromiidae and its classification. By elevating Metafruticicolini to subfamily level (Metafruticicolinae), we determined that four subfamilies comprise Hygromiidae and restricted Leptaxinae to two tribes, Leptaxini and Cryptosaccini. We illustrate the necessity of well-resolved molecular phylogenies to gain adequate classifications in this family of land snails since the use of genital morphology is limited due to the presence of homoplasies. Our results set the origin of Leptaxinae in the Early Miocene in the Iberian Peninsula from which the Macaronesian Islands were posteriorly colonized. The deep split found between the continental and the Macaronesian lineages poses two colonization bias for Macaronesia: throughout the seamounts located between Macaronesia and the continents, now submerged but at one time exposed, or through the oldest actually emerged islands of Macaronesia. In the Iberian Peninsula, the climatic change of the Miocene from subtropical climate towards the present-day Mediterranean climate had great importance shaping the diversification of Cryptosaccini, as well as the Pleistocene glacial cycles.

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# References


Abdel-Monem, A.A., Fernandez, L.A., Boone, G.M., 1975. K-Ar ages from the eastern Azores group (Santa Mafia, Miguel and the Formigas Islands). Lithosphere 8, 247–254. doi:10.1016/0024- 4937(75)90008-0

Aigoin, D.A., Devos, N., Huttunen, S., Ignatov, M.S., Gonzalez-Mancebo, J.M., Vanderpoorten, A., 2009. And if Engler was not completely wrong? Evidence for multiple evolutionary origins in the moss flora of Macaronesia. Evolution 63, 3248–3257. doi:10.1111/j.1558-5646.2009.00787.x

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Automat. Contr. 19, 716–723. doi:10.1109/TAC.1974.1100705

Barley, A.J., Spinks, P.Q., Thomson, R.C., Shaffer, H.B., 2010. Fourteen nuclear genes provide phylogenetic resolution for difficult nodes in the turtle tree of life. Mol. Phylogenet. Evol. 55, 1189–1194. doi:10.1016/j.ympev.2009.11.005

Bauer, A.M., Parham, J.F., Brown, R.M., Stuart, B.L., Grismer, L., Papenfuss, T.J., Böhme, W., Savage, J.M., Carranza, S., Grismer, J.L., Wagner, P., Schmitz, A., Ananjeva, N.B., Inger, R.F., 2010. Availability of new Bayesian-delimited gecko names and the importance of character-based species descriptions. Proc. R. Soc. B 278, 490–492. doi:10.2307/1467045

Bidegaray-Batista, L., Ferrández, M.Á., Arnedo, M.A., 2014. Winter is coming: Miocene and Quaternary climatic shifts shaped the diversification of Western-Mediterranean *Harpactocrates* (Araneae, Dysderidae) spiders. Cladistics 30, 428–446.

Böhme, M., 2003. The Miocene Climatic Optimum: Evidence from ectothermic vertebrates of Central Europe. Palaeogeogr. Palaeoclimatol. Palaeoecol. 195, 389–401. doi:10.1016/S0031-0182(03)00367-5

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A., Drummond, A.J., 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. PLOS Comput. Biol. 10, e1003537. doi:10.1371/journal.pcbi.1003537

Cardoso, P., Arnedo, M.A., Triantis, K.A., Borges, P.A.V., 2010. Drivers of diversity in Macaronesian spiders and the role of species extinctions. J. Biogeogr. 37, 1034–1046. doi:10.1111/j.1365-2699.2009.02264.x

Caro, A., Gómez-Moliner, B.J., Madeira, M.J., 2019. Integrating multilocus DNA data and 3D geometric morphometrics to elucidate species boundaries in the case of *Pyrenaearia* (Pulmonata: Hygromiidae). Mol. Phylogenet. Evol. 132, 194–206.

Casanovas-Vilar, I., García-Paredes, I., Alba, D.M., van den Hoek Ostende, L.W., Moyà-Solà, S., 2010. The European Far West: Miocene mammal isolation, diversity and turnover in the Iberian Peninsula. J. Biogeogr. 37, 1079–1093. doi:10.1111/j.1365-2699.2010.02286.x

Chueca, L.J., Gómez-Moliner, B.J., Madeira, M.J., Pfenninger, M., 2018. Molecular phylogeny of *Candidula* (Geomitridae) land snails inferred from mitochondrial and nuclear markers reveals the polyphyly of the genus. Mol. Phylogenet. Evol. 118, 357–368. doi:10.1016/j.ympev.2017.10.022

Cook, L.M., 1996. Habitat, isolation and the evolution of Madeiran landsnails. Biol. J. Linn. Soc. 59, 457–470. doi:10.1006/bijl.1996.0075

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772. doi:10.1038/nmeth.2109

Dépraz, A., Cordellier, M., Hausser, J., Pfenninger, M., 2008. Postglacial recolonization at a snail's pace (*Trochulus villosus*): confronting competing refugia hypotheses using model selection. Mol. Ecol. 17, 2449–2462. doi:10.1111/j.1365-294X.2008.03760.x

Emerson, B.C., Oromí, P., Hewitt, G.M., 2000. Interpreting colonization of the *Calathus* (Coleoptera: Carabidae) on the Canary Islands and Madeira through the application of the parametric bootstrap. Evolution 54, 2081–2090. doi:10.1111/j.0014-3820.2000.tb01251.x

Férnandez-Palacios, J.M., de Nascimento, L., Otto, R., Delgado, J.D., García-del-Rey, E., Arévalo, J.R., Whittaker, R.J., 2011. A reconstruction of Palaeo-Macaronesia, with particular reference to the long-term biogeography of the Atlantic island laurel forests. J. Biogeogr. 38, 226–246. doi:10.1111/j.1365-2699.2010.02427.x

Geldmacher, J., van den Bogaard, P., Hoernle, K., Schmincke, H.U., 2000. The 40 Ar/ 39 Ar age dating of the Madeira Archipelago and hotspot track (eastern North Atlantic). Geochemistry, Geophys. Geosystems 1. doi: 10.1029/1999GC000018.

Gittenberger, E., Piel, W.H., Groenenberg, D.S.J., 2004. The Pleistocene glaciations and the evolutionary history of the polytypic snail species *Arianta arbustorum* (Gastropoda, Pulmonata, Helicidae). Mol. Phylogenet. Evol. 30, 64–73. doi:10.1016/S1055-7903(03)00182-9

Gittenberger, E., Ripken, T.E.J., 1985. Seven Late Miocene species of terrestrial gastropods (Mollusca: Gastropoda: Pulmonata) from the island of Lanzarote, Canary Islands. Proc. K. Ned. Akad. van Wet. Ser. C 88, 397–406.

Gómez-Moliner, B.J., Elejalde, A.M., Arrébola, J.R., Puente, A.I., Martínez-Ortí, A., Ruiz, A., Madeira, M.J., 2013. Molecular phylogeny of the Helicodontidae and Trissexodontidae (Gastropoda). Zool. Scr. 42, 170–181. doi:10.1111/j.1463-6409.2012.00571.x

Groenenberg, D.S.J., Neubert, E., Gittenberger, E., 2011. Reappraisal of the "Molecular phylogeny of Western Palaearctic Helicidae *s.l.* (Gastropoda: Stylommatophora)": When poor science meets GenBank. Mol. Phylogenet. Evol. 61, 914–923. doi:10.1016/j.ympev.2011.08.024

Harl, J., Duda, M., Kruckenhauser, L., Sattmann, H., Haring, E., 2014. In search of glacial refuges of the land snail *Orcula dolium* (Pulmonata, Orculidae) - An integrative approach using DNA sequence and fossil data. PLoS One 9, e96012. doi:10.1371/journal.pone.0096012

Hedges, S.B., Kumar, S., 2004. Precision of molecular time estimates. Trends Genet. 20, 242–247. doi:10.1016/j.tig.2004.03.004

Holyoak, D.T., Holyoak, G.A., 2014. An undescribed species of *Cryptosaccus* (Gastropoda: Hygromiidae) from the south-west of the province of León, NW Spain. Iberus 32, 27–34.

Hugall, A.F., Stanisic, J., 2011. Beyond the prolegomenon: a molecular phylogeny of the Australian camaenid land snail radiation. Zool. J. Linn. Soc. 161, 531–572. doi:10.1111/j.1096-3642.2010.00644.x

Jiménez-Moreno, G., Fauquette, S., Suc, J.P., 2010. Miocene to Pliocene vegetation reconstruction and climate estimates in the Iberian Peninsula from pollen data. Rev. Palaeobot. Palynol. 162, 403–415. doi:10.1016/j.revpalbo.2009.08.001

Jordaens, K., Van Riel, P., Frias Martins, A.M., Backeljau, T., 2009. Speciation on the Azores islands: congruent patterns in shell morphology, genital anatomy, and molecular markers in endemic land snails (Gastropoda, Leptaxinae). Biol. J. Linn. Soc. 97, 166–176.

Jörger, K.M., Schrödl, M., 2013. How to describe a cryptic species? Practical challenges of molecular taxonomy. Front. Zool. 10, 59. doi:10.1186/1742-9994-10-59

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. doi:10.1093/bioinformatics/bts199

Köhler, F., Criscione, F., 2015. A molecular phylogeny of camaenid land snails from north-western Australia unravels widespread homoplasy in morphological characters (Gastropoda, Helicoidea). Mol. Phylogenet. Evol. 83, 44–55. doi:10.1016/j.ympev.2014.11.009

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33, 1870–1874. doi:10.1093/molbev/msw054

Landis, M.J., Matzke, N.J., Moore, B.R., Huelsenbeck, J.P., 2013. Bayesian analysis of biogeography when the number of areas is large. Syst. Biol. 62, 789–804. https://doi.org/10.1093/sysbio/syt040

Lanfear, R., Calcott, B., Ho, S.Y.W., Guindon, S., 2012. PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol. Biol. Evol. 29, 1695–1701. doi:10.1093/molbev/mss020

Larrea, P., Wijbrans, J.R., Galé, C., Ubide, T., Lago, M., França, Z., Widom, E., 2014. 40Ar/39Ar constraints on the temporal evolution of Graciosa Island, Azores (Portugal). Bull. Vocanology 76, 796. doi:10.1007/s00445-014-0796-8

Lerner, H.R.L., Meyer, M., James, H.F., Hofreiter, M., Fleischer, R.C., 2011. Multilocus resolution of phylogeny and timescale in the extant adaptive radiation of Hawaiian honeycreepers. Curr. Biol. 21, 1838–1844. doi:10.1016/j.cub.2011.09.039

Linder, H.P., Hardy, C.R., Rutschmann, F., 2005. Taxon sampling effects in molecular clock dating: An example from the African Restionaceae. Mol. Phylogenet. Evol. 35, 569–582. doi:10.1016/j.ympev.2004.12.006

Löytynoja, A., Goldman, N., 2005. An algorithm for progressive multiple alignment of sequences with insertions. Proc. Natl. Acad. Sci. USA. 102, 10557–62. doi:10.1073/pnas.0409137102

Löytynoja, A., Goldman, N., 2008. Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. Science 320, 1632–1635. doi:10.1126/science.1158395

Matzke, N.J., 2013a. BioGeoBEARS: BioGeography with Bayesian (and Likelihood) evolutionary analysis in R scripts. University of California, Berkeley, CA. Available at: https://github.com/nmatzke/BioGeoBEARS

Matzke, N.J., 2013b. Probabilistic historical biogeography: new models for founder-event speciation, imperfect detection, and fossils allow improved accuracy and model-testing. Front. Biogeogr. 5, 242–248. https://doi.org/10.21425/F5FBG19694

Matzke, N.J., 2014. Model selection in historical biogeography reveals that founder-event speciation is a crucial process in island clades. Syst. Biol. 63, 951–970. https://doi.org/10.1093/sysbio/syu056

McGowen, M.R., 2011. Toward the resolution of an explosive radiation - A multilocus phylogeny of oceanic dolphins (Delphinidae). Mol. Phylogenet. Evol. 60, 345–357. doi:10.1016/j.ympev.2011.05.003

Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees, in: 2010 Gateway Computing Environments Workshop (GCE). IEEE, pp. 1–8. doi:10.1109/GCE.2010.5676129

Mouret, V., Guillaumet, A., Cheylan, M., Pottier, G., Ferchaud, A.L., Crochet, P.A., 2011. The legacy of ice ages in mountain species: post-glacial colonization of mountain tops rather than current range fragmentation determines mitochondrial genetic diversity in an endemic Pyrenean rock lizard. J. Biogeogr. 38, 1717–1731. doi:10.1111/j.1365-2699.2011.02514.x

Neiber, M.T., Razkin, O., Hausdorf, B., 2017. Molecular phylogeny and biogeography of the land snail family Hygromiidae (Gastropoda: Helicoidea). Mol. Phylogenet. Evol. 111, 169–184. doi:10.1111/zsc.12218

Nordsieck, H., 1993. Das System der paläarktischen Hygromiidae (Gastropoda: Stylommatophora: Helicoidea). Arch. für Molluskenkd. 122, 1–23.

Nordsieck, H., 2014. Annotated check-list of the genera of fossil land snails (Gastropoda: Stylommatophora) of western and central Europe (Cretaceous – Pliocene), with description of new taxa. Arch. für Molluskenkd. 143, 153–185. doi:10.1127/arch.moll/1869-0963/143/153-185

Pauls, S.U., Lumbsch, H.T., Haase, P., 2006. Phylogeography of the montane caddisfly *Drusus discolor*: evidence for multiple refugia and periglacial survival. Mol. Ecol. 15, 2153–2169. doi:10.1111/j.1365-294X.2006.02916.x

Pérez-Losada, M., Harp, M., Høeg, J.T., Achituv, Y., Jones, D., Watanabe, H., Crandall, K.A., 2008. The tempo and mode of barnacle evolution. Mol. Phylogenet. Evol. 46, 328–346. doi:10.1016/j.ympev.2007.10.004

Perez, K.E., Defreitas, N., Slapcinsky, J., Minton, R.L., Anderson, F.E., Pearce, T.A., 2014. Molecular phylogeny, evolution of shell shape, and DNA barcoding in Polygyridae (Gastropoda: Pulmonata), an endemic North American clade of land snails. Am. Malacol. Bull. 32, 1–31. doi:10.4003/006.032.0103

Porter, M.L., Pérez-Losada, M., Crandall, K.A., 2005. Model-based multi-locus estimation of decapod phylogeny and divergence times. Mol. Phylogenet. Evol. 37, 355–369. doi:10.1016/j.ympev.2005.06.021

Poux, C., Madsen, O., Glos, J., De Jong, W.W., Vences, M., 2008. Molecular phylogeny and divergence times of Malagasy tenrecs: Influence of data partitioning and taxon sampling on dating analyses. BMC Evol. Biol. 8, 102. doi:10.1186/1471-2148-8-102

Puente, A.I., 1994. Estudio taxonómico y biogeográfico de la superfamilia Helicoidea Rafinesque, 1815 (Gastropoda: Pulmonata: Stylommatophora) de la Península Ibérica e Islas Baleares. Leioa, PhD thesis, University of the Basque Country (EHU/UPV), Spain.

Razkin, O., Gómez-moliner, B.J., Prieto, C.E., Martínez-ortí, A., Arrébola, J.R., Muñoz, B., Chueca, L.J., Madeira, M.J., 2015. Molecular phylogeny of the western Palaearctic Helicoidea (Gastropoda, Stylommatophora). Mol. Phylogenet. Evol. 83, 99–117. doi:10.1016/j.ympev.2014.11.014

Ree, R.H., Moore, B.R., Webb, C.O., Donoghue, M.J., 2005. A likelihood framework for inferring the evolution of geographic range on phylogenetic trees. Evolution (N. Y). 59, 2299–2311.

Ree, R.H., Smith, S.A., 2008. Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. Syst. Biol. 57, 4–14. https://doi.org/10.1080/10635150701883881

Ronquist, F., 1997. Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. Syst. Biol. 46, 195–203.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542. doi:10.1093/sysbio/sys029

Rutschmann, S., Detering, H., Simon, S., Funk, D.H., Gattolliat, J.L., Hughes, S.J., Raposeiro, P.M., DeSalle, R., Sartori, M., Monaghan, M.T., 2017. Colonization and diversification of aquatic insects on three Macaronesian archipelagos using 59 nuclear loci derived from a draft genome. Mol. Phylogenet. Evol. 107, 27–38. doi:10.1016/j.ympev.2016.10.007

Schileyko, A.A., 1978. Fauna SSSR. Mollyuski. Tom. III, vyp. 6. Nazemnye molljuski nadsemejstva Helicoidea. Nauka, Leningrad.

Schileyko, A.A., 1991. Taxonomic status, phylogenetic relations and system of the Helicoidea sensu lato (Pulmonata). Arch. für Molluskenkd. 120, 187–236.

Schileyko, A.A., 2006. Treatise on recent terrestrial pulmonate molluscs. Part 14. Helicodontidae, Ciliellidae, Hygromiidae. Ruthenica Suppl. 2, 1907–2047.

Soares, A.E.R., Schrago, C.G., 2015. The influence of taxon sampling on Bayesian divergence time inference under scenarios of rate heterogeneity among lineages. J. Theor. Biol. 364, 31–39. doi:10.1016/j.jtbi.2014.09.004

Stamatakis, A., 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313. doi:10.1093/bioinformatics/btu033

Suc, J.P., 1984. Origin and evolution of the Mediterranean vegetation and climate in Europe. Nature 307, 429–432.

Swofford, D.L., 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sunderland, MA, Sinauer Associates.

Van Riel, P., Jordaens, K., Van Houtte, N., Frias Martins, A.M., Verhagen, R., Backeljau, T., 2005. Molecular systematics of the endemic Leptaxini (Gastropoda: Pulmonata) on the Azores islands. Mol. Phylogenet. Evol. 37, 132–143. doi:10.1016/j.ympev.2005.03.019

Vasconcelos, T.N.C., Proença, C.E.B., Ahmad, B., Aguilar, D.S., Aguilar, R., Amorim, B.S., Campbell, K., Costa, I.R., De-carvalho, P.S., Faria, J.E.Q., Giaretta, A., Kooij, P.W., Lima, D.F., Mazine, F.F., Peguero, B., Prenner, G., Santos, M.F., Soewarto, J., Wingler, A., Lucas, E.J., 2017. Myrteae phylogeny, calibration, biogeography and diversification patterns: Increased understanding in the most species rich tribe of Myrtaceae. Mol. Phylogenet. Evol. 109, 113–137. doi:10.1016/j.ympev.2017.01.002

Weir, J., Schluter, D., 2008. Calibrating the avian molecular clock. Mol. Ecol. 17, 2321–2328.

# Supplementary material

**Table S1.** List of the specimens studied in this work sorted according to the new systematics with voucher number, locality information and GenBank accession numbers (EHUMC: Zoology and Animal Cell Biology Department, University of the Basque Country, Vitoria-Gasteiz, Spain; FW: private collection of F. Walther, Essen, Germany; HNHM: Hungarian Natural History Museum, Budapest, Hungary; MN: private collection of Marco T. Neiber, Sehnde, Germany; MVHN: Museum of Natural History, Valencia, Spain; SMNS: State Museum of Natural History, Stuttgart, Germany; ZMH: Zoological Museum of the University Hamburg, Hamburg, Germany; ZMMU: Zoological Museum of Moscow State University, Moscow, Russian Federation). As GenBank does not accept submitting DNA sequences less than 200 base pairs, we highlight sequences shorter than that length with grey shades and an asterisk (*) and we provided the sequences after the table.

| Taxon | Voucher | Locality | Province | Country | HUSO | X | Y | GenBank accession number | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | COI | 16s | ITS1-5.8s-ITS2-28s | 60SL17 | 60SL13 | 60SL7 | RPL14 |
| **Hygromiidae** | | | | | | | | | | | | | | |
| **Leptaxinae** | | | | | | | | | | | | | | |
| **Cryptosaccini** | | | | | | | | | | | | | | |
| *Pyrenaearia cantabrica* (2) | EHUMC-1927 | Desfiladero de las Xanas | Asturias | Spain | 30T | 257582 | 4795207 | MG013581 | MG585408 | MG585449 | MG585508 | * | MG585601 | MG585644 |
| *Pyrenaearia cantabrica* (1) | EHUMC-1932 | La Horadada | Burgos | Spain | 30T | 464603 | 4735039 | MG013586 | MG585409 | MG585450 | MG585509 | MG585557 | MG585602 | MG585645 |
| *Pyrenaearia parva* | EHUMC-1974 | Pedraforca, subida por el canal | Barcelona | Spain | 31T | 393016 | 4676882 | MG013628 | MG585417 | MG585458 | MG585517 | MG585565 | MG585609 | -- |
| *Pyrenaearia carascalopsis* (1) | EHUMC-0991 | Port de Vielha | Lleida | Spain | 31T | 316007 | 4723973 | EU310008 | MG585406 | MG585439 | MG585498 | MG585549 | MG585594 | MG585637 |
| *Pyrenaearia carascalopsis* (2) | EHUMC-1951 | Port de Salau | Ariège | France | 31T | 347150 | 4733559 | MG013605 | MG585412 | MG585453 | MG585512 | MG585560 | MG585605 | MG585648 |
| *Pyrenaearia organiaca* | EHUMC-1971 | Congost del Pont de la Torre | Lleida | Spain | 31T | 363704 | 4679023 | MG013625 | MG585416 | MG585457 | MG585516 | MG585564 | MG585608 | MG585651 |
| *Pyrenaearia navasi* | EHUMC-1963 | Moncayo, fuente de San Gaudioso | Zaragoza | Spain | 30T | 598446 | 4626990 | MG013617 | MG585415 | MG585456 | MG585515 | MG585563 | -- | -- |
| *Pyrenaearia molae* | EHUMC-1961 | Mola de Colldejou | Tarragona | Spain | 31T | 321089 | 4553127 | MG013615 | MG585414 | MG585455 | MG585514 | MG585562 | MG585607 | MG585650 |
| *Pyrenaearia guillenae* | EHUMC-1946 | Aigüestortes, Canal des Estanyeres | Lleida | Spain | 31T | 339763 | 4718950 | MG013600 | MG585411 | MG585452 | MG585511 | MG585559 | MG585604 | MG585647 |
| *Pyrenaearia cotiellae* | EHUMC-1958 | Ibón de Armeña | Huesca | Spain | 31T | 282718 | 4710237 | MG013612 | MG585413 | MG585454 | MG585513 | MG585561 | MG585606 | MG585649 |
| *Pyrenaearia carascalensis* (2) | EHUMC-1945 | Monte Perdido | Huesca | Spain | 31T | 256128 | 4728518 | MG013599 | MG585410 | MG585451 | MG585510 | MG585558 | MG585603 | MG585646 |
| *Pyrenaearia carascalensis* (1) | EHUMC-2064 | Aldamin | Bizkaia | Spain | 30 T | 518240 | 4765250 | MG585386 | MG585419 | MG585460 | MG585519 | MG585567 | MG585611 | MG585653 |
| *Cryptosaccus asturiensis* (1) | EHUMC-1020 | Somiedo A13 | Asturias | Spain | 29T | 722864 | 4776145 | KY818338 | KY818454 | MG585442 | MG585501 | MG585552 | MG585595 | MG585639 |
| *Cryptosaccus asturiensis* (3) | EHUMC-2065 | Pola de Somiedo 2Km N | Asturias | Spain | 29T | 723198 | 4776898 | MG585387 | MG585420 | MG585461 | MG585520 | MG585568 | MG585612 | MG585654 |
| *Cryptosaccus asturiensis* (2) | EHUMC-2066 | Pola de Somiedo 2Km N | Asturias | Spain | 29T | 723198 | 4776898 | MG585388 | MG585421 | MG585462 | MG585521 | MG585569 | MG585613 | MG585655 |
| *Fractanella cabrerensis* | EHUMC-1977 | Alto del Peñon | León | Spain | 29T | 701788 | 4675552 | MG013631 | MG585418 | MG585459 | MG585518 | MG585566 | MG585610 | MG585652 |
| *Mengoana jeschaui* (1) | EHUMC-1029 | Between Cangas and Llanes | Asturias | Spain | 30T | 342944 | 4799854 | KY818339 | KY818505 | MG585445 | MG585503 | MG585555 | MG585598 | MG585641 |
| *Mengoana jeschaui* (2) | EHUMC-2067 | Los Camapanarios, Parque Natural de los Collados del Asón | Cantabria | Spain | 30T | 451587 | 4783373 | MG585389 | MG585422 | MG585463 | MG585522 | MG585570 | MG585614 | MG585656 |
| *Mengoana jeschaui* (3) | EHUMC-2068 | Sotres (below the village) | Asturias | Spain | 30T | 358099 | 4788493 | MG585390 | MG585423 | MG585464 | MG585523 | * | MG585615 | MG585657 |
| **Leptaxini** | | | | | | | | | | | | | | |
| *Leptaxis groviana* | MVHN-2189 | Praia Punta São Llorenço, Madeira | Madeira | Portugal | 28S | 339170 | 3624247 | KY818343 | KY818489 | MG585487 | MG585543 | * | MG585629 | MG585667 |
| *Leptaxis nivosa* | MN 0251-Hyg | Camacha towards Pico do Castelo, Porto Santo | Madeira | Portugal | 28S | 375299 | 3661318 | KY818349 | KY818551 | MG585484 | MG585541 | MG585586 | MG585628 | -- |
| *Leptaxis simia* | MVHN-2191 | São Vicente, Madeira | Madeira | Portugal | 28S | 307749 | 3630133 | KY818351 | KY818496 | MG585488 | -- | * | MG585630 | -- |
| *Leptaxis membranacea* | MN 0250-Hyg | Vereda dos Balcões, Madeira | Madeira | Portugal | 28S | 322857 | 3623737 | KY818350 | KY818495 | MG585483 | MG585540 | MG585585 | MG585627 | -- |
| *Leptaxis caldeirarum* | ZMH-96815 | W of Várzea, São Miguel | Açores | Portugal | 26S | 602812 | 4191727 | KY818346 | KY818492 | MG585494 | -- | * | -- | -- |
| *Leptaxis sanctaemariae* (2) | EHUMC-2069 | Terres do Raposo, Santa Maria | Açores | Portugal | 26S | 675441 | 4090244 | -- | -- | MG585465 | MG585524 | MG585571 | MG585616 | -- |
| *Leptaxis sanctaemariae* (1) | EHUMC-2070 | Gloria, Santa Maria | Açores | Portugal | 26S | 672747 | 4090887 | -- | -- | MG585466 | MG585525 | MG585572 | MG585617 | -- |
| *Leptaxis drouetiana* | EHUMC-2071 | Ribeirinha Farol, Faial | Açores | Portugal | 26S | 360374 | 4273053 | MG585391 | MG585424 | MG585467 | -- | MG585573 | * | -- |
| *Leptaxis terceirana* | EHUMC-2072 | Fontinhas, Terceira | Açores | Portugal | 26S | 490799 | 4287582 | MG585392 | MG585425 | MG585468 | MG585526 | MG585574 | * | -- |
| *Leptaxis minor* | EHUMC-1903 | Miradouro das Fontinhas, Santa Maria | Açores | Portugal | 26S | 671326 | 4092220 | KY818347 | KY818493 | KY818601 | MG585507 | -- | -- | -- |
| *Leptaxis azorica* (4) | EHUMC-2073 | Bordes da Sete Cidades, São Miguel | Açores | Portugal | 26S | 605915 | 4190189 | MG585393 | MG585426 | MG585469 | MG585527 | MG585575 | -- | -- |
| *Leptaxis azorica* (1) | EHUMC-2074 | Salto do Cavalo, São Miguel | Açores | Portugal | 26S | 651034 | 4183499 | MG585394 | MG585427 | MG585470 | MG585528 | -- | -- | -- |
| *Leptaxis azorica* (3) | ZMH-96812-1 | Sete Cidades, 500 m from dam towards Lagoa de Santiago, São Miguel | Açores | Portugal | 26S | 607086 | 4190025 | KY818344 | KY818490 | MG585492 | MG585546 | * | MG585634 | -- |
| *Leptaxis azorica* (2) | ZMH-96812-2 | Sete Cidades, 500 m from dam towards Lagoa de Santiago, São Miguel | Açores | Portugal | 26S | 607086 | 4190025 | KY818345 | KY818491 | MG585493 | MG585547 | MG585591 | MG585635 | -- |
| *Portugala inchoata* (2) | EHUMC-2075 | Ruins of Conimbriga | Coimbra | Portugal | 29T | 543186 | 4438887 | MG585395 | MG585428 | MG585471 | MG585529 | MG585576 | MG585618 | * |
| *Portugala inchoata* (3) | EHUMC-1035 | Ruins of Conimbriga | Coimbra | Portugal | 29T | 543186 | 4438887 | KY818340 | KY818530 | MG585448 | MG585506 | MG585556 | MG585600 | MG585643 |
| *Portugala inchoata* (1) | EHUMC-2076 | Ordes | A Coruña | Spain | 29T | 582390 | 4756937 | MG585396 | MG585429 | MG585472 | MG585530 | MG585577 | MG585619 | MG585658 |

| Taxon | Voucher | Locality | Province | Country | HUSO | X | Y | GenBank accession number | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | COI | 16s | ITS1-5.8s-ITS2-28s | 60SL17 | 60SL13 | 60SL7 | RPL14 |
| **Trochulinae** | | | | | | | | | | | | | | |
| **Ciliellini** | | | | | | | | | | | | | | |
| *Ciliella ciliata* | EHUMC-2077 | Bosque de Gresolet, Saldes | Barcelona | Spain | 31T | 394886 | 4677247 | MG585397 | MG585430 | MG585473 | MG585531 | MG585578 | MG585620 | MG585659 |
| **Archaicini** | | | | | | | | | | | | | | |
| *Archaica (Euarchaica) heptapotamica* | ZMMU Lc-21736 | Talas valley N of Kyzyl-Say | Talas | Kyrgyzstan | 42T | 741048 | 4716197 | KY818366 | KY818441 | MG585497 | MG585548 | MG585593 | -- | -- |
| *Odontotrema diplodon* | ZMMU Lc-20481 | Chatkal mountains, valley of Piazty-say | Jalal-Abad | Kyrgyzstan | 42T | 746728 | 4624423 | KY818373 | KY818518 | MG585496 | -- | -- | -- | -- |
| *Paedhoplita (Paedhoplita) caelestimontana* | FW-1426 | South coast of Issyk Kul, 7 km E of Kara Talaa | Ysyk-Köl | Kyrgyzstan | 43T | 622218 | 4684459 | KY818374 | KY818520 | MG585480 | MG585537 | * | MG585624 | -- |
| **Ganulini** | | | | | | | | | | | | | | |
| *Ganula lanuginosa* | EHUMC-1024 | Andratx-Sant Elm | Mallorca | Spain | 31S | 446047 | 4381346 | KY818385 | KY818469 | MG585443 | -- | MG585553 | MG585596 | -- |
| **Urticicolini** | | | | | | | | | | | | | | |
| **Trochulini** | | | | | | | | | | | | | | |
| *Trochulus (Trochulus) hispidus* | EHUMC-2078 | Road in front of Quirón Hospital | Bizkaia | Spain | 30T | 503781 | 4797329 | MG585398 | MG585431 | MG585474 | -- | MG585579 | MG585621 | MG585660 |
| *Trochulus (Trochulus) striolatus* | EHUMC-2079 | Cambridge University Botanic Gardens | England | UK | 31U | 303556 | 5786528 | MG585399 | MG585432 | MG585475 | MG585532 | MG585580 | -- | -- |
| **Caucasigenini** | | | | | | | | | | | | | | |
| **Ashfordiini** | | | | | | | | | | | | | | |
| *Ashfordia granulata* | EHUMC-1015 | Ordes | A Coruña | Spain | 29T | 582390 | 4756937 | KY818376 | KY818444 | MG585441 | MG585500 | MG585551 | -- | -- |
| **Halolimnohelicini** | | | | | | | | | | | | | | |
| **Monachini** | | | | | | | | | | | | | | |
| *Monacha (Monacha) cantiana* | EHUMC-1030 | Sopelana | Bizkaia | Spain | 30T | 499783 | 4802424 | KX507234 | KX495428 | MG585446 | MG585504 | -- | MG585599 | -- |
| *Monacha (Monacha) cartusiana* (2) | EHUMC-1031 | Cañon del Río Dulce | Guadalajara | Spain | 30T | 533182 | 4541079 | KX507235 | KX495429 | MG585447 | MG585505 | -- | -- | MG585642 |
| *Monacha (Monacha) cartusiana* (1) | EHUMC-2080 | Arkaia | Araba | Spain | 30T | 529813 | 4743612 | MG585400 | MG585433 | MG585476 | MG585533 | MG585581 | -- | MG585661 |
| *Monacha (Paratheba) bithynica* | ZMH-119331 | Bilecik towards Eski?ehir, rocky slope near junction towards Pazaryei | Bilecik | Turkey | 36S | 243964 | 4430906 | KX507221 | KX495411 | MG585495 | -- | MG585582 | MG585636 | MG585672 |
| **Hygromiinae** | | | | | | | | | | | | | | |
| **Hygromiini** | | | | | | | | | | | | | | |
| *Hygromia (Riedelia) limbata* (1) | EHUMC-1027 | Queralbs, Daió | Girona | Spain | 31T | 432084 | 4690529 | KY818322 | KY818481 | MG585444 | MG585502 | MG585554 | MG585597 | MG585640 |
| *Hygromia (Riedelia) limbata* (2) | EHUMC-2081 | Beriain, S slope | Nafarroa | Spain | 30T | 581861 | 4749064 | MG585401 | MG585434 | MG585477 | MG585534 | * | MG585622 | MG585662 |
| **Perforatellini** | | | | | | | | | | | | | | |
| *Monachoides vicinus* | ZMH-96747 | Codlea, E slope of Vârful Magura Codlei | Brasov | Romania | 35T | 376280 | 5062809 | KY818333 | KY818513 | KY818622 | -- | -- | -- | -- |
| *Lindholmomneme altaica* | MN 0124-Hyg | Choya district, Karasuk, near the road 6 km NE of the village | Altai Republic | Russia | 45U | 444247 | 5749917 | KY818329 | KY818500 | KY818609 | -- | -- | -- | -- |
| **Metafruticicolinae** | | | | | | | | | | | | | | |
| *Metafruticicola (Metafruticicola) pellita* | ZMH-50281 | Lasíthi, Kalamafka 0.5 km towards Kalo Chorio | Kríti | Greece | 35S | 378091 | 3882671 | KY818359 | KY818507 | MG585491 | MG585545 | MG585590 | MG585633 | MG585671 |
| *Metafruticicola (Westerlundia) naxiana* | ZMH-29313 | Tombrouk 0.5 km towards Vathianos Kampos | Kríti | Greece | 35S | 337949 | 3911560 | KY818361 | KY818509 | MG585490 | -- | MG585589 | MG585632 | MG585670 |
| *Cyrnotheba corsica* | SMNS ZI0105001 | Truggia, Vallée du Liamone, stone oak forest southwest of camping area | Corsica | France | 32T | 484637 | 4662315 | KX507212 | KX495401 | MG585489 | MG585544 | MG585588 | MG585631 | MG585669 |
| *Hiltrudia kuzmici* | HNHM-98877 | N side of the castle hill | Shkodër | Albania | 34T | 375291 | 4656241 | KY818355 | KY818478 | MG585481 | MG585538 | MG585583 | MG585625 | MG585664 |
| *Hiltrudia mathildae* | MN 0127-Hyg | Brač Island, environment of Mirca, valley of dry brook Murvica | Split-Dalmatia | Croatia | 33T | 623024 | 4802376 | KY818357 | KY818480 | MG585482 | MG585539 | MG585584 | MG585626 | MG585665 |
| **Geomitridae** | | | | | | | | | | | | | | |
| *Actinella (Actinella) lentiginosa* | MVHN-2190 | São Vicente, Madeira | Madeira | Portugal | 28S | 307896 | 3629172 | MG585405 | KJ458482 | KJ458580 | -- | -- | -- | MG585668 |
| *Trochoidea (Trochoidea) elegans* | EHUMC-2082 | IVEF, Gasteiz | Araba | Spain | 30T | 525559 | 4742196 | MG585402 | MG585435 | MG585478 | MG585535 | -- | MG585623 | MG585663 |
| *Trochoidea (Trochoidea) elegans* | EHUMC-1895 | IVEF, Gasteiz | Araba | Spain | 30T | 525559 | 4742196 | -- | -- | -- | -- | KY850614 | -- | -- |
| *Microxeromagna lowei* | EHUMC-1899 | Valdestillas | Valladolid | Spain | 30T | 352254 | 4594705 | KY850419 | KY850467 | KY850557 | KY850713 | KY850632 | KY850790 | KY850862 |
| *Helicella itala* | EHUMC-1898 | Gopegi, NE road | Araba | Spain | 30T | 521917 | 4757495 | KY850415 | KY850465 | KY850555 | KY850709 | KY850628 | KY850788 | KY850859 |
| *Cernuella (Cernuella) virgata* | EHUMC-1883 | Leioa, University | Bizkaia | Spain | 30T | 503193 | 4797318 | KY850412 | KY850519 | KY850605 | KY850757 | KY850679 | -- | -- |
| **Helicidae** | | | | | | | | | | | | | | |
| *Otala (Otala) lactea* | EHUMC-2083 | Bardenas Reales, perimetral | Nafarroa | Spain | 30T | 625228 | 4676260 | MG585403 | MG585436 | MG585479 | MG585536 | MG585582 | -- | -- |
| *Massylaea vermiculata* | EHUMC-1011 | Murchante, el Aspra | Nafarroa | Spain | 30T | 609993 | 4653191 | MG585385 | MG585407 | MG585440 | MG585499 | MG585550 | -- | MG585638 |
| *Corneola desmoulinsii atricha* | MVHN-2164 | Congost de Mont-rebei | Lleida | Spain | 31T | 308064 | 4660706 | MG585404 | MG585438 | MG585486 | -- | MG585587 | -- | MG585666 |
| *Marmorana (Murella) muralis* | MVHN-1274 | Napoli Castle | Campania | Italy | 33T | 436988 | 4521070 | -- | MG585437 | MG585485 | MG585542 | -- | -- | -- |

**60SL13**

(note: similar to 60S ribosomal protein L13)

> ***Pyrenaearia cantabrica*(2) (EHUMC-1927) (182 bp)**
AATACAACACTAAAATCAGGCTTGGACGTGGATTCACACTGGACGAGTTGAAGGCCGCAGGTGTCAACAAGAAAGTTGCACGCAC
CATTGGCATCTCTGTTGACTACCGCAGACGAAGCCTGTCTATTGAGTCACTCCAGCAGAACGTTCAGCGACTGAAGGAGTACAAGA
CCAAGCTGGTT

> ***Mengoana jeschaui*(3) (EHUMC-2068) (188 bp)**
GAGTTGAAGGCAGCAGGTGTCAACAAGAAAGTTGCACGCACCATTGGCATCTCTGTTGACTACCGCAGACGAAACCTGTCTATTGA
ATCMCTCCAGCAGAACGTTCAGCGACTGAAGGAGTACAAGACCAAGCTGGTTGTCTTCCCCCGCAAGGAGGGCAAGCCTGGCAAG
GGAGATGCTTCACCTGA

> ***Leptaxis groviana* (MVHN-2189) (166 bp)**
TGGATTCACACTGGACGAGTTGAAGGCTGCAGGTGTCAACAAGAAAGTTGCACGCACCATTGGCATCTCTGTTGACTACCGCAGAC
GAASCCTGTCTATTGAGTCACTTCAGCAGAACGTTCAGCGACTGAAGGAGTACAAGACCAAGCTGGTTGGAGGGCAAGCC

> ***Leptaxis simia* (MVHN-2191) (183 bp)**
AATACAACACTAAACTCAGGCTTGGACGTGGATTCACATTGGACCAGTTGAAGGCTGCAGGTGTCAACAAGAAAGTTGCACGCAC
CATTGGCATCTCTGTTGACTACCGCAGACGAAACCTGTCTTTTGAGTCACTCCAGCAGAACGTTCAGCGACTCAAGAAGTACAAGA
CCAAGCTGGTTG

> ***Leptaxis caldeirarum* (ZMH-96815) (183 bp)**
AATACAACACTAAAATCAGGCTTGGACGTGGATTCACACTGGACGAGTTGAAGGCTGCAGGTGTCAACAAGAAAGCTGCACGCAC
CATTGTCGTCTCTGTTTACCGTAGACGAAGTCTGTCTATTGAGTCACTCCAACAGAACGTTCAGCAACTGAAGGAGTATAAGATCA
AGCTGGTCGTCT

> ***Leptaxis azorica*(3) (ZMH-96812-1) (150 bp)**
TGGACGAGTTGAAGGCTGCAGGTGTCAACAAGAAAGCTGCACGCACCATTGTCATCTATGTTTACCGTAGACGAAGTCTGTCTATT
GAGTCACTCCAACAGAACGTTCAGCAACTGAAGGAGTATAAGATCAAGCTGGTTGTCTTCCCCC

> ***Paedhoplita caelestimontana* (FW-1426) (95 bp)**
GCATGCACCWTTKGCATCTCTGTTGACTACCGCAGACGAAGCCTGYCTATTGAATCACTCCAASAGAACGTTCAGCRACTGAAGGA
ATACAAGAC

> ***Hygromia limbata*(2) (EHUMC-2081) (175 bp)**
AATACAACACTTTAATCAGGCTTGGACGTGGATTCACACTGGATGAGTTAAAGGCAGCAGGCGTCAACAAGAAAGTTGCACGCAC
CATTGGCATCTCTGTTGACTACCGTAGACGAAGCCTGTCTATTGAATCACTCCAGCAGAACGTTCAACGGCTGAAGGAATACAAGA
CCAA

**60SL7**

(note: similar to 60S ribosomal protein L7)

> ***Leptaxis drouetiana* (EHUMC-2071) (165 bp)**
CATGTTGAAGATTGCYGAGCCCTATGTTGCTTGGGGGGTTCCCAACATGAAGACCGTCCGAGAGCTCATCTACAAGAGAGGCTTTG
GGAAGTCAACCACCAGAGGATTGCCCTGGCAGAGAACKCCATTGTTGAAAAGGAACTAGGCAAGAGGGACATCATKTGT

> ***Leptaxis terceirana* (EHUMC-2072) (165 bp)**
CATGTTGAAGATTGCYGAGCCCTATGTTGCTTGGGGGGTTCCCAACATGAAGACCGTCCGAGAGCTCATCTACAAGAGAGGCTTTG
GGAAGTCAACCACCAGAGGATTGCCCTGGCAGAGAACKCCATTGTTGAAAAGGAACTAGGCAAGAGGGACATCATKTGT

**RPL14**

(note: similar to 60S ribosomal protein L14)

> ***Portugala inchoata*(2) (EHUMC-2075) (199 bp)**
TTGTTGATGGCCCATGTTCTGGTGTAAACCGCAAGGACCTGAACTTCAAGGCTCTCCAYCTGACACAGTTTACAGTTKGTATTGCYC
ACTCCGCTASAGTAGGAACTGTGAGGAAGGCCTGAGAGAAGGCAGATATTAACAAGAAGTGGGAACAGACCACATGGGCAGAGA
AACTGGCCACAAGTGAAAGGAGGGCACA

**Table S2.** Primers used for amplification and sequencing.

| Gene | Primer | Sequence | Annealing temperature | Reference |
|---|---|---|---|---|
| COI | LCO1490 (Fw) | GGTCAACAAATCATAAAGATATTGG | 55°C | Folmer et al. (1994) |
| | HCO2198 (Rv) | TAAACTTCAGGGTGACCAAAAAATCA | 55°C | Folmer et al. (1994) |
| 16S | 16Scs1 (Fw) | AAACATACCTTTTGCATAATGG | 55°C | Chiba (1999) |
| | 16Scs2 (Rv) | AGAAACTGACCTGGCTTACG | 55°C | Chiba (1999) |
| | 16SarI (Fw) | CGCCTGTTTATCAAAAACAT | 55°C | Palumbi et al. (1991) |
| | 16SbrH (Rv) | CCGGTCTGAACTCAGATCACGT | 55°C | Palumbi et al. (1991) |
| ITS1-5.8S | ITS1L (Fw) | TCCGTAGGTGAACCTGCGGAAGGAT | 55°C | Hillis and Dixon (1991) |
| | 58C (Rv) | TGCGTTCAAGATATCGATGTTCAA | 55°C | Hillis and Dixon (1991) |
| 5.8S-ITS2-28S | LSU-1 (Fw) | CTAGCTGCGAGAATTAATGTGA | 55°C | Wade et al. (2006) |
| | LSU-3 (Rv) | ACTTTCCCTCACGGTACTTG | 55°C | Wade et al. (2006) |
| 28S | LSU-2 (Fw) | GGGTTGTTTGGGAATGCAGC | 55°C | Wade et al. (2006) |
| | LSU-5 (Rv) | GTTAGACTCCTTGGTCCGTG | 55°C | Wade et al. (2006) |
| 60SL17 | 9Fw60SL17 | TGGGTCATACCTGCGTGTTC | 58°C | Chueca et al. (2018) |
| | 9Rv60SL17 | CTTCTCGGCCAGGACAACTT | 58°C | Chueca et al. (2018) |
| | 31Fw60SL17 | AAATACYYGTGAAACTGCANA | 58°C | Chueca et al. (2018) |
| | 31Rv60SL17 | CATGTATGGRTTRAWGCGTCC | 58°C | Chueca et al. (2018) |
| 60SL13 | 2Fw60SL13 | GCAGCGCATGGTCAAAACAT | 60°C | Chueca et al. (2018) |
| | 2Rv60SL13 | CAGCTTGGCGTTGATTCGTG | 60°C | Chueca et al. (2018) |
| | 30Fw60SL13 | CCCATGAGAAAGAAGNGRAG | 60°C | Chueca et al. (2018) |
| | 30Rv60SL13 | CANAAGCGTTGTGGTTACGC | 60°C | Chueca et al. (2018) |
| | 300Fw60SL13 | SGTCAGATGYCCTACCTTCA | 62°C | This study |
| | 300Rv60SL13 | GTTTCACCAGCTTYAGYTCT | 62°C | This study |
| 60SL7 | 11Fw60SL7 | AGCGAGCGGAGAAATATGCC | 58°C | Chueca et al. (2018) |
| | 11Rv60SL7 | TCTGTCTGACCAGGGCATCA | 58°C | Chueca et al. (2018) |
| | 32Fw60SL7 | AACTTCNATGTCCCNGCNGA | 58°C | Chueca et al. (2018) |
| | 32Rv60SL7 | CCRTARTCACCACCATCRTT | 58°C | Chueca et al. (2018) |
| | 302Fw60SL7 | CCAGCTGTTCAGAYTGAGGC | 62°C | This study |
| | 302Rv60SL7 | GAGAGGACAGYTTGAATGGC | 62°C | This study |
| RPL14 | 14FwRPL14 | CCTACATTGCCCACGGAGAT | 60°C | Chueca et al. (2018) |
| | 14RvRPL14 | TTTCACGGCTTTCTTGGTTGG | 60°C | Chueca et al. (2018) |
| | 33FwRPL14 | AGATGACAAAGGCAAACTYGTK | 60°C | Chueca et al. (2018) |
| | 33RvRPL14 | CTTGGTTGGYTTCTTRGGCT | 60°C | Chueca et al. (2018) |
| | 303FwRPL14 | AAACTYGTKGCCATTGTT | 61°C | This study |
| | 303RvRPL14 | RGCTTGTTTRGCCTTCATT | 61°C | This study |
| | 3003FwRPL14 | TTGTTGAYGGCCCATGTT | 64°C | This study |
| | 3003RvRPL14 | TCCAGCAGCTTYCCTCAGTT | 64°C | This study |

**References:**

Chiba, S., 1999. Accelerated evolution of land snails *Mandarina* in the oceanic Bonin Islands: Evidence from mitochondrial DNA sequences. Evolution 53, 460–471.

Chueca, L.J., Gómez-Moliner, B.J., Madeira, M.J., Pfenninger, M., 2018. Molecular phylogeny of *Candidula* (Geomitridae) land snails inferred from mitochondrial and nuclear markers reveals the polyphyly of the genus. Mol. Phylogenet. Evol. 118, 357–368

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Hillis, D.M., Dixon, M.T., 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q. Rev. Biol. 66, 411–453.

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. The Simple Fool's Guide to PCR, Version 2.0. Department of Zoology, University of Hawaii, Honolulu, HI.

Wade, C.M., Mordan, P.B., Naggs, F., 2006. Evolutionary relationships among the Pulmonate land snails and slugs (Pulmonata, Stylommatophora). Biol. J. Linn. Soc. 87, 593–610.

**Table S3.** Apomorphic substitutions supporting the family-groups and subfamily-groups here defined for each *locus*. Nucleotides before arrows indicate plesiomorphies, nucleotides after arrow apomorphies and in parenthesis the position of the nucleotide in the alignment. Consistency index (CI) values are provided too. nrDNA refers to ITS1-5.8S-ITS2-28S gene cluster. Does *locus* without any apomorphic substitution are not included.

| | *Locus* | | | | |
|---|---|---|---|---|---|
| **Taxa** | **16S** | **nrDNA** | **60SL17** | **60SL7** | **RPL14** |
| **Metafruticicolinae** | A → T (565) CI = 0.75 | T → C (868) CI = 1.00<br>C → T (1739) CI = 0.75 | None | G → A (1) CI = 1.00<br>C → T (43) CI = 1.00<br>G → A (200) CI = 1.00<br>C → T (349) CI = 1.00 | None |
| **Hygromiinae** | None | G → C (1879) CI = 1.00<br>T → C (2411) CI = 1.00 | None | None | None |
| **Trochulinae** | T → A (567) CI = 0.75 | G → C (1626) CI = 1.00<br>C → T (1674) CI = 1.00<br>G → A (1720) CI = 1.00 | None | None | None |
| **Leptaxinae** | None | C → A (181) CI = 0.67<br>T → G (823) CI = 1.00<br>A → C (1631) CI = 0.75 | None | None | C → A (207) CI = 0.67 |
| Leptaxini | None | G → A (711) CI = 0.67<br>C → T (1600) CI = 1.00 | C → T (13) CI = 0.75 | None | None |
| Cryptosaccini | None | C → T (2115) CI = 1.00<br>C → T (2250) CI = 0.67 | None | None | G → A (164) CI = 1.00 |

**Table S4.** Results of the Likelihood Ratio Tests performed on the nested models. alt: alternative model; null: null model; Ln*L*alt: log-likelihood of the alternative model; Ln*L*null: log-likelihood of the null model; DFalt: number of parameters in the alternative model; DFnull: number of parameters in the null model; DF: degrees of freedom; Dstatistic: value of the statistic (comparison of the likelihood scores of the models); pval: p-value.

| **alt** | **null** | **Ln*L*alt** | **Ln*L*null** | **DFalt** | **DFnull** | **DF** | **Dstatistic** | **pval** |
|---|---|---|---|---|---|---|---|---|
| DEC+j | DEC | -32.54 | -44.26 | 3 | 2 | 1 | 23.43 | 0.0000 |
| DIVALIKE+j | DIVALIKE | -32.40 | -39.74 | 3 | 2 | 1 | 14.67 | 0.0001 |
| BayAreaLIKE+j | BayAreaLIKE | -34.09 | -55.55 | 3 | 2 | 1 | 42.92 | 0.0000 |

# II. ARTIKULUA

## Leptaxinae (Gastropoda: Hygromiidae) lur barraskilo subfamiliaren filogenia molekularra eta biogeografia

- **Laburpena**
- **Emaitzak eta eztabaida**
- **Ondorioak**

**Amaia Caro,** Marco T. Neiber, Benjamín J. Gómez-Moliner & María José Madeira

*Molecular phylogenetics and evolution* (under review)

# Leptaxinae (Gastropoda: Hygromiidae) lur barraskilo subfamiliaren filogenia molekularra eta biogeografia

## Laburpena

Leptaxinae subfamilia Hygromiidae barraskilo familia oso dibertsoaren barruan kokatzen da. Ezberdintasun morfologiko diagnostiko argirik gabe, bere estatusa informazio molekularrean bakarrik oinarritzen da eta banaketa ez-jarraia daukaten hiru tribuz osaturik dago, Leptaxini, Cryptosaccini eta Metafruticicolini. Hala ere, tribu hauen arteko erlazio filogenetikoak ez daude ebatzita eta genero batzuen taldekatzea tribuetara ez dago estatistikoki sostengatua. Leptaxinae-ren barruko erlazioak argitzeko eta Hygromiidae-ren barruan daukaten posizioa argitzeko, haien filogenia eraiki genuen *multilocus* hurbiltze baten bidez, bi gene mitokondrial eta zortzi markatzaile nuklear erabiliz. Gainera, taldearen historia biogeografikoa inferitzeko, filogenia kalibratu genuen eta arbasoen banaketaren estimazio analisi bat burutu genuen. Metafruticicolini-ri subfamilia maila esleitu genion (Metafruticicolinae) eta Leptaxinae Cryptosaccini eta Leptaxini tribuetara mugatu genuen. *Portugala* genero lusitaniarra Leptaxini-ra pasa zen, aurretik Macaronesiako *Leptaxis* generoa bakarrik biltzen zuena. Cryptosaccini-ren barruan Sierra de la Cabrera-ra mugatzen den genero berria deskribatu da, *Fractanella* gen. nov. Gure emaitzen arabera, Leptaxinae Iberiar penintsulan sortu zen Behe Miozenoan eta bertatik Irla Macaronesikoak kolonizatu zituen. Macaronesiako eta Iberiako leinuen arteko banaketarentzat estimatutako adin zaharra dela eta, kolonizazio hau artxipelagoen eta kontinente europarraren eta afrikarraren artean kokatzen diren, eta behinola ur-gainean egon ziren, urazpiko-medien bitartez gertatu zela hipotetizatzen dugu, nahiz eta gaur egun ur-gainean dauden Macaronesiako irla zaharrenen bitartez ere gertatu ahal izan zen. Iberiar penintsulan Erdi Miozenoan hasitako aldaketa klimatikoa, klima subtropikaletik gaur egungo klima mediterraneora igaroz, subfamiliaren dibertsifikazioan faktore garrantzitsua izan zela identifikatu zen, Pleistozenoko ziklo glaziarrekin batera.

## Gako-hitzak

Hygromiidae; Leptaxinae; sistematika; filogeografia; *multilocus*; azkon-aparatua

# 3. Emaitzak eta eztabaida

## 3.1. Filogenia eta sistematika

Gene guztiak konbinatzen zituen azken lerrokatzeak 66 banako biltzen zituen eta 7428 karaktere, zeintzuetatik 1650ek (22,2%) parsimoniarako informazioa zeukaten (GenBank kodeak **S1 Taula** Osagarrian kontsultatu daitezke). **1. taulan** zerrendatzen dira lerrokatzearen luzera, parsimoniarako informazioa daukaten lekuak eta batez besteko base frekuentzia markatzaile bakoitzarentzat.

Informazio mitokondriala eta nuklearra konbinatzen zituen datu-basea erabiliz burututako analisietatik lortutako filogenia **1. irudian** ikusi daiteke. Topologia Egiantz Handieneko (EgH) analisiaren emaitzetan oinarritzen da, baina nodoetan bai Egiantz Handieneko *bootstrap* balioak eta bai Inferentzia Bayestarraren (IB) ondorengo probabilitateak azaltzen dira, bi analisiek topologia bera lortu baitzuten. Are gehiago, bi metodoek ia nodo guztiak sostengatu zituzten (*bootstrap* balioak (BB) > 70, ondorengo probabilitateak (OP) > 0,95). Horrela, Hygromiidae-ren erlazio filogenetikoen ezagutza hobetu egin zen aurreko ikerketa molekularrekiko eta hainbat *locus* mitokondrial eta nuklear erabiltzearen onura agerian utzi zen, beste talde batzuetan dokumentatu izan den bezala (adibidez Barley et al., 2010; Lerner et al., 2011; McGowen, 2011). Hala ere, organismo talde batzuentzat, barraskiloak barne, genomaren zati ezberdinak anplifikatzeko primer bateria zabal baten eskuragarritasuna mugatua izan da. Hemen, Geomitridae familiako *Candidula* generoarentzat identifikatutako lau markatzaile nuklear (Chueca et al., 2018) sekuentziatu ahal izan ditugu, ez bakarrik hertsiki erlazionaturiko Hygromiidae familian, baizik eta baita lausoki erlazionaturiko Helicidae familian ere. Beraz, markatzaile hauek Helicoidea barruko beste taldeetan ere erabilgarriak izan daitezke, superfamiliarentzat eskuragarri dauden markatzaile molekular kopurua nabarmenki handituz.

Gure emaitza filogenetikoek Hygromiidae talde monofiletiko gisa guztiz sostengatu zuten eta baita Geomitridae bere talde haurride gisa ere (**1. irudia**). Neiber et al.-en (20017) eta Razkin et al.-en (2015) filogenien arabera Geomitridae eta Canariellidae familiak biltzen dituen kladoa Hygromiidae-ren talde haurridea da. Tamalez, ezin izan genuen Canariellidae familiaren espezierik analisietan sartu eta, beraz, ezin izan genuen aztertu zer nolako erlazio daukan Hygromiidae eta Geometridae-rekin. Bestalde, Geomitridae-rentzat lortu genituen erlazioek

bat egin zuten Chueca et al.-ek (2018), Neiber et al.-ek (2017) eta Razkin et al.-ek (2015) aurretik egindako ikerketa molekularretan lortutakoekin.

Hygromiidae-rentzat lau klado nagusi lortu ziren eta subfamilia maila esleitu genien: Metafruticicolinae, Hygromiinae, Trochulinae eta Leptaxinae. Inferentzia Bayestarrak bakarrik sostengatu zuen Trochulinae izan ezik (BB = 49, OP = 0,99), gainontzeko subfamiliak eta haien arteko erlazioak bai IB-k eta bai EgH-k sendoki sostengatu zituzten.

**Metafruticicolinae** (BB = 90, OP = 1), hemen mugatzen den bezala, Neiber et al.-en (2017) Metafruticicolini tribuarekin bat dator, hau da, *Hiltrudia*, *Cyrnotheba* eta *Metafruticicola* generoak biltzen ditu. Autore hauek klado hau Cryptosaccini-ren talde haurride gisa berreskuratu zuten eta, batera, Leptaxini-ren klado haurridea osatzen zuten. Arrazoi hau dela, Leptaxinae-ren tributzat hartu zuten. Hala ere, erlazio hauek ez zeukaten sostengu estatistikorik eta *Hiltrudia* taldearen parte zen ere ez zegoen argi. Hemen, *Hiltrudia* generoa *Cyrnotheba* eta *Metafruticicola* generoen haurridea dela sostengu sendoarekin berresten dugu baina, beste aldetik, kladoa gainontzeko higromidoen talde haurridea dela berreskuratu genuen. Beraz, ezin da Leptaxinae-ren tributzat hartu eta subfamilia mailara pasa da. Kontuan hartu behar da gainera gure Metafruticicolinae ez dela Nordsieck-ek (1993) eta Schileyko-k (2006, 1978) mugatutako Metafruticicolinae-ren parekoa (sailkapenen arteko konparaketa sakona Neiber et al.-en, 2017 jasotzen da). Gure Metafruticolinae-ren parte diren taxon guztiek ez daukate organo kopulatzaile osagarririk eta, beraz, subfamiliaren ezaugarri sinapomorfikoa da. Hala ere, organo kopulatzaile osagarria galtzea hainbat aldiz gertatu da independenteki Hygromiidae-n.

**Hygromiinae** (BB = 91, OP = 1) eta **Trochulinae** (BB = 49, OP = 0,99) talde haurrideak direla sendoki sostengatu zen (BB = 91, OP = 1). Bi subfamilia hauek bat egiten dute hurrenez hurren Neiber et al.-ek (2017) mugatu zituzten Hygromiinae eta Trochulinae-rekin. Hygromiinae-n bi klado nagusi lortu genituen, Hygromiini eta Perforatellini, autore hauek dagoeneko proposatuak eta, ondorioz, haien sailkapena berretsiz. Ashfordiini (*Ashfordia* Taylor, 1917), Ganulini (*Ganula* Gittenberger, 1970), Monachaini (*Monacha*) eta Trochulini (*Trochulus* Chemnitz, 1786) *sensu* Neiber et al. (2017) guztiz sostengatutako klado batean bilduta berreskuratu ziren, autore hauekin bat eginez. Are gehiago, nahiz eta ezin izan genuen Urticicolini, Caucasigenini eta Halolimnohelicini tribuen ordezkaririk erabili analisietan, gure emaitzak bat datoz autore hauek

klado honetarako deskribatutako erlazioekin. *Odontotrema diplodon* Lindholm, 1927, *Archaica heptapotamica* (Lindholm, 1927) eta *Paedhoplita caelestimontana* (Tzvetkov, 1940) espezieak biltzen zituen kladoa guztiz sostengatu zen, Archaicini tribuaren baliozkotasuna berretsiz. Nahiz eta sostengurik gabe, *Ciliella ciliata* (Hartmann, 1821), Ciliellini-ren espezie bakarra, Archaicini-ren talde haurride gisa lortu zen eta, batera, Trochulinae-ren gainontzeko tribuen haurride ziren, Neiber et al.-ek (2017) aurkitu ez zituzten erlazioak.

Emaitzek **Leptaxinae** sendoki sostengatu zuten (BB = 99, OP = 1) eta Hygromiinae eta Trochulinae biltzen zituen kladoaren talde haurridea zela sostenguarekin berreskuratu zuten (BB = 87, OP = 1). Metafruticicolini subfamiliatik kanpo utzita, Razkin et al.-en (2015) Leptaxinae berreskuratu genuen. Subfamiliak bi klado nagusi zituen eta tribu maila esleitu genien: Leptaxini eta Cryptosaccini.

Leptaxini-k (BB = 83, OP = 0,99) bi genero biltzen ditu, Macaronesiako *Leptaxis* generoa eta *Portugala* genero iberiar monotipikoa. Tribuaren osaketa beraz Neiber et al.-ek (2017) definitutako Leptaxini-tik ezberdintzen da, beraiek *Leptaxis* bakarrik sartu baitzuten. *Portugala*-ren eta *Leptaxis*-en arteko erlazioa Nordsieck-ek (1993) dagoeneko proposatu zuen, bi generoek antzeko aparatu genitala daukatelako, zaku-osagarririk gabeko azkon-zaku oso zabalarekin eta aurkako aldeetan kokatzen diren eta nabarmenki adarkatzen diren bi muki-guruinekin. Hala ere, autore honek *Lindholmomneme* Haas, 1936, *Chilanodon* Westerlund, 1897, *Pseudotrichia* Schileyko, 1970, *Monachoides* Gude eta Woodward, 1921, *Perforatella* Schlüter, 1838, *Urticicola* Lindholm, 1927 eta *Mengoana* generoak ere biltzen zituen Leptaxini-n (= *Perforatella-Leptaxis* taldea). Gure emaitzetan *Portugala Leptaxis*-en talde haurridea dela lortzeak Macaronesia Iberiar penintsularen mendebaldetik kolonizatu zela iradokitzen du. Eskualde mediterraneoarekin batera, luzaroan iradoki da Europa eta batez ere Iberiar penintsula Maraconesiaren kolonizazio iturri izan zitezkeela, bertako taxonen arteko afinitatea dela eta (Cardoso et al., 2010; Cook, 1996; Férnandez-Palacios et al., 2011). Hainbat ikerketa molekularrek espezie europarren eta macaronesiarren artean talde haurride erlazioa lortu dute, besteak beste, kakalardo karabidoetan (Emerson et al., 2000), ur-gezako intsektuetan (Rutschmann et al., 2017) eta lur barraskiloetan (Razkin et al., 2015). Azoreetako artxipelagoan endemikoak diren *Leptaxis* espezie guztiak erabili ziren analisietan (*L. minor*, *L. azorica*, *L. caldeirarum*, *L. sanctaemariae*, *L. terceirana* eta *L. Drouetiana*) eta ondo sostengatutako kladoa osatu zuten (BB = 96, OP = 1), Madeirako espezieen artean txertatuta. Azoreetako *Leptaxis* espezieen arteko erlazioak sendoki sostengatu ziren eta aurretiko lanekin

bat egin zuten (Jordaens et al., 2009; Van Riel et al., 2005). Tamalez, ezin izan genuen Cabo Verdeko *Leptaxis bollei* (Albers, 1856) analisietan sartu eta, ondorioz, ezin izan genuen generoaren barruan betetzen duen lekua zehaztu.

Guztiz sostengatutako Cryptosaccini-k *Mengoana*, *Cryptosaccus* eta *Pyrenaearia* genero iberiarrak bildu zituen. *Cryptasaccus asturiensis* Prieto eta Puente, 1994 eta orain dela gutxi deskribatutako *C. cabrerensis*-ek Holyoak eta Holyoak, 2014 ez zuten klado monofiletikorik osatu. Horren ordez, *C. cabrerensis Pyrenaearia*-ren espezie haurride gisa berreskuratu zen (BB = 100, OP = 1), *C. asturiensis* biekiko haurride zen bitartean (BB = 94, OP = 0,92). Holyoak eta Holyoak-ek (2014) *C. cabrerensis* bi arrazoiengatik erlazionatu zuten estuago *C. asturiensis*-ekin beste higromidoekin baino: azkenengo kiribilaren periferian ezkata moduko mikro-eskultura daukan oskol forma orokorra partekatzen dutelako eta biek zaku-osagarri txikiago bat daukan azkon-zaku bakuna daukatelako haien ugaltze-aparatuan. Hala ere, haien artean, bai oskolean eta bai genitalaren morfologian ere, desberdintasun nabarmenak zeudela ere ohartarazi zuten. Desberdintasun nagusienetako bat zaku-osagarrian dago; izan ere, *C. asturiensis*-en azkon-zakuarekin guztiz fusionatua dago eta ezin da kanpotik ikusi eta *C. cabrerensis*-en, ordea, partzialki bakarrik dago fusionatuta eta kanpotik ikusi daiteke. Zaku-osagarriaren ezaugarri hau dela eta, autore hauek *C. cabrerensis*-en eta *Pyrenaearia*-ren artean afinitate posible bat iradoki zuten, azken genero honen azkon-aparatua azkon-zaku batez eta guztiz banatutako antzeko tamainako zaku-osagarriaz osatuta dagoelako. Gure analisi filogenetikoek hiru taxon hauen artean erlazio estua dagoela berresten dute. Hala ere, guk lortutako erlazio filogenetikoak eta aipatutako ezberdintasun morfologikoak direla eta, genero berri bat, *Fractanella* gen. nov., proposatzen da *C. cabrerensis*-entzat (deskribapen formala beherago). *Pyrenaearia* generoarentzat lortutako erlazio filogenetikoak Caro et al.-ek (2019) lortutakoekin bat zetozen eta are sostengu gehiago eman genien haien nodoei. *Mengoana* generoa aurrez aipaturiko Cryptosaccini generoen talde haurride gisa lortu zen. *Mengoana*-ren azkon-aparatua apendize batek baino ez du osatzen, zaku-osagarri bat zena Puente-ren (1994) arabera. Beraz, Cryptosaccini tribuaren genero bakoitzak azkon-aparatu morfologia ezberdina dauka. Hala ere, guztiek daukate amankomunean azkon-aparatua bakuna dela eta beti zaku-osagarriz osatuta dagoela, eta adarkatutako lau muki-guruin dituztela. Ondorioz, tribu hau Leptaxini-tik ezberdindu daiteke azken tribu honetako espezieek bi muki-guruin dituztelako eta ez daukatelako zaku-osagarririk, nahiz eta bi tribuek azkon-aparatu bakuna partekatzen duten.

Taxon berri bat deskribatzen denean, aurretik ezagutzen ziren espezieetatik ezberdintzen duten ezaugarri diagnostikoak xedatu behar dira (Bauer et al., 2010). Lan hau ohituraz ezaugarri morfologikoetan oinarritu izan da; hala ere, heredagarria den edozein ezaugarri erabili daiteke taxon bat diagnostikatzeko, besteak beste, jokabide-ezaugarriak edo ezaugarri fisiologikoak edo molekularrak (Jörger eta Schrödl, 2013). Ondorioz, ezaugarri morfologikoez gain diagnosi molekularra ere gehitzea praktika baliagarria izan daiteke, batez ere homoplasia morfologikoak taxonomia nahastu dezakeen kasuetan. Hemen definitutako Hygromiidae familia eta subfamilien aldaketa apomorfikoak **S3 taula** osagarrian zerrendatzen dira.

## 3.2. Taxon berriaren deskribapena

***Fractanella*** *Caro eta Madeira gen. nov.*

Espezie tipoa: *Cryptosaccus cabrerensis* Holyoak eta Holyoak, 2014

Diagnosia: **3. taulan** Cryptosaccini genero hau sostengatzen duten koherentzia indize maximoa (CI = 1,00) daukaten aldaketa apomorfikoak zerrendatzen dira. Diagnosi morfologiko xehatu batentzako Holyoak eta Holyoak (2014) kontsultatu daiteke, baina lehen aipatu bezala, generoaren ezaugarri morfologiko nabarienak bi dira. Alde batetik aparatu-estimulatzailea adarkatutako lau muki-guruinez eta azkon-zakua eta zaku-osagarria partzialki fusionatuta dituen azkon-aparatu bakun batez osatuta dago. Bestetik, azkenengo kiribilaren periferian ezkata moduko mikro-eskultura daukan oskol malgu, zeharrargi eta oso gutxi kaltzifikatua dauka.

Barne hartzen dituen taxonak: *Fractanella cabrerensis* (Holyoak eta Holyoak, 2014)

Etimologia: latineko fracta (= zartatua) hitzetik eratorria, bere oskolak daukan mikro-eskulturari egiten dio erreferentzia, zartatu itxura ematen baitio.

3.3. Dibertsifikazio-datak eta patroi biogeografikoak

BEAST bidez informazio mitokondriala eta nuklearra konbinatzen zituen datu-basetik inferitutako dibertsifikazio-data kalkuluek ondo ebatzitako zuhaitz ultrametrikoa lortu zuten (**2. irudia**). EgH-enean eta IB-ean oinarritutako analisi filogenetikoen topologiekiko ezberdintasunak Metafruticicolinae-ren barruko eta Madeirako *Leptaxis* espezieen arteko erlazioetara mugatu ziren, baina hauek edo ez zeuden ondo sostengatuak kronograman edo analisi guztietan. Zuhaitz

ultrametiko honen Leptaxinae-ak bakarrik kontuan hartuz oinarritutako BioGeoBEARS programarekin egindako arbasoen banaketaren estimazio analisiek DIVALIKE+j modeloa lortu zuten sostengu estatistiko sendoenarekin (**4. taula**). Modelo biogeografiko honek estimatu zuen arbasoen banaketa **2. irudian** laburtzen da.

Gure datazio analisien arabera, batez beste, Geomitridae eta Hygromiidae-ren arteko banaketa orain dela 53,6 milioi urte (Ma) gertatu zen (%95-eko ondorengo dentsitate tarte altuena (HPD): 58,7–49,5 Ma) eta gaur egungo Hygromiidae-ren dibertsifikazioa orain dela 27,0 Ma hasi zen (%95 HPD: 35,6–18,4 Ma). Data hauek Razkin et al.-ek (2015) eta Neiber et al.-ek (2017) kalkulatutako dibergentzia-datak baino gazteagoak dira. Laginketa taxonomikoa dibergentzia-daten kalkuluan eragiten duen faktore garrantzitsu bat dela ondorioztatu da (Linder et al., 2005; Poux et al., 2008; Soares eta Schrago, 2015). Beraz, gure laginketa taxonomikoaren eta Razkin et al.-en (2015) zein Neiber et al.-en (2017) laginketen artean dauden ezberdintasun handiak izan daitezke Hygromiidae-rentzat analisi ezberdinetan lortutako dibergentzia-data estima desberdinen iturri. Bestalde, Neiber et al.-ek (2017) lortutako topologia, lan honetan lortutako topologiatik desberdina da klado garrantzitsuenen arteko erlazioei dagokionez, bereziki Metafruticicolinae-renposizioari dagokionez, eta honek dibergentzia-daten inferentzian ere eragina izan dezake eta data estima ezberdinen erantzule izan daiteke. Datazio molekularrean, gainera, beste problema batzuek ere izan dezakete eragina, adibidez, erabilitako kalibrazio puntuen aukeraketak eta kokapenak (Hedges eta Kumar, 2004; Vasconcelos et al., 2017). Ondorioz, dibergentzia inferentzian eragina izan dezaketen faktore guztiek izan dezaketen efektuak ondo ulertu gabe, kontuz ibili behar da data estimak interpretatzerakoan (Linder et al., 2005). Azken finean gure xedea den Leptaxinae-rentzat lortutako data estimak, Macaronesiako historia geologikoarekin koherente dira eta ez daude kontraesanean erregistro fosilarekin. Beraz, gure dibergentzia inferentziak zentzuzkoa dirudi, nahiz eta ezin den baztertu desbiderapen gradu baten posibilitatea.

Gure analisi kronologiko eta biogeografikoen arabera, Leptaxinae-ren dibertsifikazioa Behe Miozenoan hasi zen (orain dela 18,8 Ma; %95 HPD: 26,4–11,9 Ma) Iberiar penintsulan. *Portugala*-ren eta *Leptaxis*-en arteko banaketa orain dela 17,5 Ma datatu zen (%95 HPD: 24,0–10,3 Ma). Kalkulu hau Madeira artxipelagoko irla zaharrenaren, Porto Santoren, agerpena baino zaharragoa da, bere metakin azpi-aereo zaharrenak orain dela 14,2–13,1 Ma bitartean

datatuta baitaude (Geldmacher et al., 2000). Bestalde, Madeirako *Leptaxis* espezieen dibertsifikazioa orain dela 9,2 Ma hasi zela kalkulatu zen, Porto Santo dagoeneko ur-gainean zegoenean (%95 HPD: 13,2–5,4 Ma). *Leptaxis*-en eta *Portugala*-ren arteko banaketa zaharrak, orain dela 17,5 Ma, lehenengo Selvagens artxipelagoa edo Kanaria uharteak kolonizatu zirela iradoki dezake, orduan ur-gainean baitzeuden, eta, gerora, bertatik Madeira artxipelagora iritsi zirela. Ondoren, generoa iraungitu egingo zen Selvagens artxipelagoan eta/edo Kanaria uharteetan. Kanaria uharteetan aurkitutako orain dela 6–12 milioi urteko *Leptaxis* fosil batek (Gittenberger eta Ripken, 1985) hain zuzen ere artxipelago honetan generoaren presentzia berresten du. Hala ere, gero eta informazio geologiko gehiagok, Férnandez-Palacios et al.-en (2011) lanean bilduta, iradokitzen du Madeira eta Kanaria artxipelagoen eta kontinente europarraren eta afrikarraren artean kokatzen diren urazpiko-mendiak behinola ur-gainean egon zirela eta, beraz, kolonizaziorako erabilgarri, orain dela 60 milioi urtetik orain dela 5 milioi urte arte. Honek esan nahi du bi Leptaxini leinu nagusien arteko banaketa gertatu zenean, Selvagens artxipelagoko irlez eta Kanaria uharteetako irla zaharrenez gain, irla gehiago egon zitezkeela erabilgarri, Iberiar penintsulatik gertuago gainera. Beraz, Madeira eta Kanaria uharteen kolonizazioa, gaur egun ur-azpian dauden irla hauen bitartez ere gertatu ahal izan zen. Madeiran endemikoa den *Brachythecium percurrens* goroldioarentzat ere gaur egungo irlak baino zaharragoak diren dibergentzia-datak lortu dira; izan ere, bere familiaren (Helicodontioideae) baitako arbaso komunetan berrienarekiko dibergentzia orain dela 40 milioi urte izan zela kalkulatu zen (Aigoin et al., 2009), Macaronesia osoko irla zaharrenaren agerpena baino lehenago beraz. Ezin da baztertu, ezta ere, bi Leptaxini leinuak kontinentean hasi izana dibertsifikatzen, geroago kolonizatuz Macaronesia.

Madeirako eta Azoreetako *Leptaxis* espezieak orain dela 7,4 Ma (%95 HPD: 10,8–4,4 Ma) banatu ziren gure dibergentzia inferentziaren arabera. Honek bat egiten dut Santa Maria irlaren agerpenarekin, orain dela 8,1 Ma (Abdel-Monem et al., 1975). Guk lortutako filogeniak eta kronogramak ez dute progresio arau argia sostengatzen Azoreetako *Leptaxis* espezieentzat (**3. irudia**) eta gure arbasoen banaketaren kalkuluak ere progresio falta berresten du. Ordea, gure emaitzek Santa Marian bi leinu (A1 eta A2, **3. irudia**) sortu zirela sostengatu zuten eta bertatik bi aldiz independenteki kolonizatu zela São Miguel, hurrengo irla zaharrena, orain dela 4,0 milioi urte sortua (Abdel-Monem et al., 1975). Gerora, eredu biogeografikoaren arabera, A2 leinuak Terceira (3,5 Ma) eta/edo Graciosa (2,5 Ma) kolonizatuko

zituen Santa Mariatik; izan ere, *L. terceirana* (Terceira eta Graciosan endemikoa) eta *L. drouetiana* (Faial eta Picon endemikoa) biltzen dituen kladoa eta Santa Mariako *L. sanctaemariae*ren arteko banaketa gertatu zenean, orain dela 1,1 Ma (%95 HPD: 2,0–0,3 Ma), Teceira eta Graciosa ziren *Leptaxis*-en presentzia daukaten eta garai hartan ur-gainean zeuden irla bakarrak (Santa Maria eta São Miguelez gain). Azkenik, Faial (0,9 Ma) Terceiratik edo Graciosatik kolonizatuko zen, *L. terceirana*-ren eta *L. drouetiana*-ren arteko banaketarekin orain dela 0,4 Ma (%95 HPD: 0,8–0,1 Ma) bat datorrena. Picoren (0,3 Ma) kolonizazioa Faialetik gertatuko zen seguruenik.

Gure dibergentzia-data eta arbasoen banaketa kalkuluetan oinarrituta, gaur egungo Cryptosaccini-en dibertsifikazioa orain dela 9,9 Ma (%95 HPD: 14,7–5,6 Ma), Goi Miozenoan, hasi zen Iberiar penintsularen iparraldean eta tribuaren generoen arteko banaketa Pliozenoaren aurretik gertatu zen. Cryptosaccini-ren eta bere talde haurridearen arteko banaketa Behe Miozenoan gertatu zenez, badirudi tribuaren barruan dibertsifikaziorik gabeko 9 milioi urte egon zirela. Honek tribuaren sorreraren eta gaur egungo espezieen arteko dibertsifikazioaren artean iraungipen handi bat edo hainbat iraungipen garrantzitsu egon zirela iradokitzen du. Miozenoan zehar, eskualde mediterraneoaren mendebaldean sekulako aldaketa klimatikoa gertatu zen. Erdi Miozenoan hasita (orain dela 15–7 Ma), orokorrean subtropikala zen klima pixkanaka aldatzen hasi zen baldintza hotzagoetara eta lehorragoetara, azkenik udako lehorte nabaria bereizgarri duen gaur egungo klima mediterraneoan bilakatuz orain dela 3,2 Ma (Jiménez-Moreno et al., 2010; Suc, 1984). Cryptosaccini espezie guztiek inguru laiotzak behar dituzte eta ez dituzte baldintza lehorrak jasaten. Beraz, baliteke aldaketa klimatiko handi honen eragina egundokoa suertatu izana eta guk kalkulatutako dibergentzia-datek tribuaren eboluzioaren hasierarako proposatzen duten iraungipenaren eragile garrantzitsua izana. Aldaketa klimatiko hau Mediterraneoko organismo askoren iraungipenarekin erlazionatu izan da, adibidez, landareetan (Férnandez-Palacios et al., 2011), ornodunetan (Böhme, 2003; Casanovas-Vilar et al., 2010) eta artropodoetan (Bidegaray-Batista et al., 2014). Biziraun zuen Cryptosaccini leinuak haien bizileku diren mendikateek Neogenoaren amaieran eskaintzen zituzten baldintza hezeagoei esker iraun ahal izan zuen (Jiménez-Moreno et al., 2010), Mediterraneoko mendikateetan bizi den eta higrofiloa den *Harpactocrates* armiarma generoarentzat proposatu izan den bezala (Bidegaray-Batista et al., 2014).

Azkenik, *Pyrenaearia* Pliozenoan (orain dela 4,4 Ma; %95 HPD: 6,3–2,5 Ma) dibertsifikatzen hasi zela eta hiru klado nagusiak Pleistozenoko ziklo glazialak hasi aurretik dagoeneko existitzen zirela kalkulatu zen. Hala ere, espeziazio gertaera gehienak Pleistozenoko ziklo glaziarrak gertatu bitartean jazo zirela kalkulatu genuen. Ondorioz, mendietan bizi diren beste organismo askotan bezala (Bidegaray-Batista et al., 2014; Dépraz et al., 2008; Gittenberger et al., 2004; Harl et al., 2014; Mouret et al., 2011; Pauls et al., 2006), badirudi Pleistozenoko aldaketa klimatikoak eragile garrantzitsuak izan direla *Pyrenaearia*-ren espeziazioan ere.

BioGeoBEARS programa erabiliz eredu biogeografiko ezberdinen artean egindako konparaketa estatistikoan ikusi zenez, datuak hobeto egokitzen ziren gertakari-fundatzailea (+j) deskribatzen zuen parametroa kontuan hartzen zenean, parametro hau erabiltzen zuten ereduek *log-likelihood* balio baxuagoak baitzituzten. Are gehiago, eredu-habiaratuen arteko Erlazio Probabilitate Test-ek, errefusatu egin zuten gertakari-fundatzailea zeukaten eta ez zeukaten ereduek datuentzat probabilitate berdina zeukatenaren hipotesi nulua (**S4 taula osagarria**). Honek analisi biogeografikoetan gertakari-fundatzailea kontuan hartu beharreko prozesua dela nabarmentzen du, baina, gainera, eredu biogeografiko onenean (DIVALIKE+j), Leptaxinae subfamiliaren dibertsifikazioan gertakari-fundatzailea funtsezko prozesua izan zela jaso zen (j = 0.043, d = 0.002 eta e = 0.007 izan zenean, **4. taula**). Honek bat egiten du beste ikerketa biogeografiko batzuekin, non erakutsi baiten gertakari-fundatzailea prozesu erabakigarria dela irletako kladoak dituzten sistemetan, gure kasuan bezala (Matzke 2013b, 2014).

# 4. Ondorioak

Lan honetan, *multilocus* hurbiltze baten bidez, Leptaxinae barraskilo lurtarren subfamiliaren filogenia ebatzi da sostengu sendoarekin eta, gainera, Hygromiidae-ren erlazioen eta sailkapenaren ezagutza hobetu da. Metafruticicolini-ri subfamilia maila esleituta, Hygromiidae lau subfamiliak osatzen dutela ondorioztatu genuen eta Leptaxinae bi tributara, Leptaxini eta Cryptosaccini-ra, mugatu genuen. Barraskilo familia honetan, sailkapen egokiak lortzeko ondo ebatzitako filogenia molekularren garrantzia agerian utzi dugu, homoplasiek mugatu egiten baitute ugaltze-aparatuaren morfologiaren erabilera. Gure emaitzen arabera, Leptaxinae Behe Miozenoan sortu zen Iberiar penintsulan eta bertatik Macaronesia kolonizatu zen. Kontinenteko eta Macaronesiako leinuen artean lortutako antzinako banaketa dela eta, Macaronesia bi bidetatik kolonizatu ahal

izan zen: Macaronesiaren eta kontinenteen artean kokatzen diren eta behinola ur-gainean egon ziren urazpiko-mendien bitartez, edo gaur egun ur-gainean dauden Macaronesiako irla zaharrenen bitartez. Iberiar penintsulan Miozenoan gertatu zen aldaketa klimatikoak, klima subtropikaletik gaur egungo klima mediterraneora, Cryptosacini-ren dibertsifikazioan garrantzi handia izan zuela dirudi, Pleistozenoko ziklo glaziarrekin batera.

## Esker onak

L.J. Chueca, J. Corbella eta G. Guillén-en laguntza eskertu nahi dugu lagin bilketan. Eskerrik asko Geraldine eta David Holyoak-i bidalitako laginengatik eta egindako ohar taxonomiko baliagarriengatik. Lan hau Eusko Jaurlaritzak "*Systematics, Biogeography and Population Dynamics*" Ikerketa Taldeari emandako diru-laguntzarekin (IT575-13) finantzatua izan da partzialki. A. Caro-k Eusko Jaurlaritzaren Hezkuntza, Unibertsitate eta Ikerketa Sailaren doktoretza bekaren babesarekin burutu zuen lana (Ref. PRE_2015_2_0191).

PAPER III

# A new species of *Pyrenaearia* (Gastropoda: Hygromiidae) from the Pyrenees

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# A new species of *Pyrenaearia* (Gastropoda: Hygromiidae) from the Pyrenees

## Abstract

A new species of the land snail genus *Pyrenaearia* from the Pyrenees, identified combining multilocus genetic data with techniques of 3D geometric morphometrics on the shell, is described here and named as *Pyrenaearia guillenae* spec. nov. We provide information about the species diagnosis, distribution and habitat and assess its threat status as Near Threatened according to IUCN criteria.

# 1. Introduction

The land snail genus *Pyrenaearia* Hesse, 1921 is endemic to the northern Iberian Peninsula with a discontinuous distribution across the Cantabrian Mountains, the Pyrenees, the pre-litoral mountain system of Catalonia and the Mount Moncayo (Ortiz de Zárate, 1956; Puente, 1994). The genus is a rock-dwelling specialist further restricted to shady environments and, therefore, their populations are limited to north faces or very abrupt terrains and cliffs of mountainous areas of calcareous substrate (Prieto, 1986; Puente, 1994), with the exception of *P. navasi* (Fagot, 1907) which inhabits in siliceous substrate (Prieto, 1991).

Because the genus is anatomically conservative (Ortiz de Zárate, 1956), and due to its recent and rapid speciation (Elejalde, Madeira, Prieto, Backeljau & Gómez-Moliner, 2009), solving its taxonomy is challenging. First, morphological differentiation of the species is restricted to shell characters which are known to be highly correlated with environmental factors and can led to local adaptations that may blur taxonomy (Fiorentino, Salomone, Manganelli & Giusti, 2008; Razkin, Gómez-Moliner, Vardinoyannis, Martínez-Ortí & Madeira, 2016; Stankowski, 2011, 2013). Secondly, the rapid and recent speciation of the genus may also hinder species delimitation due to incomplete lineage sorting (Edwards & Knowles, 2014). To overcome these problems, Caro, Gómez-Moliner & Madeira (2019) carried out an integrative species delimitation approach on the genus combining multilocus genetic data with 3D geometric morphometrics techniques on the shell. In that work the authors reviewed the genus taxonomy and identified ten species distributed in clades G1 to G9 (with two molecularly indiscernible but morphologically distinct species in clade G1). There was no available name for one of the species of *Pyrenaearia* from the Pyrenees distributed in Aigüestortes and Andorra (clade G8), and the purpose of the present paper is to provide a formal name for its taxonomic recognition.

The integration of multiple lines of evidence is widely recognized as indispensable to properly delimit species (Padial, Miralles, De la Riva & Vences, 2010) and, as its application increased, so does the discovery of new species. However, it has been noticed that several of these new species are not accompanied by formal species description (Pante, Schoelinck & Puillandre, 2015). Naming new species is fundamental to link the data from different sources referring to them and to build new knowledge about them (Patterson, Cooper, Kirk, Pyle & Remsen, 2010), as it is also essential to describe biodiversity (Pante et al., 2015). Here we provide a formal description, determine the distribution and assess the threat status of the newly discovered species of *Pyrenaearia*, *P. guillenae* spec. nov., to ensure its recognition by scientific community, governments (environmental managers) and non-scientist audience.

## 2. Materials and Methods

For the anatomical study of the species, four specimens from the type locality drowned in water and preserved in 70% ethanol were dissected using a Nikon SMZ-U (Zoom 1:10) and drawings were made with the assistance from a Nikon drawing tube. Their shells were photographed with a Nikon D3100 (objective Tamron SP AF 90mm f/2.8 Di Macro) at different depth and then they were merged with CombineZM (Hadley, 2008) to obtain fully focused photos. Shells from all the known populations (n = 38) were measured with a digital caliper (Ratio 6369H15) for the maximum and minimum diameter and for height to record the variability of the shell across all the distribution range. Shell whorls were counted following Kerney & Cameron (1979).

To diagnose the species molecularly, we searched for synapomorphies in DNA sequences. Nucleotide apomorphic substitutions were mapped along the consensus tree obtained by Caro et al. (2019) based on their sequence alignment containing the mitochondrial cytochrome c oxidase subunit I (*COI*) and 16S RNA ribosomal subunit (*16S*), along with the nuclear gene cluster consisting of the loci *ITS1-5.8S-ITS2-28S*, using the command 'describetrees / plot=cladogram opt=deltran apolist = yes' in PAUP* (Swofford, 2002).


**Figure 1.** Frontal (left), superior (middle) and inferior (right) views of *Pyrenaearia guillenae* spec. nov. shells. A: Holopyte; B: Paratype 1; C: Paratype 2, all from the type locality.

We applied the IUCN criteria (IUCN, 2012; 2017) to assess the threat status of *P. guillenae* spec. nov. Extent of occurrence (EOO) was calculated as the minimum convex polygon (the smallest polygon in which no internal angles exceeds 180 degrees and which contains all the sites of occurrence). Area of occupancy (AOO) was estimated by considering an area of 2x2 km for each of the occupied UTM grid cells (IUCN, 2012).

## 3. Systematics

Family HYGROMIIDAE Tryon, 1866

Subfamily LEPTAXINAE Boettger, 1909

Genus *Pyrenaearia* Hesse, 1921

***Pyrenaearia guillenae*** spec. nov.

**Type material:** Holotype, collected the 30th of July of 2014 by Glòria Guillén and Jordi Corbella, is deposited in the collection of the Museum of Natural Sciences of Madrid with catalog number MNCN15.05.200023H; forepart of the body in 70% ethanol, a small portion of foot in 96% ethanol for molecular analyses and shell kept separately; GenBank accession numbers of amplified DNA sequences: MG593766 (cytochrome c oxidase subunit I, mitochondrial), MG593767 (16S RNA ribosomal subunit, mitochondrial) and MG593768 (ITS1-5.8S-ITS2-28S gene cluster, nuclear).

**Paratypes:** All deposited in the collection of the Museum of Natural Sciences of Madrid. From the type locality (MNCN15.05.200023P): 3 individuals with forepart of the body in 70% ethanol, a small portion of foot in 96% and shell kept separately (Paratypes 1-3); 1 specimen in 96% ethanol (Paratype 4); and 6 shells (Paratype 5). From Lo Forcallet, Montanyó de Llacs (MNCN15.05.200024): 1 specimen in 96% ethanol (Paratype 6); and 1 shell (Paratype 7). From Muntanya de Casamanya (MNCN15.05.200025): 2 individuals in 96% ethanol (Paratypes 8-9).

**Type locality:** Canal de les Estanyeres, Aigüestortes (Serra de les Agudes, Pyrenees, Lleida province, Spain); UTM: 31T 0339763 4718950; altitude: 2300 m; glacier bucket in which marble clasts predominate, with little vegetation and a slight slope facing towards northeast.

**Etymology:** The species epiphet *guillenae* is a noun formed from the personal name Guillén after Glòria Guillén who along with Jordi Corbella found two of the known populations, including the type locality.

## Description:

Adult shell (**Fig. 1**) relatively thin, globular to somewhat depressed, with 4.75–5.5 whorls (n = 14). Maximum breadth 10.5–12.5 mm, minimum breadth 9.1–10.5 mm and height 6.1–8.2 mm (n = 38 for all measures). Body world expanded regularly, rounded to slightly angulated at the beginning, rounded near the aperture and slightly descending towards the opening. Umbilicus very narrow, circular and deep, representing 1/10 of maximum shell width, partially covered by columellar edge. Oblique and oval aperture, with marginal ends somewhat convergent. Peristome thin throughout, plane except at the base, being widely reflected towards the umbilicus. Peristome is sometimes slightly thickened inside, close to the opening. Shell surface with irregular transversal striae and with a faint spiral microsculpture. Background color light brown to brown, with many radial, narrow, whitish bands, varying in density all along the teloconch. There is a conspicuous whitish carenal band in the periphery of the shell. Protoconch uniformly brown.

Genital anatomy (**Fig. 2**), based on dissection of four mature animals. Genital atrium short, with similar width to vagina. Penis moderately long (5–7 mm), cylindrical, enlarged in the central part indicating the position of the verge. Penial retractor muscle inserted at the proximal end of penis indicating the separation with epiphallus. Epiphallus as long as penis, coiled, slightly narrower than penis, becoming narrower towards its distal end. Flagellum (4–6 mm) shorter and narrower than epiphallus, with the half distal portion very narrow. Vagina cylindrical, of similar length to penis, often somewhat narrower or with the same width than penis. Dart sac complex showing a double structure, with two muscular sacs of similar size, closely linked together and placed on the same side of vagina. External sac containing a dart. Six to eight digitiform glands in four groups (usually bifurcated) inserting near the proximal part of vagina. Duct of bursa copulatrix cylindrical, long (10–12 mm), moderately wide. Bursa oval, thin walled.

## Diagnosis:

The genital anatomy described here unequivocally places *P. guillenae* spec. nov. within *Pyrenaearia*, but as it has been reported for the genus (Ortiz de Zárate, 1956), it is unable to distinguish it from the other genus species. The shell is easily differentiated from those of *P. cantabrica* (Hidalgo, 1873), *P. oberthueri* (Ancey, 1884) and *P. daanidentata* Raven, 1988 which instead of a narrow umbilicus present a wide one and also from the more flattened shell of *P. molae* Haas, 1924 and *P. organiaca* (Fagot, 1905). Further, it can not be confused with the shell of *P. parva* Ortiz de Zárate, 1956 smaller, with a wider umbilicus and strong striation, nor with the fragile and uniformly brown *P. navasi* (Fagot, 1907). However, with the naked eye the shell of this species and those of *P. cotiellae* (Fagot, 1906), *P. carascalensis* (Férussac,

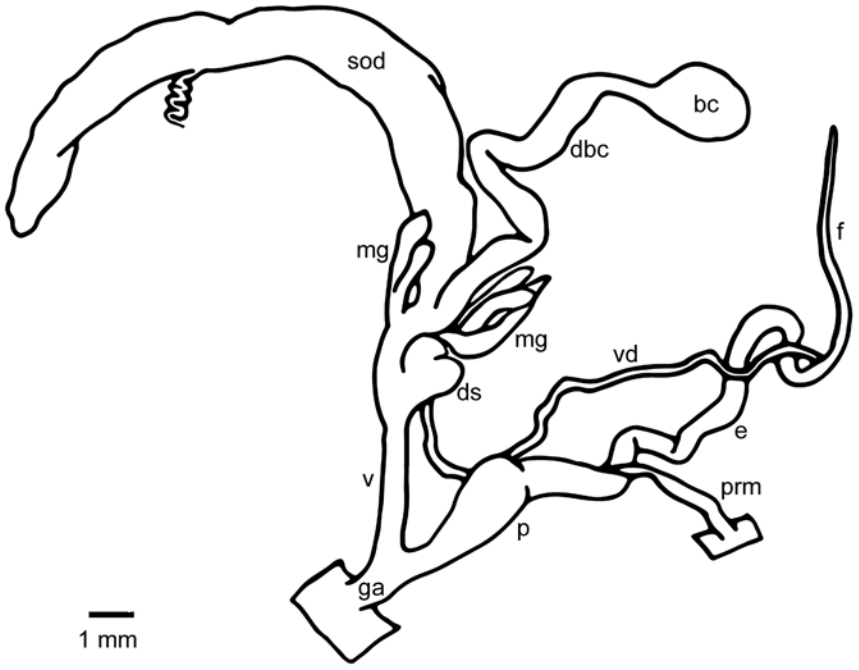


**Figure 2.** Anatomy of genitalia in *Pyrenaearia guillenae* spec. nov. based on the holotype. Abbreviations, **bc**: bursa copulatrix; **dbc**: duct of bursa copulatrix; **ds**: dart sac; **e**: epiphallus; **f**: flagellum; **ga**: genital atrium; **mg**: mucus gland; **p**: penis; **prm**: penial retractor muscle; **sod**: spermoviduct; **v**: vagina; **vd**: vas deferens.

1821) and *P. carascalopsis* (Bourguignat in Fagot, 1884) are not discernible since they share the same overall shape and colouration and striation patterns and these species can only be delimited morphologically when fine geometric morphometric techniques are applied (Caro et al., 2019). Nevertheless, molecular characters are able to unequivocally diagnose *P. guillenae* spec. nov. and separate it from the rest. Within the genus, considering only the apomorphic nucleotide substitutions with hight consistency index (CI > 0.65), *P. guillenae* spec. nov. is characterized by apomorphic changes in cytochrome c oxidase subunit I gene: A → T in 78 base pair (bp) (CI = 1.00), T → C in 114 bp (CI = 0.75) and T → C in 189 bp (CI = 0.67). Besides, the species can be distinguished from its sibling species *P. cotiellae* due to the apomorphic substitution of T → C (CI = 1.00) this last species presents in the 946 position of the ITS1-5.8S-ITS2-28S nuclear gene cluster.

**Distribution:** The species is known from four populations (**Fig. 3**). Three are present in Aigüestortes (Lleida) in Canal de les Estanyeres, Rocablanca and Lo Forcallet and the fourth is located in Andorra in Muntanya de Casamanya. Exact location of the populations is listed in **Table 1**.

**Habitat:** Shady screes and rock walls of calcareous materials, under stones or directly glued to the rocks. It inhabits above 2100 m.

**Assessment of threat status:**

From the five IUCN criteria used to evaluate the threat category of a taxon, (A: Population size reduction; B: Geographic range; C: Small population size and decline; D: Very small or restricted population; E: Quantitative analysis), there is only information of criteria B and D. There are no studies about population size or trends, but field observations indicate that population trend is stable. Besides, the largest population of the species (three of the four known sites) lives in a protected area, the National Park of Aigüestortes i Estany de Sant Maurici, meaning that all these sites are under the protection of national and regional environmental guidelines.

For criterion B three conditions have to be met simultaneously: small geographic range (EOO and/or AOO) together with severely fragmented population or small number of locations, continuous decline or extreme fluctuations. EOO and AOO are very restricted (185.4 km$^2$ and 12 km$^2$, respectively) while the number of locations is limited to 4 sites and the population is fragmented (living above 2100 m). Nevertheless, there are no evidences of continuous decline or extreme fluctuations in EOO, AOO, number of subpopulations or number of mature individuals. Hence, the species only meets two conditions of this criterion. On the other hand, the species covers the criterion D2 to be assessed as Vulnerable: AOO less than 20 km$^2$ or number of locations equal to or less than five (IUCN, 2012). However, the restricted area of occupancy under criterion D2 (IUCN, 2017, page 69) "is defined such that the population is prone to the effects of human activities or

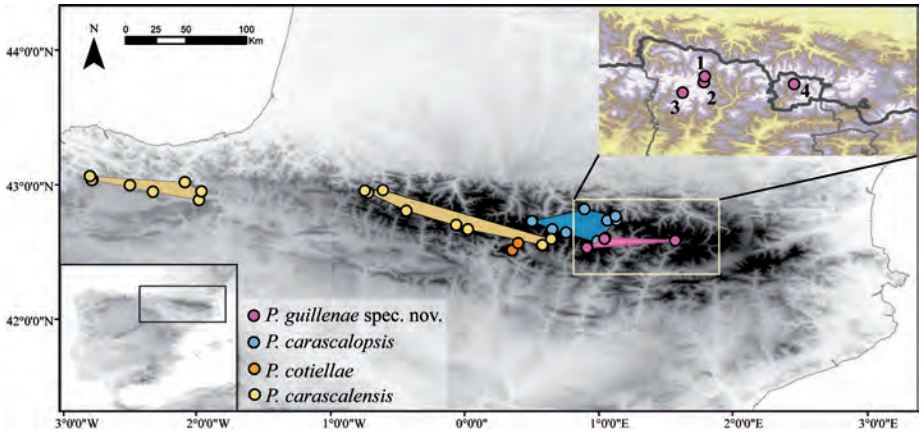


**Figure 3.** Distribution of the *Pyrenaearia* species from the Axial Pyrenees. On the top right zoom with the distribution of *Pyrenaearia guillenae* spec. nov. 1: Canal de les Estanyeres, Aigüestortes; 2: Rocablanca, Aigüestortes; 3: Lo Forcallet, Montanyó de Llacs, Aigüestortes; 4: Muntaya de Casamanya.

stochastic events in an uncertain future, and is thus capable of becoming Critically Endangered or even Extinct in a very short time period (e.g., within one or two generations after the threatening event occurs)". It is not plausible that *P. guillenae* spec. nov. could become Critically Endangered or Extinct in a short time. Further, IUCN guidelines (IUCN, 2017, page 69) state that "if the taxon is highly restricted, and there are plausible threats that can cause the species to become Vulnerable or Endangered in a short time, then the taxon should be considered for listing as Near Threatened". Being a species living only above 2100 m, global warming could represent a major threat for *P. guillenae* spec. nov. Thus, according to criterion D2, this species is assessed as Near Threatened.

Some of the records historically assigned to *P. carascalensis* (Altimira, 1965, 1994; Elejalde et al., 2009; Puente, 1994) are now attributed to *P. guillenae* spec. nov. (see **Fig. 3** with the distribution of the *Pyrenaearia* species from the Axial Pyrenees). Even though *P. carascalensis* has been splitted into two species and its geographical range is now restricted to the western Pyrenees and East Cantabrian Mountains (EOO = 1659.2 km$^2$, estimated adding the EOO areas of the two populations as shown in **Fig. 3**), we propose to maintain its current threat status as Least Concern (Gómez-Moliner, 2011).

**Table 1.** U.T.M. and altitude of the known populations of *Pyrenaearia guillenae* spec. nov.

| Locality | Province | Country | HUSO | X | Y | Altitude (m) |
|---|---|---|---|---|---|---|
| Canal de les Estanyeres, Aigüestortes | Lleida | Spain | 31T | 339763 | 4718950 | 2300 |
| Rocablanca, Aigüestortes | Lleida | Spain | 31T | 339326 | 4718268 | 2519 |
| Lo Forcallet, Montanyó de Llacs, Aigüestortes | Lleida | Spain | 31T | 328536 | 4711093 | 2184 |
| Muntaya de Casamanya | Ordino | Andorra | 31T | 382653 | 4715934 | 2600 |

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# References


Altimira C. 1965. Notas malacológicas. 1. Moluscos interesantes del Parque Nacional de Aigues Tortes. *Miscellania Zoológica,* 2: 19–22.

Altimira C. 1994. Moluscos del Parque Nacional de Aigüestortes. *Butlletí de la Institució Catalana d'Història Natural*, 62: 53–64.

Caro A., Gómez-Moliner B.J. & Madeira M.J. 2019. Integrating multilocus DNA data and 3D geometric morphometrics to elucidate species boundaries in the case of *Pyrenaearia* (Pulmonata: Hygromiidae). *Molecular Phylogenetics and Evolution*, 132: 194–206.

Edwards D.L. & Knowles L.L. 2014. Species detection and individual assignment in species delimitation: can integrative data increase efficacy? *Proceedings of the Royal Society B*, 281: 20132765.

Elejalde M.A., Madeira M.J., Prieto C.E., Backeljau T. & Gómez-Moliner B.J. 2009. Molecular phylogeny, taxonomy, and evolution of the land snail genus *Pyrenaearia* (Gastropoda, Helicoidea). *American Malacological Bulletin*, 27: 69–81.

Fiorentino V., Salomone N., Manganelli G. & Giusti F. 2008. Phylogeography and morphological variability in land snails: the Sicilian *Marmorana* (Pulmonata, Helicidae). *Biological Journal of the Linnean Society*, 94: 809–823.

Gómez-Moliner B.J. 2011. *Pyrenaearia carascalensis*. The IUCN Red List of Threatened Species 2011: e.T156706A4986329. Downloadable from: http://dx.doi.org/10.2305/IUCN.UK.2011-1.RLTS.T156706A4986329.en.

Hadley A. 2008. CombineZM. Downloadable from: http://www.hadleyweb.pwp.blueyonder.co.uk

IUCN. 2012. *IUCN Red List Categories and Criteria: Version 3.1*. IUCN, Gland (Switzerland) and Cambridge (UK), Second edition, 32pp.

IUCN Standards and Petitions Subcommittee. 2017. *Guidelines for Using the IUCN Red List Categories and Criteria*. Version 13. Prepared by the Standards and Petitions Subcommittee. Downloadable from: http://www.iucnredlist.org/documents/RedListGuidelines.dpf

Kerney M.P. & Cameron R.A.D. 1979. *Land snails of Britain & north-west Europe*. HarperCollins, London (UK), 288 pp., 24 pls.

Ortiz de Zárate A. 1956. Observaciones anatómicas y posición sistemática de varios Helícidos españoles. IV. Género *Pyrenaearia* Hesse, 1921. *Boletín de La Real Sociedad Española de Historia Natural*, 54: 35–61.

Padial J.M., Miralles A., De la Riva I. & Vences M. 2010. The integrative future of taxonomy. *Frontiers in Zoology*, 7: 16.

Pante E., Schoelinck C. & Puillandre N. 2015. From integrative taxonomy to species description: one step beyond. *Systematic Biology*, 64 (1): 152–160.

Patterson D.J., Cooper J., Kirk P.M., Pyle R.L. & Remsen D.P. 2010. Names are key to the big new biology. *Trends in Ecology & Evolution*, 25 (12): 686–691.

Prieto C.E. 1986. *Estudio sistemático y biogeográfico de los Helicidae sensu Zilch, 1959-60 (Gastropoda: Pulmonata: Stylommatophora) del País Vasco y regiones adyacentes*. PhD thesis, University of the Basque Country (EHU/UPV).

Prieto C.E. 1991. P*yrenaearia navasi sylvatica* ssp.nov., una subespecie originada por una regresión glaciar cuaternaria en el macizo del Moncayo. *Kobie (Serie Ciencias Naturales)*, 20: 69–75.

Puente A.I. 1994. *Estudio taxonómico y biogeográfico de la superfamilia Helicoidea Rafinesque, 1815 (Gastropoda: Pulmonata: Stylommatophora) de la Península Ibérica e Islas Baleares*. PhD thesis, University of the Basque Country (EHU/UPV).

Razkin O., Gómez-Moliner B.J., Vardinoyannis K., Martínez-Ortí A. & Madeira M.J. 2016. Species delimitation for cryptic species complexes: case study of *Pyramidula* (Gastropoda, Pulmonata). *Zoologica Scripta*, 46 (1): 55–72.

Stankowski S. 2011. Extreme, continuous variation in an island snail: local diversification and association of shell form with the current environment. *Biological Journal of the Linnean Society*, 104 (4): 756–769.

Stankowski S. 2013. Ecological speciation in an island snail: evidence for the parallel evolution of a novel ecotype and maintenance by ecologically dependent postzygotic isolation. *Molecular Ecology*, 22: 2726–2741.

Swofford D.L. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sunderland, MA, Sinauer Associates.

# III. ARTIKULUA

## Pirinioetako *Pyrenaearia* (Gastropoda: Hygromiidae) espezie berri bat

- **Laburpena**
- **Sistematika**

**Amaia Caro,** María José Madeira & Benjamín J. Gómez-Moliner

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# Pirinioetako *Pyrenaearia* (Gastropoda: Hygromiidae) espezie berri bat

## Laburpena

*Multilocus* DNA datuak eta 3D morfometria geometrikoa elkartuz identifikatu den Pirinioetako *Pyrenaearia* lur barraskilo generoko espezie berri bat deskribatzen da hemen, *Pyrenaearia guillenae* spec. nov. izenpean. Espeziearen diagnosiaren, banaketaren eta habitataren inguruko informazioa biltzeaz gain, Ia Arriskuan kategoria esleitu diogu Natura eta Baliabide Naturalak Kontserbatzeko Nazioarteko Batasunaren (NKNB) irizpideen arabera.

# 3. Sistematika

HYGROMIIDAE Tryon, 1866 familia
LEPTAXINAE Boettger, 1909 subfamilia
*Pyrenaearia* Hesse, 1921 generoa
***Pyrenaearia guillenae*** spec. nov.

**Eredu materiala:** Holotipoa, Glòria Guillén-ek eta Jordi Corbella-k bildua 2014ko uztailaren 30ean, Madrileko Natur Zientzien Museoan dago, MNCN15.05.200023H katalogo zenbakiarekin; animaliaren gorputza %70eko etanolean kontserbatua dago, oinaren zatitxo bat %96eko etanolean analisi molekularrentzat eta oskola, lehor, bereizita gordeta dago; anplifikatutako DNA sekuentzien GenBank kodeak: MG593766 (zitokromo c oxidasa I, mitokondriala), MG593767 (16S RNA erribosomala, mitokondriala) eta MG593768 (ITS1-5.8S-ITS2-28S gene multzoa, nuklearra).

**Paratipoak:** guztiak Madrileko Natur Zientzien Museoan gordeta daude. *Locus typicus*-etik (MNCN15.05.200023P): 3 norbanako daude animaliaren gorputza %70eko etanolean eta oinaren zatitxo bat %96eko etanolean kontserbatuta daukatenak, oskola, lehor, bereizita gordeta (1-3 Paratipoak); norbanako bat %96eko etanolean gordeta dago (4 Paratipoa); eta 6 oskol daude (5 Paratipoa). *Lo Forcallet, Montanyó de Llacs* ingurutik (MNCN15.05.200024): norbanako bat dago %96eko etanolean (7 Paratipoa); eta oskol bat (8 Paratipoa). *Muntanya de Casamanya* ingurutik (MNCN15.05.200025): 2 norbanako daude %96eko etanolean (8-9 Paratipoak).

***Locus typicus:*** Canal de les Estanyeres, Aigüestortes (Serra de les Agudes, Pirinioak, Lleida, Espainia); UTM-ak: 31T 0339763 4718950; altitudea: 2300 m; batez ere marmol klastoz osatutako aldapa xumeko pertz glaziarra, landaretza gutxirekin eta ipar-ekialderako orientazioarekin.

Etimologia: *guillenae* epitetoa Guillén abizenetik eratorri dugu, espeziea Glòria Guillén-i eskainita baitago, berak aurkitu dituelako, Jordi Corbellarekin batera, espeziearen bi populazio, *locus typicus*-a barne.

## Deskribapena:

Oskola nahiko mehea da (**1. irudia**), borobildutik apur bat galkatura, 4,75–5,5 bueltarekin (n = 14). Bere zabalera maximoa 10,5–12,5 mm bitartekoa da, zabalera minimoa 9,1–10,5 mm artekoa eta altuera 6,1–8,2 mm artekoa (n = 38 neurri guztientzat). Kiribiltzean erregularki handitzen da: hasieran era borobilduan edo ertz xumeak aurkeztuz; amaierara hurbildu ahala guztiz era borobilean; eta irekieran xumeki beheratuz. Oso zilbor estua dauka, borobila eta sakona, zabalera maximoaren 1/10 hartzen duena eta kolumelaren ertzak partzialki estalita. Irekiera zeiharra eta obalatua da, ertz elkartu samarrekin. Peristoma mehea eta laua da behealdean izan ezik, non zilborrerantz nabarmenki tolesten den. Batzuetan peristoma are estuagoa egiten da barnealdean, irekieratik gertu. Oskolaren azalerak zeharkako ildaska irregularrak eta mikroeskultura espiral ahula aurkezten ditu. Atzealdeko koloreak marroixkatik marroira doan gradientea aurkezten du eta banda radial estu eta txuri asko dauzka, haien dentsitatea aldatu egiten delarik telomaskorrean zehar. Oskolaren periferian zehar banda karenal txuri eta nabarmena dauka. Protomaskorra homogeneoki marroia da.

Genitalaren anatomia (**2. irudia**) lau norbanako helduen disekzioan oinarritzen da. Atrio genitala motza da, baginaren tamainaren antzekoa. Zakila nahiko luzea (5–7 mm) eta zilindrikoa. Erdialdean zabaldu egiten da, papila dagoen tokian. Zakilaren muskulu erretraktorea zakilaren ertz proximalean txertatzen da, epifaloarekiko banaketa erakutsiz. Epifaloa zakila bezain luzea da, baina meharragoa, ertz distalean oraindik estuagoa egiten delarik; gainera kiribildua agertzen da. Flageloa epifaloa baino motzagoa (4–6 mm) eta meharragoa da, erdialdetik amaierara oso estua delarik. Bagina zilindrikoa da eta gutxi gorabehera zakilaren luzera dauka, pixka bat meharragoa edo zabalera berdinekoa izan daitekeelarik. Azkon-aparatuak estruktura bikoitza du, antzeko tamaina daukaten bi zaku muskularrez osatuta, elkarri estuki lotuak eta baginaren alde batean. Kanpokoak azkon bat gordetzen du. Lau taldetan biltzen diren sei–zortzi glandula digitiforme ditu, baginaren zati proximaletik gertu txertatzen direnak. *Bursa copulatrix*-aren hodia zilindrikoa da, luzea (10–12 mm) eta nahiko zabala. *Bursa* obalatua eta pareta estukoa da.

## Diagnosia:

Hemen deskribatzen den ugaltze-aparatuaren morfologiak zalantzarik gabe kokatzen du *P. guillenae* spec. nov. *Pyrenaearia* generoaren barruan, baina generoarentzat dagoeneko jakina denez (Ortiz de Zárate, 1956), ez da gai generoko beste espezieetatik

bereizteko. Bere oskola erraz desberdindu daiteke *P. cantabrica*-ren (Hidalgo, 1873), *P. oberthueri*-ren (Ancey, 1884) eta *P. daanidentata*-ren Raven, 1988 oskoletatik hauek zilbor zabala daukatelako estua beharrean eta baita askoz zapalagoak diren *P. molae*-ren Haas, 1924 eta *P. organiaca*-ren (Fagot, 1905) oskoletatik ere. Are gehiago, ezin da nahastu ildaska nabarmenak eta zilbor zabalagoa daukan *P. parva*-ren Ortiz de Zárate, 1956 oskol txikiarekin, eta ezta *P. navasi*-ren (Fagot, 1907) oskol ahul eta marroi uniformearekin ere. Hala ere, begi hutsez espezie honen oskola ezin da *P. cotiellae*-ren (Fagot, 1906), *P. carascalensis*-en (Férussac, 1821) eta *P. carascalopsis*-en (Bourguignat in Fagot, 1884) oskoletatik bereizi, forma orokorra eta kolore eta ildaska patroiak oso antzekoak direlako, espezie hauek geometria morfometriko teknikak erabiliz bakarrik mugatu daitezkeelarik morfologikoki (Caro et al., 2019). Karaktere molekularrak, aitzitik, gai dira *P. guillenae* spec. nov. zalantzarik gabe diagnostikatzeko eta besteetatik bereizteko. Koherentzia indize (CI) balio altua daukaten nukleotido aldaketa apomorfikoak bakarrik kontuan hartuz (CI > 0,65), *P. guillenae* spec. nov. zitokromo c oxidasa I geneari esker bereiz daiteke generoaren gainerako espezieengandik: 78. base-parean (bp) A → T (CI = 1,00), 114. bp-an T → C (CI = 0,75) eta 189. bp-an T → C (CI = 0,67). Gainera, *P. guillenae* spec. nov. espeziea bere espezie haurride den *P. cotiellae*-tik bereiz daiteke, bigarren honek ITS1-5.8S-ITS2-28S gene nuklear multzoaren 946. base-parean aurkezten duen T → C (CI = 1,00) aldaketa apomorfikoari esker.

**Banaketa:** espeziea lau tokitan aurkitu daiteke (**3. irudia**). Hiru populazio Aigüestortes-en (Lleida) barruan daude, Canal de les Estanyeres-en, Rocablanca-n eta Lo Forcallet-en. Laugarrena Andorran dago, Muntanya de Casamanya inguruan. Populazioen kokapen zehatzak **1. taulan** zerrendatzen dira.

*Habitata*: Material karetsuko harritza eta arroka-pareta laiotzak, harri azpian edo zuzenean arroketara itsatsita egon daitezkeelarik. 2100 m-tik gora bizi da.

**Mehatxu egoeraren ebaluazioa:**

Taxon baten mehatxu egoera ebaluatzeko erabiltzen diren NKNB-ren irizpideetatik (A: Populazio tamainaren murrizketa; B: Banaketa geografikoa; C: Populazio tamaina txikia eta beherakada; D: Oso populazio txikiak edo mugatuak; E: Analisi kuantitatiboa), B eta D irizpideentzat baino ez dago informaziorik. Ez dago populazio tamainaren ez joeraren inguruko ikerketarik, baina landa laneko behaketek populazioen joera egonkorra dela adierazten dute. Gainera, espeziearen

populaziorik handiena (ezagutzen diren lau tokietatik hiru) babestutako inguru batean kokatzen da, Aigüestortes i Estany de Sant Maurici Parke Nazionalean, eta beraz, bai ingurumen arau nazionalek eta bai eskualdekoek babesten dute.

B irizpidea aplikatzeko, 3 baldintza bete behar dira batera: banaketa geografikoa mugatua izatea (EOO eta/edo AOO), populazioa oso zatikatua egotea edo toki gutxitara mugatuta, eta beherakada jarraitua edo aldaketa handiak aurkeztea. EOO eta AOO oso mugatuta daude (185,4 $km^2$ eta 12 $km^2$, hurrenez hurren) eta 4 tokitan bakarrik ezagutzeaz gain, populazioa zatikatuta dago (2100 m-tik gora bakarrik biziz). Hala ere, ez dirudi EOO-k, AOO-k, subpopulazio kopuruak edo norbanako helduen kopuruak beherakadarik edo aldaketa handirik jasan dutenik. Ondorioz, espezieak irizpide honen bi baldintza bakarrik betetzen ditu. Bestalde, espezieak D2 irizpidea betetzen du Ahula kategorian sartzeko: AOO 20 $km^2$ baino txikiagoa izatea edo populazio kopurua bost edo hortik beherakoa izatea (IUCN, 2012). Hala ere, D2 irizpidean okupazio-area mugatua deskribatzean zehazten denez (IUCN, 2017, 69 or.), "populazioak etorkizun ez zehatz batean giza jardueren edo gertaera estokastikoen efektuak jasan ahal ditu eta ondorioz Arrisku Larrian edo Arriskuan kategorietara pasa daiteke oso denbora tarte laburrean (adibidez, mehatxua gertatu eta belaunaldi bat edo bi pasa eta gero)". Ez da probablea *P. guillenae* spec. nov. Arrisku Larrian edo Arriskuan kategorietara pasatzea denbora tarte laburrean. Bestalde, NKNB-ren irizpideen arabera (IUCN, 2017, 69 or.) "taxon bat oso mugatuta baldin badago eta denbora tarte laburrean espeziea Ahula edo Arriskuan kategorietara pasarazi dezaketen mehatxuak existitzen baldin badira, orduan taxon-a Ia Arriskuan zerrendatu behar da". 2100 m-tatik gora bakarrik bizi den espeziea izanda, aldaketa klimatikoa mehatxu garrantzitsua izan daiteke *P. guillenae* spec. nov.-arentzat. Ondorioz, D2 irizpideari jarraituz, espezie honi Ia Arriskuan kategoria esleitzen zaio.

Orain arte *P. carascalensis* espezieari esleitzen zitzaizkion aipu batzuk (Altimira, 1965, 1994; Elejalde et al., 2009; Puente, 1994), *P. guillenae* spec. nov.-ari dagozkio orain (ikusi Pirinio Axialetako *Pyrenaearia* espezieen banaketa **3. irudian**). *P. carascalensis* bi espezietan banatzean bere banaketa mendebaldeko Pirinioetara eta ekialdeko Kantauriar Mendietara mugatu den arren (EOO = 1659,2 $km^2$, **3. irudian** erakusten diren bi *P. carascalensis* populazioen EOO area batuz kalkulatuta), espezie honentzat Arrisku Txikian mehatxu kategoria mantentzea proposatzen dugu (Gómez-Moliner, 2011).

## Esker onak

N. Abad, Z. Cancho, A. Caro-Aramendia, L.J. Chueca, J. Corbella, I. Fernández, M. Gartzia, R. Gibaja, G. Guillén, J. Suso eta N. Suso-ren laguntza eskertu nahi dugu lagin bilketan. Lan hau Eusko Jaurlaritzak "*Systematics, Biogeography and Population Dynamics*" Ikerketa Taldeari emandako diru-laguntzarekin (IT575-13) finantzatua izan da partzialki. A. Caro-k Eusko Jaurlaritzaren Hezkuntza, Unibertsitate eta Ikerketa Sailaren doktoretza bekaren babesarekin burutu zuen lana (Ref. PRE_2015_2_0191).

# CHAPTER 4

## Population demographics and basic biological traits

# 4. KAPITULUA

## Populazio demografia eta oinarrizko ezaugarri biologikoak

PAPER IV

# Population monitoring of *Pyrenaearia carascalensis* (Gastropoda: Hygromiidae) in Gorbeia Natural Park

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*In preparation*

# Population monitoring of *Pyrenaearia carascalensis* (Gastropoda: Hygromiidae) in Gorbeia Natural Park


## Abstract

Despite mountainous areas have remained relatively well preserved even in densely populated territories, long-range transport of contaminants and especially climate change are now affecting these areas and pose new challenges in the conservation of mountains' diversity. Under this scenario, a proper understanding of the ongoing processes leading to biodiversity loss in these areas is necessary to implement appropriate management and conservation measures, which can only be achieved by long-term monitoring on key taxa restricted to mountains. In this study, we started a population monitoring in the Gorbeia Natural Park of the mountain endemic land snail *Pyrenaearia carascalensis* using capture-recapture techniques to investigate species' population demographics and basic biological traits. Here we present the results of the data obtained throughout four years, where we assessed the variation on survival and recapture probability over time on adult and juvenile age classes independently, and studied the possible effect of climatic variables and sampling effort in both survival and recapture. We found differences in adult and juvenile survival and, while adult survival was high and constant, juvenile survival decreased with the increment of autumn freezing days. We reported a long lifespan and a long lapse to reach adulthood for the species as well as possibly the production of large offspring, typical traits of slow life histories which are common among alpine species. Recapture probability varied over time and, although we showed the importance of sampling at least in two occasions in spring and two more times in autumn, we were not able to explain its variation. We further estimated a population size of 434 individuals in the study area and proved a low active displacement of the individuals. This way, we lay the foundation of a long-term monitoring on a species that will allow tracking the changes that are taking place in mountainous areas and will help launching effective measures to protect mountain diversity.



## Keywords

population monitoring; *Pyrenaearia carascalensis*; capture-recapture; population demographics; sentinel species; Gorbeia

# 1. Introduction

Increasing evidence is showing that we are facing a deep biodiversity loss (Cardinale et al., 2012). The actual trend of species extinction rate is approaching to surpass the background extinction rate found in the fossil record, which has led to suggest that Earth may face the sixth mass extinction within a few years' time (Hooper et al., 2012). Through co-opting resources, fragmenting habitats, introducing non-native species, spreading pathogens, releasing pollutants and killing species directly, human activities are responsible for the increase in the extinction rate (Barnosky et al., 2011; MEA, 2005). Climatic change triggered by anthropogenic activities has also been identified among the drivers threatening global biodiversity (MEA, 2005). Indeed, despite the projections yielded by the different approaches applied to predict the effect of climate change on biodiversity have differed greatly, most of them have indicated alarming consequences for biodiversity (e.g. Beaumont et al., 2011; Dawson et al., 2011; Gilman et al., 2010; Hillebrand et al., 2018; Pereira et al., 2010; Selwood et al., 2015; Thomas et al., 2004).

Due to their inaccessibility, mountainous areas have remained relatively well preserved even in densely populated territories. However, long-range transport of contaminants and climatic change are now affecting these areas and pose new challenges in the conservation of mountains' biodiversity (Catalan et al., 2017). Moreover, the steeply altitudinal gradient in mountains promotes contrasting environmental conditions in a short distance (Becker and Bugmann, 2001; Lloret, 2017) which makes them highly sensitive to climate change (Catalan et al., 2017). The effect of climate change in mountains is indeed already being reported (e.g. Jordan et al., 2016; Lin et al., 2018; Menéndez et al., 2014; Nakashizuka et al., 2016). Under this scenario, a proper understanding of the ongoing processes leading to biodiversity loss in these areas is necessary to implement appropriate management and conservation measures and to adequately allocate resources. Long-term monitoring on key taxa restricted to mountains will provide essential information on the undergoing dynamics, which will allow projecting the ecological responses of the organisms (Margalida, 2017; Zamora et al., 2017), and, hence, will be decisive to successfully preserve mountain diversity. So far, monitoring works have been mainly focused on vertebrates, however, apart from being more diverse and abundant, the use of invertebrates to assess biodiversity patterns and environmental processes at some scales can provide more relevant information than that obtained from vertebrates (Yen and Butcher, 1997) and, thus, it is important to direct efforts to monitor invertebrate taxa too.

The land snail genus *Pyrenaearia* Hesse, 1921 is a taxon restricted to mountains distributed exclusively throughout the northern mountain systems of the Iberian Peninsula (Ortiz de Zárate, 1956; Puente, 1994). The genus is strictly rock-dwelling

and, since it is confined to shady environments, it is limited to north-facing rock outcrops or very abrupt terrains and mountain cliffs (Prieto, 1986; Puente, 1994). Thus, its habitat exhibits a discontinuous distribution which, combined to the low active dispersal capabilities of land snails, mean that *Pyrenaearia* species will not be able to adapt to rapid environmental changes by quickly shifting their geographic distribution and colonising new suitable areas in different altitudes or latitudes (Fernández-Chacón et al., 2011). Therefore, the genus could act as an excellent sentinel taxon of the changes that are taking place in mountainous areas, especially those related to climate change.

Nevertheless, despite the genus has been subject of several molecular works (Caro et al., 2019; Elejalde et al., 2009) and its shell morphology has been studied in detail with 3D geometric morphometrics (Caro et al., 2019), basic biological traits and population demographics of all *Pyrenaearia* species remain completely unknown. Since the way an organism interacts with its environment and other organisms is primarily determined by its biological traits (McGill et al., 2006), they are key to understand the mechanisms behind biodiversity patterns and the functioning of the ecosystems (Petchey and Gaston, 2006; Verberk et al., 2010). On the other hand, a sound knowledge of population demographics is required to assess the species involved in a process of decline and to determine its causes (e.g. Duangchantrasiri et al., 2016; Esteban et al., 2016; Guimarães et al., 2014). All in all, a proper understanding of these traits is necessary to forecast the future responses of the species and, hence, to launch effective approaches for their management and conservation. It is striking, however, the little attention and resources that have been assigned to study these characters. Indeed, even in well-known fauna, a severe shortage of these data has already been reported and it has been recognised that this information gap is a drag to adequately study ecosystems (Tyler et al., 2012).

Here, we performed a capture-recapture study on a population of *P. carascalensis* (Férussac, 1821), one of the most widely distributed *Pyrenaearia* species, which occupies the highest summits of the Basque Country and the west and central Pyrenees, with its eastern limit in Noguera Ribagoçana Valley. Through this approach, we aim to shed some light upon the species' population demographics across years and year-round, as well as basic biological traits such as growth, maturity age, lifespan and dispersion abilities. Moreover, we also assess the correlation between life-history parameters and local climate variability to decipher how the climatic variables affect the population. This way, we pursuit to lay the foundation of a long-tern monitoring on the species that will allow monitoring the changes that are taking place in mountainous areas and will help launching effective approaches to protect mountain diversity.

# 2. Materials and methods

## 2.1. Study area and population

The studied *P. carascalensis* population occupies a calcareous rock outcrop formed by seven rocks located at an altitude of 1334 m on the north face of the Mount Gorbeia the highest in the area (N Spain, 30T, X: 517739, Y: 4765043) (**Figure 1**). Rocks' height ranges from 1.5 m to 5 m, which combined with their terrace-like structure, allow an exhaustive sampling of their complete surface without the need of any belay device. The study area is located within the limits of Gorbeia Natural Park and far from the main paths crossing the Park. Its legal protection and isolation ensure a lack of direct human disturbances on the population and enable us studying the population dynamics and its correlation with environmental variables such as climate variability without the confusing effect of direct human stressors (Parmesan and Yohe, 2003). The population may be considered effectively isolated due to the low dispersion capacity of the species and that the outcrop is surrounded by a large extension of not suitable habitat, consisting on grass and siliceous substrate. Further, since the population is close to the summit, the individuals cannot migrate upwards.

## 2.2. Data collection

The monitoring was carried out from 2013 to 2016. Once it was verified that during the winter the individuals hibernate within crevices and therefore they were not detectable in that season, visit schedule was set to 4–5 sampling occasions from spring to autumn (**Supplementary material 1**), except in 2015 when only 3 visits could be carried out.

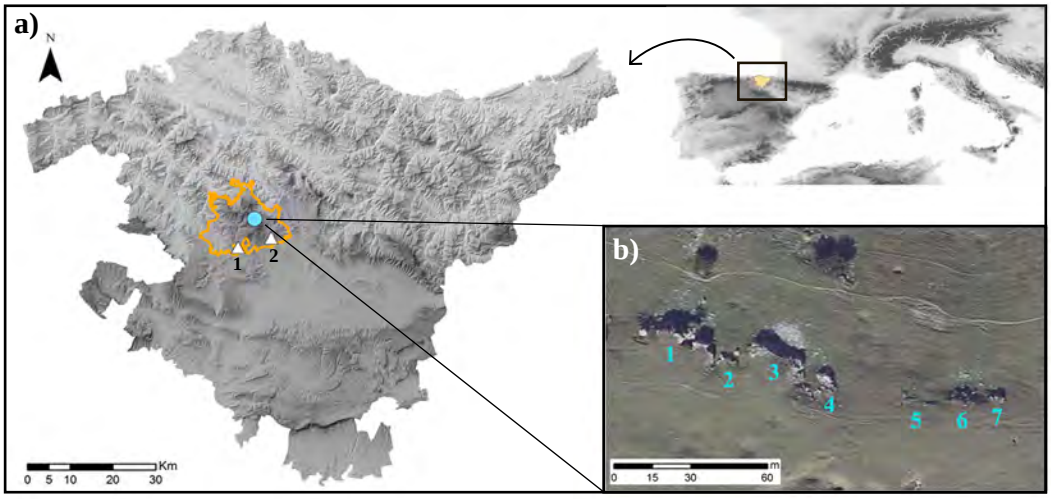


**Figure 1.** Study area: a) Gorbeia Natural Park delimited by orange line, blue dot indicates the location of the monitored *P. carascalensis* population and white triangles the location of the meteorological stations of Sarria (1) and Murua damming (2); b) Detail of the study area where the seven calcareous rocks can be distinguished.

Individuals were marked with a specific method developed for snail shells that did not affect their survival (**Supplementary material 2**). The marking procedure was as follows: (1) when an individual was captured for the first time (i.e. had no mark) and it exceeded 7 mm of diameter (smaller snails could suffer severely by manipulation), it was marked with an individual number and its stadium (juvenile or adult) and the shell diameter for the juveniles were recorded, and (2) for already marked individuals (i.e. recaptures) their presence was noted and if juveniles they were measured again. The passage from youthful to adulthood, indicated by a fully formed peristome slightly reflected toward the umbilicus, was also recorded. Individuals with no identifiable tags, were marked with a new mark, nevertheless, they were excluded from the analysis since they could not be fitted in the model.

To study the relationship between climatological conditions and life-history parameters as well as population trend, climatic variables were obtained from the meteorological station located on the damming of Gorbeia (30T, X: 521589, Y: 4760576) (**Figure 1**), which is one of the closest station to the population and displays the most similar climatic conditions to the studied site. The validity of the values of the climatic variables were checked with those of the nearby meteorological station of Sarria (30T, X: 513845, Y: 4758373).

## 2.3. Matrix construction

This work aims to study both the temporal trend of the population and the year-round variability of the life-history parameters associated to the seasonal climatic oscillations typical of the climate of the area. Being a hibernating species, the data from one activity period to the next can be used to achieve a temporal trend. On the other hand, since the species is associated to mountainous areas and shady environments, the hottest period could also be critical for the population and, therefore, we were also interested in whether there are differences in the life-history parameters through the activity period before and after the period of maximum heat. Thus, the presence/absence data obtained from the monitoring along the four years were grouped to get two sets of matrices: (1) data grouped by activity periods which coincide with years (annual matrices), and (2) data grouped in two seasons, from spring to the hottest period and from the hottest period to autumn (seasonal matrices).

For annual matrices, the observations were coded into encounter histories by grouping all the visits performed in a year, so that if an individual appeared at least in one of the visits of a year it was noted as '1' while if it did not appear in any visit it was considered as a recapture failure and noted as '0'. As the data were collected during four years, the encounter occasions (k) were four (k = 4). For seasonal matrices, first the hottest fortnight of the year was delimited using the meteorological variables

of Gorbeia station. Then, the encounter histories were built by sorting the visits of a year in two groups, before and after the warmest fortnight (hereafter spring and autumn seasons respectively for simplicity) and, similarly to annual matrices, the observations were coded as '1' if they appeared in at least one of the visits of a season and as '0' if they were not detected. In this case, eight encounter occasions were recorded, two for each year (k = 8). The two sets of matrices were independently constructed for juvenile and adult snails to analyse possible differences among both age classes which, in addition, helped avoiding problems that could hinder modelling due to possible different behaviours of each group.

When a snail dies, the shell endured enabling us to detect a proportion of marked death animals. Using the absence '0' in binomial matrices for both death and detection failure supposes a statistical problem (Tavecchia et al., 2016). In order to avoid this error, the information of death individuals was included in the matrices so that there was no uncertainty about the significance of the '0' after the last '1' of the encounter histories of death individuals, helping the modelling.

## 2.4. Capture-recapture modelling

Our capture-recapture datasets were analysed using Comarck-Jolly-Seber (CJS) and related plausible models to obtain separate estimates of two parameters: survival probability ($\phi$) and recapture probability ($p$) (Lebreton et al., 1992). Before performing any modelling, goodness-of-fit (GOF) tests were carried out in each of our matrices using U-CARE 2.3.4 software (Choquet et al., 2009) to verify whether there was any systematic deviations from CJS model assumptions (Lebreton et al., 1992).

First, we performed four GOFs to verify whether there was any transient dependence (TEST3.SR and TEST3.SM) or trap dependence (TEST2.CT and TEST2.CL) in our data. Then, the Global Test was used to achieve the variance inflation factor (ĉ-hat) that gives information about possible over-dispersion and overall adequacy of the data. Under the hypothesis that all the individuals have the same recapture probability implied in CJS model, encounter histories with several consecutive capture failures between two recapture events are not expected and they behave as outliers. These individuals can contribute largely to the significance of the GOF tests and are convenient to be eliminated (Lebreton et al., 1992; Pérez-Mellado et al., 2015). We considered outliers the specimens with annual encounter histories with a recapture gap of two consecutive years ('1001'). In the same way, in seasonal matrices, we treated as outliers individuals with four or more absences between presences, '100001' or '1000001', which accounted also for a gap of at least two years. All these individuals were erased to get more appropriate datasets.

### 2.1.1. Basic models

We initially modelled apparent survival and recapture probabilities solely allowing them to be constant (.) or time dependent (t) (i.e. they varied along encounter occasions) to establish a baseline model structure (Dybala et al., 2013). In seasonal datasets, survival from autumn to spring (winter survival) and survival from spring to autumn (i.e. the transition of the hottest period and hereafter summer survival for simplicity) were represented, as well as spring and autumn recapture probabilities. Since these parameters could show seasonal dependence, we constructed some basic models where $\phi$ and $p$ were set as constant for the same season but different among seasons (2t). **Table 1** lists all the models tested for both seasonal and annual matrices. The same models were tested separately for adult and juvenile datasets.

### 2.4.2. External covariates: climatic variables and sampling effort

Once the best basic models were established, new models were constructed introducing external covariates for the parameters that showed temporal variation (i.e. they were time dependent) in order to determine if any of them could explain their variation. Survival and recapture probabilities may not be affected by the same variables and, hence, distinct hypotheses were tested for each of them.

In the case of survival, we focused on the effects of environmental factors such as climatic variables, which are common to all snails but vary over the year and from year to year (Catchpole et al., 2000). The survival of *P. carascalensis* might be affected by extreme temperature events rather than mean tendencies and, thus, we analysed its correlation with the minimum temperatures of the coldest month ($\phi$H1) and the maximum temperatures of the hottest month ($\phi$H2). Since during the hibernation the individuals are sheltered, we expected that freezing days (< 0 ºC) in winter would not affect them heavily ($\phi$H5). Instead, freezing days in which individuals could be unprotected out of the shelters, such as in autumn ($\phi$H3) and spring ($\phi$H4), might greatly influence their survival. The amount of hot days in summer (> 25 ºC) ($\phi$ H6) was also considered as a factor that could have a great effect on the survival of *P. carascalensis*. Precipitation amount was not supposed to be important for the survival of individuals. On the contrary, humidity on the rocks and soil may play a key role and affect the survival of individuals differently depending on the season. Thus, precipitation days (> 0 L/m$^2$) instead of precipitation amount was selected as an appropriate measure of humidity ($\phi$H7, $\phi$H8, $\phi$H9, $\phi$H10). All hypotheses affecting survival probability are shown in **Table 2**.

For recapture probability, rather than using over-year climatic variables, we took climatic conditions of the previous three days of each visit, since individuals' activity is probably influenced by daily conditions. We used the average of

**Table 1.** Basic models for seasonal and annual matrices.

| No | Model | Period |
|---|---|---|
| 1 | $\phi(.)\ p(.)$ | Seasonal and annu |
| 2 | $\phi(t)\ p(.)$ | Seasonal and annu |
| 3 | $\phi(.)\ p(t)$ | Seasonal and annu |
| 4 | $\phi(t)\ p(t)$ | Seasonal and annu |
| 5 | $\phi(.)\ p(2t)$ | Seasonal |
| 6 | $\phi(t)\ p(2t)$ | Seasonal |
| 7 | $\phi(2t)\ p(.)$ | Seasonal |
| 8 | $\phi(2t)\ p(t)$ | Seasonal |
| 9 | $\phi(2t)\ p(2t)$ | Seasonal |

**Table 2.** Hypotheses tested to explain the variation in the survival of *P. carascalensis* and their notation in the models.

| Hypotheses | Representative variables | Notation |
|---|---|---|
| $\phi$H1. Minimum temperature | Average of the minimum temperatures of the coldest month | (.tmin) |
| $\phi$H2. Maximum temperature | Average of the maximum temperatures of the hottest month | (.tmax) |
| $\phi$H3. Freezing days in autumn | Amount of freezing days in autumn | (.freeze autumn) |
| $\phi$H4. Freezing days in spring | Amount of freezing days in spring | (.freeze spring) |
| $\phi$H5. Freezing days in winter | Amount of freezing days in winter | (.freeze winter) |
| $\phi$H6. Hot days in summer | Amount of days in summer > 25 ºC | (.25 summer) |
| $\phi$H7. Precipitation days in spring | Amount of days in spring > 0 L/m$^2$ | (.pre days spring) |
| $\phi$H8. Precipitation days in summer | Amount of days in summer > 0 L/m$^2$ | (.pre days summer) |
| $\phi$H9. Precipitation days in autumn | Amount of days in autumn > 0 L/m$^2$ | (.pre days autumn) |
| $\phi$H10. Precipitation days in winter | Amount of days in winter > 0 L/m$^2$ | (.pre days winter) |

**Table 3.** Hypotheses tested to explain the recapture probability of *P. carascalensis* and their notation in the models.

| Hypotheses | Representative variables | Notation |
|---|---|---|
| *Climatic variables* | | |
| *p*H1. Minimum temperature | Average of the previous 3 days before the visits | (.min) |
| *p*H2. Maximum temperature | Average of the previous 3 days before the visits | (.max) |
| *p*H3. Humidity | Average of the previous 3 days before the visits | (.hum) |
| *p*H4. Precipitation | Average of the previous 3 days before the visits | (.pre) |
| *Sampling effort* | | |
| *p*H5. Annual sampling effort | 3 visits = 1 and 4–5 visits = 2 | (.esf12a) |
| *p*H6. Seasonal sampling effort | 1 visits = 1 and 2–3 visits = 2 | (.esf12b) |
| *p*H7. Number of visits per season | 1, 2 or 3 | (.esf123) |

minimum temperature (*p*H1), maximum temperature (*p*H2), humidity (*p*H3) and precipitation (*p*H4) of the previous three days of every visit as covariates which, similarly to survival, were expected to be the principal drivers of the activity of the specimens. Besides the climatic variables, sampling effort was also considered as a plausible variable influencing recapture probability (*p*H5–*p*H7). As stated before, we did not achieve the same number of visits for each year or season. Hence, it was thought that the number of visits could be the source of the time dependence of the recapture probability. For annual matrices, we achieved 4–5 visits per year except for 2015, in which only three visits were performed. To incorporate that information on the models, 4–5 visits were given a value of '2', while a value of '1' was set for the three visits of 2015 (*p*H5). For seasonal matrices, we accomplished 2–3 visits per season except for autumn of 2015, with only one visit. In this case, we performed a model where for the one sampling occasion a value of '1' was set and a value of '2' was given to 2–3 visits (*p*H6). An additional model was built for seasonal matrices differentiating between one, two and three visits in which we respectively assign '1', '2' and '3' values to each of them (*p*H7). This approach modelling sampling effort also allowed us to investigate the minimum optimal number of visits to ensure representative datasets. All the covariates tested for recapture probability are listed in **Table 3.**

### 2.4.3. Model building and model selection

Models were built in program MARK v8.1 (White et al., 2001; White and Burnham, 1999) using 2ndPart algorithm, which computes the information matrix directly using central difference approximation. This method provides the most accurate estimates of standard errors and it is the default and preferred method (White and Burnham, 1999). For those datasets with a slightly high variance inflation factor, ĉ-hat = 1–3 (Choquet et al., 2009), we adjusted model construction to accommodate the lack of fit. MARK is known to erroneously count the number of parameters in some time dependent models. In these cases, the number of parameters were manually corrected.

Model selection was based on the corrected Akaike´s Information Criterion accounting for over-dispersion (QAICc) (Lebreton et al., 1992). We selected our best model as the one with the lowest QAICc value, while models differing less than 2 values of ΔQAICc (ΔQAICc < 2) were considered statistically equivalent (Fernández-Chacón et al., 2011, 2013). Finally, if the selected best model had QAICc-Weight ($\omega_i$) ≥ 0.6, the estimates of the survival and recapture probabilities yielded by the model were considered adequate. On the contrary, if $\omega_i$ < 0.6, estimates were calculated by model averaging.

## 2.5. Population size

Exhaustive counts of all the individuals within a population are not possible and, therefore, population size must be derived from partial sampling by analytical methods that account for detection uncertainty. By performing sampling occasions within a short time interval, a closed population without emigration, immigration, dead and birth can be assumed so that only detection probability has to be considered. In 2016, three consecutive monthly visits beginning on May were performed considering that over that period deaths and recruitment would be negligible. We addressed population size estimation following the models of Otis et al. (1978) which are based on the parametrization of three parameters: the number of individuals not encountered ($f_0$), the probability of capture and marking for the very first time ($p_0$) and the probability of being recaptured ($c$). Three models were built where $f_0$ was always supposed to be constant: (1) $f_0$, $p_0(.) = c(.)$ in which there is no temporal variation in either $p_0$ or $c$ and both are set equal, (2) $f_0$, $p_0(t) = c(t)$ where although the parameters are equal they are time dependent, and (3) $f_0$, $p_0(.)$, $c(.)$ with both parameters constant but different between them. Then, the population size estimate (N-hat) was derived from the best model.

## 2.6. Basic biological traits

This monitoring study also provided information about some basic biological traits of the species such as growing, maturity age, lifespan and displacement habits. To examine these traits we also included the information of two additional visits performed in 2017 spring from the ongoing monitoring. Growth dynamic was investigated by plotting the diameters of juveniles against time. Individuals measured four or fewer times were removed from the plotting because they created noise in the growth real tendency. Then, the time needed to reach adulthood was inspected considering the differences in size on the first capture. Since some adult individuals marked the first year were detected along all the years of monitoring, we were only able to estimate a minimum value for the lifespan by adding the time needed to reach maturity to the sampling years. Finally, the movements of the individuals across the study area were tracked to get an approximation of the dispersion potential of the species. With this purpose, the study area was divided into seven sectors according to the seven rocks forming the outcrop and the biggest ones (1 and 3) were further divided in subsectors (A, B, C, D, E, F, G, H and A, B, C, D respectively). While performing the monitoring, the sector in which each individual was captured was annotated and then they were returned accordingly so that changes between sectors could be recorded.

# 3. Results

## 3.1. Survival and recapture probabilities

From spring 2013 to the end of autumn 2016, we marked 1018 individuals of *P. carascalensis*. Nevertheless, after removing the individuals without identifiable marks when recaptured, we retained 958 encounter histories. The overall GOF test of the total population (adults and juveniles together) indicated that the general model fitted the data adequately both in the seasonal matrix ($\chi^2 = 47.405$, df = 25, ĉ-hat = 1.896) and in the annual one ($\chi^2 = 6.716$, df = 4, ĉ-hat = 1.679). However, transient effect was detected in the total seasonal matrix (TEST3.SR: statistic = 2.798, two-sided p-level = 0.002, one-sided p-level = 0.001). Due to the low dispersion capacity of snails and the isolation of the population studied, even a single transient individual is highly improbable. Alternatively, this deviation could be explained by two different behaviours within the population that could correspond to age classes. Indeed, when we performed the GOF tests for annual and seasonal matrices separately for adults and juveniles, no transient effect or trap dependence were detected and the general model fitted better (**Supplementary material 3**).

**Table 4.** Ranking of seasonal models performed for adult age class showing the model structure for survival ($\phi$), recapture probability ($p$), number of parameters (np), deviance (QDEV), AIC values, QAICc-Weight ($\omega i$) and model likelihood (Model L): a) seasonal basic models and b) models with external covariates. Equivalent models in bold.

| Model | $\phi$ | $p$ | np | QDEV | QAICc | ΔQAICc | $\omega_i$ | Model L |
|---|---|---|---|---|---|---|---|---|
| *a) Seasonal basic models* | | | | | | | | |
| **1** | **(.)** | **(t)** | **8** | **217.638** | **2660.371** | **0.000** | **0.848** | **1.000** |
| 2 | (2t) | (t) | 10 | 217.637 | 2664.430 | 4.060 | 0.111 | 0.131 |
| 3 | (t) | (t) | 14 | 211.480 | 2666.429 | 6.059 | 0.041 | 0.048 |
| 4 | (t) | (2t) | 10 | 261.772 | 2708.564 | 46.162 | 0.000 | 0.000 |
| 5 | (.) | (2t) | 4 | 275.681 | 2718.414 | 58.043 | 0.000 | 0.000 |
| 6 | (t) | (.) | 8 | 285.794 | 2720.445 | 60.074 | 0.000 | 0.000 |
| 7 | (2t) | (2t) | 6 | 285.739 | 2724.424 | 64.054 | 0.000 | 0.000 |
| 8 | (.) | (.) | 2 | 306.540 | 2741.191 | 80.820 | 0.000 | 0.000 |
| 9 | (2t) | (.) | 4 | 312.772 | 2743.401 | 83.030 | 0.000 | 0.000 |
| *b) Models with external covariates* | | | | | | | | |
| **1** | **(.)** | **(t)** | **8** | **217.638** | **2660.971** | **0.000** | **0.591** | **1.000** |
| **2** | **(.)** | **(.esf12b)** | **3** | **228.465** | **2661.103** | **0.733** | **0.409** | **0.693** |
| 3 | (.) | (.esf123) | 3 | 252.985 | 2685.623 | 25.253 | 0.000 | 0.000 |
| 4 | (.) | (.pre) | 3 | 264.556 | 2697.195 | 36.824 | 0.000 | 0.000 |
| 5 | (.) | (.hum) | 3 | 296.691 | 2729.329 | 68.959 | 0.000 | 0.000 |
| 6 | (.) | (.tmin) | 3 | 300.860 | 2733.497 | 73.127 | 0.000 | 0.000 |
| 7 | (.) | (.tmax) | 3 | 312.763 | 2745.401 | 85.031 | 0.000 | 0.000 |

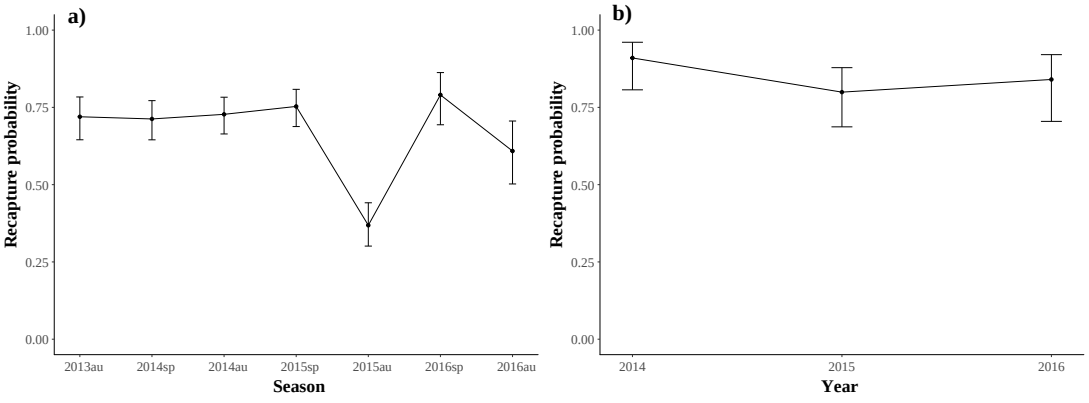


**Figure 2.** Recapture probability estimates for adult age class: a) over seasons and b) over the years.

### 3.1.1. Adult age class

For adult **seasonal** matrix the overall GOF statistic indicated that the general model described our data adequately ($\chi^2$ = 25.046, df = 23, ĉ-hat = 1.089). In this case, the basic model with constant survival throughout seasons and time dependent recapture probability, $\phi(.)p(t)$, was the best model and the only one with ΔQAICc < 2 (**Table 4a**). After modelling the recapture probability with sampling effort and climatic variables, this basic model was still the best one, although the model with sampling effort covariate, $\phi(.)p(.esf12b)$, was statistically equivalent (ΔQAICc = 0.733) (**Table 4b**). Thus, despite we were not able to fully explain time variation in recapture probability, sampling effort showed some influence over it. Recapture probability estimates of each occasion are shown in **Figure 2a** and, indeed, recapture probability decreased when the population was only visited once in autumn 2015. The survival probability of adult individuals between occasions was estimated to be 0.83 (95% confidence interval (CI): 0.81–0.85), being no differences between summer and winter survival probabilities. All parameter estimates are listed in **Supplementary material 4a.**

Adult **annual** dataset fulfilled also the general model assumptions ($\chi^2$ = 8.561, df = 4, ĉ-hat = 2.140). For the annual trend, the basic model with constant survival and time dependent recapture probability, $\phi(.)p(t)$, and the model with both parameters constant, $\phi(.)p(.)$, were statistically equivalent, although the former was more parsimonious (**Table 5a**). With survival modelled as constant by the two basic models, we then replaced the factorial 'time' effect in recapture probability by climatic variables and sampling effort, to assess their relationship with recapture probability. The analysis recovered four statistically equivalent models, those with precipitation [$\phi(.)p(.pre)$], maximum temperature [$\phi(.)p(.tmax)$] and minimum temperature [$\phi(.)p(.tmin)$] external covariates and also the time dependent basic model [$\phi(.)p(t)$] (**Table 5b**). Due to the low $\omega_i$ values of the best models, parameter estimates were calculated by model averaging (**Supplementary material 4b**).

Annual recapture probabilities, displayed in **Figure 2b**, reached their minimum in 2015. Adult survival probability was estimated to be 0.72 (95% CI: 0.66–0.77; model variation: 5.82%).

### 3.1.2. Young age class

**Table 5.** Ranking of annual models performed for adult age class showing the model structure for survival ($\phi$), recapture probability ($p$), number of parameters (np), deviance (QDEV), AIC values, QAICc-Weight ($\omega i$) and model likelihood (Model L): a) annual basic models and b) models with external covariates. Equivalent models in bold.

| Model | $\phi$ | $p$ | np | QDEV | QAICc | ΔQAICc | $\omega_i$ | Model L |
|---|---|---|---|---|---|---|---|---|
| *a) Annual basic models* | | | | | | | | |
| **1** | **(.)** | **(t)** | **4** | **-17.178** | **615.590** | **0.000** | **0.612** | **1.000** |
| 2 | **(.)** | **(.)** | **2** | **-11.358** | **617.339** | **1.749** | **0.255** | **0.417** |
| 3 | (t) | (t) | 6 | -17.265 | 619.560 | 3.968 | 0.084 | 0.138 |
| 4 | (t) | (.) | 4 | -12.131 | 620.637 | 5.047 | 0.049 | 0.080 |
| *b) Models with external covariates* | | | | | | | | |
| **1** | **(.)** | **(.pre)** | **3** | **-16.572** | **614.176** | **0.000** | **0.259** | **1.000** |
| **2** | **(.)** | **(.tmax)** | **3** | **-16.394** | **614.355** | **0.178** | **0.237** | **0.915** |
| **3** | **(.)** | **(.tmin)** | **3** | **-16.068** | **614.680** | **0.504** | **0.202** | **0.777** |
| **4** | **(.)** | **(t)** | **4** | **-17.178** | **615.590** | **1.414** | **0.128** | **0.493** |
| 5 | (.) | (.esf12a) | 3 | -14.460 | 616.289 | 2.112 | 0.090 | 0.348 |
| 6 | (.) | (.) | 2 | -11.395 | 617.339 | 3.163 | 0.053 | 0.206 |
| 7 | (.) | (.hum) | 3 | -12.308 | 618.441 | 4.264 | 0.031 | 0.119 |

Overall GOF test performed on the juvenile seasonal matrix did not deviate from the general model assumptions ($\chi^2$ = 20.932, df = 22, ĉ-hat = 0.952). When modelling juvenile **seasonal** behaviour, the basic model where survival was constant and recapture probability was time dependent, $\phi(.)p(t)$, was the most parsimonious among the other equivalent models: $\phi(2t)p(t)$ and $\phi(t)p(t)$ (ΔQAICc = 1.041 and ΔQAICc = 1.608 respectively) (**Table 6a**). With basic models indicating time dependence for both survival and recapture probabilities, we first tried modelling the recapture probability with climatic and sampling effort variables. None of the covariates used was able to explain the time dependence of recapture probability (**Supplementary material 5**) and, therefore, we proceeded to investigate the relationship of the environmental variables with survival, maintaining recapture probability time dependent. In this case, we found that freezing days in autumn was the best supported covariate, $\phi$(.freeze autumn)$p(t)$, without any other equivalent models (**Table 6b**). Estimates showed that juvenile survival was lower in winter than in summer and that it fluctuated according to the amount of freezing days in autumn:

the more the freezing days, the less the survival (**Figure 3a**). The variation of survival estimates and recapture probability are listed in **Supplementary material 6a.**

The overall GOF statistic for juvenile **annual** matrix supported the adequacy of the data ($\chi^2$ = 1.992, df = 4, ĉ-hat = 0.498). The survival of juveniles throughout years was time dependent in both the best equivalent basic models while recapture probability was recovered as constant and time dependent [$\phi$(t)$p$(.) and $\phi$(t)$p$(t) respectively] (**Table 7a**). Since the best equivalent models were not conclusive about the character (constant or time dependent) of recapture probability, we first tried modelling the parameter with the selected covariates, but none of the variables was able to explain recapture probability trend (results not showed). Thus, we continued modelling survival keeping recapture probability constant since it was the most parsimonious basic model. Similarly to seasonal results, the model accounting for the amount of freezing days in autumn, $\phi$(.freeze autumn)$p$(.), was

**Table 6.** Ranking of seasonal models performed for young age class showing the model structure for survival ($\phi$), recapture probability ($p$), number of parameters (np), deviance (QDEV), AIC values, QAICc-Weight ($\omega i$) and model likelihood (Model L): a) seasonal basic models and b) models with external covariates. Equivalent models in bold.

| Model | $\phi$ | $p$ | np | QDEV | QAICc | ΔQAICc | $\omega_i$ | Model L |
|---|---|---|---|---|---|---|---|---|
| *a) Seasonal basic models* | | | | | | | | |
| **1** | **(.)** | **(t)** | **8** | **172.124** | **1570.822** | **0.000** | **0.490** | **1.000** |
| **2** | **(2t)** | **(t)** | **10** | **169.067** | **1571.864** | **1.041** | **0.291** | **0.594** |
| **3** | **(t)** | **(t)** | **14** | **161.375** | **1572.430** | **1.608** | **0.219** | **0.448** |
| 4 | (t) | (2t) | 9 | 192.427 | 1593.172 | 22.350 | 0.000 | 0.000 |
| 5 | (t) | (.) | 8 | 207.884 | 1606.583 | 35.761 | 0.000 | 0.000 |
| 6 | (2t) | (2t) | 6 | 216.084 | 1610.706 | 39.883 | 0.000 | 0.000 |
| 7 | (.) | (2t) | 4 | 223.155 | 1613.721 | 42.898 | 0.000 | 0.000 |
| 8 | (.) | (.) | 2 | 231.749 | 1618.279 | 47.457 | 0.000 | 0.000 |
| 9 | (2t) | (.) | 4 | 231.483 | 1622.048 | 51.226 | 0.000 | 0.000 |
| *b) Models with external covariates* | | | | | | | | |
| **1** | **(.freeze autumn)** | **(t)** | **9** | **166.441** | **1567.186** | **0.000** | **0.380** | **1.000** |
| 2 | (.tmax+.tmin) | (t) | 9 | 169.146 | 1569.891 | 2.705 | 0.098 | 0.259 |
| 3 | (.tmax) | (t) | 9 | 169.310 | 1570.055 | 2.869 | 0.091 | 0.238 |
| 4 | (.tmin) | (t) | 9 | 169.663 | 1570.408 | 3.221 | 0.076 | 0.200 |
| 5 | (.freeze winter) | (t) | 9 | 169.847 | 1570.592 | 3.405 | 0.069 | 0.182 |
| 6 | (.) | (t) | 9 | 172.124 | 1570.822 | 3.636 | 0.062 | 0.162 |
| 7 | (.pre days spring+autumn) | (t) | 9 | 170.199 | 1570.944 | 3.757 | 0.058 | 0.153 |
| 8 | (.pre days summer+winter) | (t) | 9 | 170.650 | 1571.395 | 4.209 | 0.046 | 0.122 |
| 9 | (2t) | (t) | 10 | 169.067 | 1571.864 | 4.677 | 0.037 | 0.097 |
| 10 | (.freeze spring) | (t) | 9 | 171.490 | 1572.236 | 5.049 | 0.030 | 0.080 |
| 11 | (t) | (t) | 14 | 161.375 | 1572.430 | 5.244 | 0.028 | 0.073 |
| 12 | (.25 summer) | (t) | 9 | 171.793 | 1572.538 | 5.352 | 0.026 | 0.069 |

the best one (**Table 7b)**. Nevertheless, the model with factorial 'time' effect, $\phi$(t) $p$(.), was statistically equivalent. Estimates showed that juvenile survival probability decreased when the amount of freezing days in autumn increased (**Figure 3b**) (**Supplementary material 6b**). Finally, an annual recapture probability of 0.86 (95% CI: 0.76–0.93; model variation: 22.00%) was calculated.

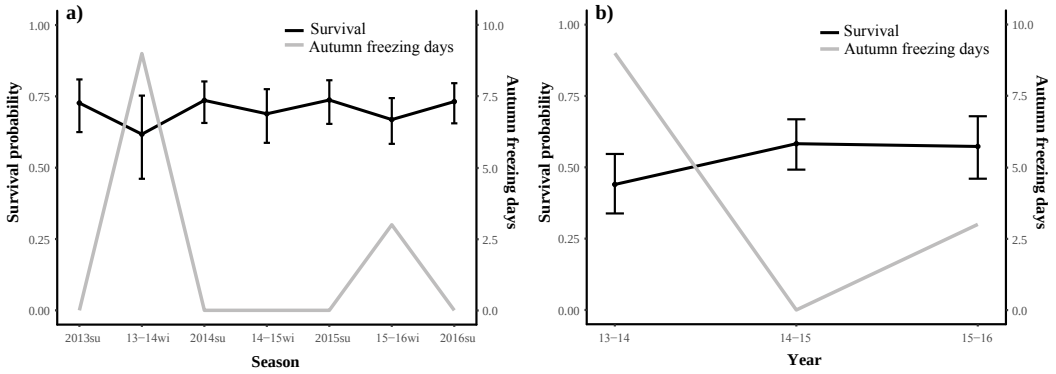


**Figure 3.** Survival probability estimates for young age class in black and amount of autumn freezing days in grey: a) over seasons and b) over the years.

**Table 7.** Ranking of annual models performed for young age class showing the model structure for survival ($\phi$), recapture probability ($p$), number of parameters (np), deviance (QDEV), AIC values, QAICc-Weight ($\omega i$) and model likelihood (Model L): a) annual basic models and b) models with external covariates. Equivalent models in bold.

| Model | $\phi$ | $p$ | np | QDEV | QAICc | ΔQAICc | $\omega_i$ | Model L |
|---|---|---|---|---|---|---|---|---|
| *a) Annual basic models* | | | | | | | | |
| **1** | **(t)** | **(.)** | **4** | **14.567** | **768.125** | **0.000** | **0.644** | **1.000** |
| **2** | **(t)** | **(t)** | **6** | **12.277** | **769.925** | **1.801** | **0.262** | **0.406** |
| 3 | (.) | (.) | 2 | 22.958 | 772.458 | 4.334 | 0.074 | 0.115 |
| 4 | (.) | (t) | 4 | 21.407 | 774.965 | 6.841 | 0.021 | 0.033 |
| *b) Models with external covariates* | | | | | | | | |
| **1** | **(.freeze autumn)** | **(.)** | **3** | **14.851** | **766.376** | **0.000** | **0.440** | **1.000** |
| **2** | **(t)** | **(.)** | **4** | **14.567** | **768.125** | **1.749** | **0.183** | **0.417** |
| 3 | (.pre days winter) | (.) | 3 | 18.267 | 769.792 | 3.416 | 0.080 | 0.181 |
| 4 | (.pre days spring) | (.) | 3 | 18.267 | 769.792 | 3.416 | 0.080 | 0.181 |
| 5 | (t) | (.) | 6 | 12.277 | 769.925 | 3.550 | 0.075 | 0.170 |
| 6 | (.tmax) | (.) | 3 | 18.603 | 770.127 | 3.752 | 0.067 | 0.153 |
| 7 | (.freeze winter) | (.) | 3 | 20.234 | 771.759 | 5.383 | 0.030 | 0.068 |
| 8 | (.pre days autumn) | (.) | 3 | 22.304 | 773.829 | 7.453 | 0.011 | 0.024 |
| 9 | (.25 summer) | (.) | 3 | 22.368 | 773.893 | 7.517 | 0.010 | 0.023 |
| 10 | (.tmin) | (.) | 3 | 22.519 | 774.044 | 7.668 | 0.010 | 0.022 |
| 11 | (.pre days summer) | (.) | 3 | 22.929 | 774.454 | 8.078 | 0.008 | 0.018 |
| 12 | (.freeze spring) | (.) | 3 | 22.929 | 774.454 | 8.078 | 0.008 | 0.018 |

## 3.2. Population size

In population size analysis, the most parsimonious model was the one with equal but time dependent $p_0$ and $c$ parameters [$f_0$, $p_0(t) = c(t)$] whereas the two other models were discarded (ΔQAICc < 2) (**Table 8**). Population size was directly estimated from the best model since $\omega_i > 0.6$. According to the estimates 434 individuals (95% CI: 405–474) comprised the population.

**Table 8.** Ranking of population size models showing the model structure for survival ($\phi$), recapture probability ($p$), number of parameters (np), deviance (QDEV), AIC values, QAICc-Weight ($\omega i$) and model likelihood (Model L).

| No | Model | np | QDEV | AICc | ΔAICc | $\omega_i$ | Model L |
|---|---|---|---|---|---|---|---|
| **1** | **$f_0$, $p_0(t) = c(t)$** | **4** | **17.497** | **-1946.386** | **0.000** | **0.726** | **1.000** |
| 2 | $f_0$, $p_0(.) = c(.)$ | 2 | 24.226 | -1943.685 | 2.701 | 0.188 | 0.259 |
| 3 | $f_0$, $p_0(.)$, $c(.)$ | 3 | 23.771 | -1942.128 | 4.257 | 0.086 | 0.119 |

## 3.3. Basic biological traits

**Figure 4** shows the shell diameter of juveniles through time. Despite it is just a temporal representation of the diameter increment, it was detected that growing rate varied through the year, growing faster during the activity period (between spring and autumn) than in winter when they hibernate.

Regarding maturity age, since we captured juveniles with a very large range of shell diameter size (7–13.5 mm), we calculated the average time required to become adult for three size groups: (a) 7–9 mm, (b) 9–11 mm, and (c) > 11 mm. These groups needed 2.72 years (standard deviation (s) = 0.72 years), 1.95 years (s = 0.65 years) and 1.71 years (s = 0.76 years) respectively to reach adulthood. The maximum time recorded to complete the transition was of 3.93 years and shell diameter mean when reaching adulthood was estimated to be 15.35 mm (s = 0.78 mm). Time needed to become adult of all individuals with initial and final diameter size appears in **Supplementary material 7**. A total of 34 specimens captured in 2013 as adults were recaptured alive in 2017. Considering that we had calculated a maximum of 3.93 years to reach maturity for the smallest juveniles, a lifespan of at least 8–9 years was estimated.

Finally, the observed dispersion was very low even within the same rock. Four individuals changed from one subsector to another within the biggest rocks: one

individual moved between 1.C and 1.F, one from 1.F to 1.C, one from 1.A to 1.F and then to 1.B, and a last one from 1.A to 1.E first and then to 1.B. Similarly, movement between rocks was minimal: five individuals moved from 3.C to 4, two from 4 to 3.C and one between 6 and 5.

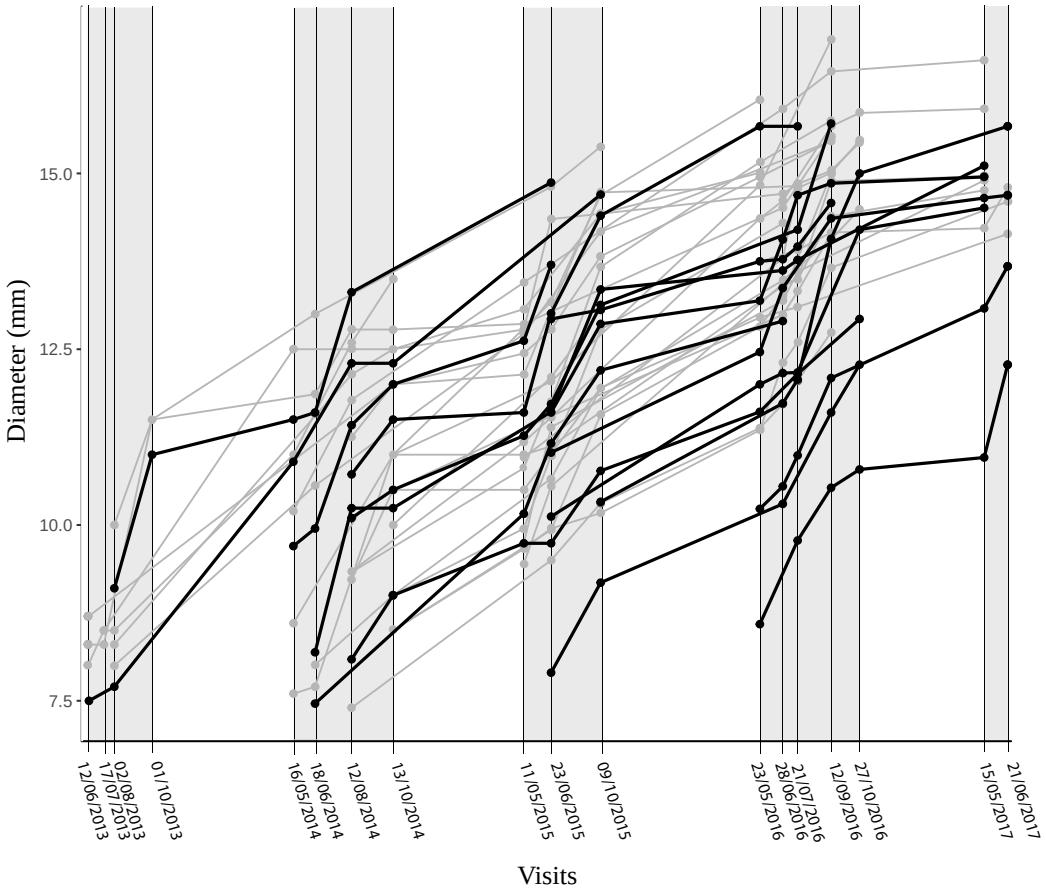


**Figure 4.** Shell diameter measurements of juvenile individuals throughout time. Individuals captured in consecutive visits and which showed clear tendency are represented in black and individuals captured in distant visits in grey to ease visualization due to overlapping.

# 4. Discussion

## 4.1. Life-history traits

This study is the first attempt ever done to unravel the population dynamics and basic biological traits of the land snail species *P. carascalensis* by capture-recapture techniques. The modelling conducted in our study, analysing adult and young age classes independently, allowed us to correctly obtain their survival probabilities since it was found that these groups display different behaviours, which was pointed

out in the first place by the transient effect detected in the GOF conducted on the matrix combining both age classes. This different behaviour led to different survival probabilities in each age classes. Adult snails showed a high and constant survival (seasonal $\phi$ = 0.83; annual $\phi$ = 0.72), whereas the juvenile survival was much lower and influenced by the amount of freezing days in autumn: the more days of freeze, the less they survive (seasonal $\phi$ = 0.61–0.73; annual $\phi$ = 0.44–0.58). Different survival probability between age classes can be the result of natural selection that progressively eliminates low-quality phenotypes, increasing the proportion of high-quality individuals through age classes and, consequently, positively varying with age (selection hypothesis; Forslund and Pärt, 1995). Alternatively, age class dependent survival can result from an age-related improvement in competence (constraint hypothesis; Pärt, 2001), because individuals of more age and experience may survive at higher rates to the same environmental stressors than juveniles (Sergio et al., 2011). We could not determine which of these hypothesis acts in the case of *P. carascalensis*, however, this fact means that it must be taken into account an effect of age-by-time interaction on vital rates not only when studying the population variation but also when proposing management measurements (Sergio et al., 2011).

Large evidences have proven that winter season is a critical period at high and temperate latitudes and that winter mortality is one of the major determinants of population dynamics in these areas (Carboneras et al., 2013). On the other hand, we also hypothesised that the hottest year period could be critical for *P. carascalensis* since it is associated to mountainous areas and shady environments. However, we did not find any variation between winter survival and summer survival in adult individuals. Moreover, adult survival trough years was also estimated to be constant which suggests a stable adult population. Notwithstanding, we only monitored the population throughout four years and, therefore, caution is need when inferring trends (Tenan et al., 2013). Instead, in the case of juveniles, winter survival probability was lower than summer survival probability and it varied negatively with the increasing amount of freezing days in autumn. The negative effect of autumn freezing days in juveniles may be related to a low experience to shelter during this extreme event so that they freeze and die. Alternatively, a high number of freezing days in autumn could be associated to more severe autumns that lead to resources scarcity, hindering the preparation for winter so that they do not achieve enough energy to survive until spring (Pilastro et al., 2003). Whatever the mechanism, according to our results, it seems that autumn variability has more impact on the winter survival of *P. carascalensis* juveniles than winter variability and severity. In the also hibernating yellow-bellied marmots (*Marmota flaviventris*), Lenihan and Van Vuren (1996) also found that winter severity was not the main responsible for winter mortality, but the mass at entry into hibernation. Nevertheless, despite the correlation found between autumn freezing days and juvenile survival, in the case of annual modelling, a model

with factorial 'time' effect, $\phi$(t)$p$(.), was statistically equivalent. Thus, there are other factors influencing the survival of juveniles, but data from more years must be necessary to fix completely and correctly their survival dynamics. Anyway, our results clearly show that juveniles are less buffered against environmental stochasticity than adults are. For the Mediterranean land snail genus *Iberus*, Polo (2015) also reported that juveniles were more sensitive than adults to environmental stochasticity, but in this case, to variables related to insolation and dessication.

Higher adult survival compared to juveniles is a typical life-history trait of long-lived species (Tavecchia et al., 2005; Tavecchia et al., 2016). On the long-lived fat dormouse and Edmondson penguin for example, Pilastro et al. (2003) and Ballerini et al. (2009) respectively, found that the mortality in the first year of life was significantly more elevated than in the next years. We found, indeed, that *P. carascalensis* is a long-lived species, since we calculated a minimum lifespan of 8–9 years. Lifespan of land snails ranges from several months to 19 years and, although over the 50% of the existing records are of short-lived species, it is thought that many terrestrial gastropods are long-lived (Heller, 2001). In the same way, we recorded a delayed maturity for the species, which needs 2.72 years on average to reach adulthood. Both long lifespan and delayed maturation are typical life-history traits of 'slow' life cycles. Slow life histories also involve slow reproductive rates and strong inversions in parental care, producing few and large offspring to enhance the change of recruitment. We still not have information about the reproductive rates of *P. carascalensis*, however, throughout the monitoring we were able to record an egg laying in which the specimen deposited 5 eggs of 2–2.5 mm in diameter. Egg size, which ultimately determines hatchling size, varies enormously among gastropod species and it is correlated with the animal size (Heller, 2001). Nevertheless, comparing the ratio between the egg size and animal size of *P. carascalensis* with the most closely related species for which egg diameter information is known (members of Hygromiidae and Geomitridae) (compiled in Heller, 2001), *P. carascalensis* lays within the group with the highest ratio. Thus, considering animal size, it seems that *P. carascalensis* produces one of the largest offspring within its closest relatives. Regarding the number of eggs, a considerable variation has been reported for land snails even within the same species and it has been related both with parent characteristics and environmental factors (e.g. Baur and Raboud, 1988; Wolda and Kreulen, 1973). Hence, with only one observation, no inferences can be done. A specific study, with an adequate sample size and controlling environmental variables, would be necessary to determine the dynamics of this trait.

Hibernation has been identified as a significant factor explaining variation in maximum lifespan, so that is has been suggested that it is a trigger to long-lived species (Pilastro et al. 2003; Wilkinson and South, 2002). More recently, Turbill et al. (2011)

performed a phylogenetically informed Generalized Least Squares modelling to test for an effect of hibernation on key attributes of life-history such as annual survival, maximum lifespan, annual reproductive output, age at maturity and generation time among mammals. They found that hibernation has a significant influence on all the life-history traits tested, but more importantly, as predicted by evolutionary theories, their results suggested that the increase in survival in hibernating species is associated to the co-evolution of a relative slow life-history. Thus, the hibernating nature of *P. carascalensis* and the traits indicative of a slow life-history that we have recovered for the species are possibly linked. According to life-history theory, slow life cycles are advantageous in variable environments since this strategy involves a limited reproductive effort that reduces the probability of investing too much in poor years, and an increase in longevity that allows attempting reproduction multiple years, so that the deleterious effects of environmental stochasticity are reduced and perturbations are buffered (Stearns, 1976). In mountainous areas, life histories are deeply influenced by elevation gradients, which led to a remarkably convergence of life-cycle traits among organisms facing the same environmental challenges (Laiolo and Obeso, 2017). Within this gradient, alpine regions are the most variable and inhospitable and, therefore, alpine populations generally adopt slow strategies, with exceptions rooted in evolutionary legacies or biogeographic history (Laiolo and Obeso, 2017). Hence, despite the studied *P. carascalensis* population lays within the montane zone, its life-history traits are those of alpine species. Indeed, the altitudinal distribution of *P. carascalensis* ranges from 460 m above sea level to 2640 m (from 1100 m to 2640 m if we excluded de unique population at 460 m), covering montane, subalpine and alpine zones. This finding further supports that *P. carascalensis* is a cold-adapted taxon with certain heat tolerance that allows the species to inhabit rock outcrops below the subalpine limit (Caro et al., 2019).

## 4.2. Recapture probability

Regarding recapture probabilities, modelling results for both adult and juvenile matrices pointed out a time dependence for the parameter. However, none of the external covariates used was able to fully explain its variation. Problems in model fitting and selection, as well as difficulties identifying the behaviour of parameters, can arise from the analysis of small and sparse datasets gathered during few sampling occasions (Tenan et al., 2013), but on the other hand, it may be also the result of an inappropriate selection of the external covariates to explain it. Thus, it is possible that the climatological conditions of the previous three days were unable to explain recapture probability because maybe the climate tendency of more days or complex interactions between variables are responsible of the individuals activity behaviour and, therefore, their detectability. Despite these problems, we were able to determine that only one sampling occasion influenced negatively recapture probability in adult

seasonal model. In view of this result, we determined that two sampling occasions per season (two in spring and two in autumn) are necessary and enough to correctly monitoring the population.

## 4.3. Population size

The analysis of the intrinsic properties (demographic parameters) of a population and their variation over time are more powerful ways to investigate population dynamics than changes in population size (Clobert et al., 1988). Even though, population size is a mayor determinant of extinction risk (Reed et al., 2003) and, therefore, we were also interested in estimating the number of individuals conforming the studied *P. carascalensis* population. Although robust estimates of population size are notoriously difficult to obtain, capture-recapture methods are accurate enough to estimate population size of closed populations of elusive species such as *P. carascalensis* which can hide in rock crevices (Tenan et al., 2013). According to our estimates, our population consists of 434 individuals in 2016 (95% CI: 405–474). This number is far below the minimum viable population size mean estimated on 4169 individuals (95% CI: 3577–5129) by Traill et al. (2007) in a meta-analysis of 30 years of publications on the subject. Nevertheless, these authors also concluded that the minimum viable population is context-specific and, among the 212 species included in their study, none was a terrestrial gastropod, nor were any species with similar characteristics enclosed. It is highly unlikely in fact that the studied area with its extension will be able to house such a high number of individuals as the one proposed by these authors and, indeed, considering the terrain load capacity, the population size estimate that we obtained for our population seems to indicate that it is not at immediate risk of extinction. On the other hand, considering that the number of individuals sighted in each visit ranges from 129 individuals to 266, it is clear that the capacity of the individuals to hide in crevices and bury is very high and reduces enormously their detectability. Besides, once again, this population size modelling also highlight that capture/recapture probabilities vary over time [$f_0$, $p_0(t) = c(t)$] despite we ignore its causes.

## 4.4. Growth and displacement

As expected, juveniles grow between spring and autumn, when they are active. A lack of growing during hibernation period is common on species that hibernate. In the land snail *Cornu aspersum*, for example, growth rate is also paused during hibernation and even a weight loss has been reported (Dupont-Nivet et al., 1997). Unfortunately, due to the variations in detectability, we were not able to obtain continuous measurements of a significant number of juveniles to infer their growth

rate and correlate it with environmental factors and, therefore, a correct analysis of this trait remains pending on a proper experimental study.

Finally, the few displacements recorded along the four years of monitoring corroborates the poor active dispersion of these land snails since even within the same rock the individuals tend to stay within an area of less than one square meter. This lack of active dispersal may be compensated by a great potential for passive dispersal by humans, animals and even wind and water (Aubry et al., 2006). This will mean that the dispersion of *P. carascalensis* and, probably the dispersion of the other species within *Pyrenaearia* genus, may depend heavily on passive dispersal as previously suggested (Caro et al., 2019; Elejalde et al., 2009).

## 5. Conclusions

Through a capture-recapture study, here we have gotten insight for the first time on the population demographics and basic biological traits of the land snail *P. carascalensis*. We reported different survivals for adult and young age classes, being juveniles less buffered against environmental stochasticity and especially affected by autumn variability, especially, by autumn freezing days. The species displays characteristics of a slow strategy including long lifespan, delayed maturity and the production of large offspring, which are the typical life-history traits of alpine species. We were not able to determine the environmental factors affecting recapture probability; however, we determined that two visits per season are necessary to correctly monitoring the population. Further, the estimated population size of 434 individuals is probably enough not to be in an immediate risk of extinction. This way, we have laid the foundation of a long-term monitoring on a species that will allow monitoring the changes that are taking place in mountainous areas and will help launching effective approaches to protect mountain diversity.

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# References


Aubry, S., Labaune, C., Magnin, F., Roche, P., Kiss, L., 2006. Active and passive dispersal of an invading land snail in Mediterranean France. J. Anim. Ecol. 75, 802–813. https://doi.org/10.1111/j.1365-2656.2006.01100.x

Ballerini, T., Tavecchia, G., Olmastroni, S., Pezzo, F., Focardi, S., 2009. Nonlinear effects of winter sea ice on the survival probabilities of Adélie penguins. Oecologia 161, 253–265. https://doi.org/10.1007/s00442-009-1387-9

Barnosky, A.D., Matzke, N., Tomiya, S., Wogan, G.O.U., Swartz, B., Quental, T.B., Marshall, C., McGuire, J.L., Lindsey, E.L., Maguire, K.C., Mersey, B., Ferrer, E.A., 2011. Has the Earth's sixth mass extinction already arrived? Nature 471, 51–57. https://doi.org/10.1038/nature09678

Baur, B., Raboud, C., 1988. Life history of the land snail *Arianta arbustorum* along an altitudinal gradient. J. Anim. Ecol. 57, 71–87.

Beaumont, L.J., Pitman, A., Perkins, S., Zimmermann, N.E., Yoccoz, N.G., Thuiller, W., 2011. Impacts of climate change on the world 's most exceptional ecoregions. Proc. Natl. Acad. Sci. U. S. A. 108, 2306–2311. https://doi.org/10.1073/pnas.1007217108

Becker, A., Bugmann, H., 2001. Global change and mountain regions. The mountain research initiative, IGBP report 49. IGBP Secretariat, Stockholm, Sweden.

Carboneras, C., Tavecchia, G., Genovart, M., Requena, S., Olivé, M., Oro, D., 2013. Inferring geographic origin, population structure and migration routes of a wintering population of Mediterranean gulls from resightings data. Popul. Ecol. 55, 343–351. https://doi.org/10.1007/s10144-013-0362-9

Cardinale, B.J., Duffy, J.E., Gonzalez, A., Hooper, D.U., Perrings, C., Venail, P., Narwani, A., MacE, G.M., Tilman, D., Wardle, D.A., Kinzig, A.P., Daily, G.C., Loreau, M., Grace, J.B., Larigauderie, A., Srivastava, D.S., Naeem, S., 2012. Biodiversity loss and its impact on humanity. Nature 486, 59–67. https://doi.org/10.1038/nature11148

Caro, A., Gómez-Moliner, B.J., Madeira, M.J., 2019. Integrating multilocus DNA data and 3D geometric morphometrics to elucidate species boundaries in the case of *Pyrenaearia* (Pulmonata: Hygromiidae). Mol. Phylogenet. Evol. 132, 194–206.

Catalan, J., Ninot, J.M., Aniz, M.M., 2017. The high mountain conservation in a changing world, in: Catalan, J., Ninot, J.M., Aniz, M.M. (Eds.), High mountain conservation in a changing world. Springer Nature, Cham, Switzerland, pp. 3–36.

Catchpole, E.A., Morgan, B.J.T., Coulson, T.N., Freeman, S.N., Albon, S.D., 2000. Factors influencing Soay sheep survival. J. R. Stat. Soc. Ser. C Appl. Stat. 49, 453–472. https://doi.org/10.1111/1467-9876.00205

Choquet, R., Lebreton, J., Gimenez, O., Reboulet, A., Pradel, R., 2009. U-CARE: utilities for performing goodness of fit tests and manipulating CApture REcapture data. Ecography 32, 1071–1074. https://doi.org/10.1111/j.1600-0587.2009.05968.x

Clobert, J., Perrins, C.M., McCleery, R.H., Gosler, A.G., 1988. Survival rate in the great tit *Parus major* in relation to sex, age, and immigration status. J. Anim. Ecol. 57, 287–306. https://doi.org/10.2307/4779

Dawson, T.P., Jackson, S.T., House, J.I., Prentice, I.C., Mace, G.M., 2011. Beyond predictions: biodiversity conservation in a changing climate. Science 332, 53–58. https://doi.org/10.1126/science.1200303

Duangchantrasiri, S., Umponjan, M., Simcharoen, S., Pattanavibool, A., Chaiwattana, S., Maneerat, S., Kumar, N.S., Jathanna, D., Srivathsa, A., Karanth, K.U., 2016. Dynamics of a low-density tiger population in Southeast Asia in the context of improved law enforcement. Conserv. Biol. 30, 639–648. https://doi.org/10.1111/cobi.12655

Dupont-Nivet, M., Mallard, J., Bonnet, J.C., Blanc, J.M., 1997. Quantitative genetics of growth traits in the edible snail, *Helix aspersa* Müller. Genet. Sel. Evol. 29, 571–587.

Dybala, K.E., Eadie, J.M., Gardali, T., Seavy, N.E., Herzog, M.P., 2013. Projecting demographic responses to climate change: adult and juvenile survival respond differently to direct and indirect effects of weather in a passerine population. Glob. Chang. Biol. 19, 2688–2697. https://doi.org/10.1111/gcb.12228

Elejalde, M.A., Madeira, M.J., Prieto, C.E., Backeljau, T., Gómez-Moliner, B.J., 2009. Molecular phylogeny, taxonomy, and evolution of the land snail genus *Pyrenaearia* (Gastropoda, Helicoidea). Am. Malacol. Bull. 27, 69–81. https://doi.org/10.4003/006.027.0206

Esteban, R., Verborgh, P., Gauffier, P., Giménez, J., Guinet, C., de Stephanis, R., 2016. Dynamics of killer whale, bluefin tuna and human fisheries in the Strait of Gibraltar. Biol. Conserv. 194, 31–38. https://doi.org/10.1016/j.biocon.2015.11.031

Fernández-Chacón, A., Bertolero, A., Amengual, A., Tavecchia, G., Homar, V., Oro, D., 2011. Spatial heterogeneity in the effects of climate change on the population dynamics of a Mediterranean tortoise. Glob. Chang. Biol. 17, 3075–3088. https://doi.org/10.1111/j.1365-2486.2011.02469.x

Fernández-Chacón, A., Genovart, M., Pradel, R., Tavecchia, G., Bertolero, A., Piccardo, J., Forero, M.G., Afán, I., Muntaner, J., Oro, D., 2013. When to stay, when to disperse and where to go: survival and dispersal patterns in a spatially structured seabird population. Ecography 36, 1117–1126. https://doi.org/10.1111/j.1600-0587.2013.00246.x

Forslund, P., Pärt, T., 1995. Age and reproduction in birds - hypotheses and tests. Trends Ecol. Evol. 10, 374–378. https://doi.org/10.1016/S0169-5347(00)89141-7

Gilman, S.E., Urban, M.C., Tewksbury, J., Gilchrist, G.W., Holt, R.D., 2010. A framework for community interactions under climate change. Trends Ecol. Evol. 25, 325–331. https://doi.org/10.1016/j.tree.2010.03.002

Guimarães, M., Munguía-Steyer, R., Doherty, P.F., Martins, M., Sawaya, R.J., 2014. Population dynamics of the critically endangered golden lancehead pitviper, *Bothrops insularis*: stability or decline? PLoS One 9, e95203. https://doi.org/10.1371/journal.pone.0095203

Heller, J., 2001. Life history strategies, in: Barker, G.M. (Ed.), The biology of terrestrial molluscs. CABI Publishing, Wallingford, UK, pp. 413–445.

Hillebrand, H., Brey, T., Gutt, J., Hagen, W., Metfies, K., Meyer, B., Lewandowska, A., 2018. Climate change: warming impacts on marine biodiversity, in: Salomon, M., Markus, T. (Eds.), Handbook on marine environment protection. Springer, Cham, Switzerland, pp. 353–373.

Hooper, D.U., Adair, E.C., Cardinale, B.J., Byrnes, J.E.K., Hungate, B.A., Matulich, K.L., Gonzalez, A., Duffy, J.E., Gamfeldt, L., O'Connor, M.I., 2012. A global synthesis reveals biodiversity loss as a major driver of ecosystem change. Nature 486, 105–108. https://doi.org/10.1038/nature11118

Jordan, S., Giersch, J.J., Muhlfeld, C.C., Hotaling, S., Fanning, L., Tappenbeck, T.H., Luikart, G., 2016. Loss of genetic diversity and increased subdivision in an endemic alpine stonefly threatened by climate change. PLoS One 11, e0157386. https://doi.org/10.1371/journal.pone.0159931

Laiolo, P., Obeso, J.R., 2017. Life-history responses to the altitudinal gradient, in: Catalan, J., Ninot, J.M., Aniz, M.M. (Eds.), High mountain conservation in a changing world. Springer Nature, Cham, Switzerland, pp. 253–283.

Lebreton, J.D., Burnham, K.P., Clobert, J., Anderson, D.R., 1992. Modeling survival and testing biological hypotheses using marked animals: a unified approach with case studies. Ecol. Monogr. 62, 67–118.

Lenihan, C., Van Vuren, D., 1996. Growth and survival of juvenile yellow-bellied marmots (*Marmota flaviventris*). Can. J. Zool. 74, 297–302. https://doi.org/10.1139/z96-037

Lin, X., Rioual, P., Peng, W., Yang, H., Huang, X., 2018. Impact of recent climate change on Lake Kanas, Altai Mountains (N.W. China) inferred from diatom and geochemical evidence. J. Paleolimnol. 59, 461–477. https://doi.org/10.1007/s10933-018-0019-y

Lloret, F., 2017. Trade-offs in high mountain conservation, in: Catalan, J., Ninot, J.M., Aniz, M.M. (Eds.), High mountain conservation in a changing world. Springer Nature, Cham, Switzerland, pp. 37–59.

Margalida, A., 2017. Importance of long-term studies to conservation practice: the case of the bearded vulture in the Pyrenees, in: Catalan, J., Ninot, J.M., Aniz, M.M. (Eds.), High mountain conservation in a changing world. Springer Nature, Cham, Switzerland, pp. 343–383.

McGill, B.J., Enquist, B.J., Weiher, E., Westoby, M., 2006. Rebuilding community ecology from functional traits. Trends Ecol. Evol. 21, 178–185. https://doi.org/10.1016/j.tree.2006.02.002

MEA (Millennium Ecosystem Assessment), 2005. Ecosystems and human well-being: biodiversity synthesis of the Millennium Ecosystem Assessment. World Resources Institute, Washington, DC.

Menéndez, R., González-Megías, A., Jay-Robert, P., Marquéz-Ferrando, R., 2014. Climate change and elevational range shifts: evidence from dung beetles in two European mountain ranges. Glob. Ecol. Biogeogr. 23, 646–657. https://doi.org/10.1111/geb.12142

Nakashizuka, T., Shimazaki, M., Sasaki, T., Tanaka, T., Kurokawa, H., Hikosaka, K., 2016. Influences of climate change on the distribution and population dynamics of subalpine coniferous forest in the Hakkoda Mountains, northern Japan, in: Kudo, G. (Ed.), Structure and function of mountain ecosystems in Japan. Springer Nature, Tokyo, Japan, pp. 1–15. https://doi.org/10.1007/978-4-431-55954-2

Ortiz de Zárate, A., 1956. Observaciones anatómicas y posición sistemática de varios Helícidos españoles. IV. Género *Pyrenaearia* Hesse, 1921. Bol. R. Soc. Esp. Hist. Nat. 54, 35–61.

Otis, D.L., Burnham, K.P., White, G.C., Anderson, D.R., 1978. Statistical inference from capture data on closed animal populations. Wildl. Monogr. 62, 3–135.

Parmesan, C., Yohe, G., 2003. A globally coherent fingerprint of climate change impacts across natural systems. Nature 421, 37–42. https://doi.org/http://www.nature.com/nature/journal/v421/n6918/suppinfo/nature01286_S1.html

Pärt, T., 2001. Experimental evidence of environmental effects on age-specific reproductive success: the importance of resource quality. Proc. R. Soc. B Biol. Sci. 268, 2267–2271. https://doi.org/10.1098/rspb.2001.1803

Pereira, H.M., Leadley, P.W., Proenca, V., Alkemade, R., Scharlemann, J.P.W., Fernandez-Manjarres, J.F., Araujo, M.B., Balvanera, P., Biggs, R., Cheung, W.W.L., Chini, L., Cooper, H.D., Gilman, E.L., Guenette, S., Hurtt, G.C., Huntington, H.P., Mace, G.M., Oberdorff, T., Revenga, C., Rodrigues, P., Scholes, R.J., Sumaila, U.R., Walpole, M., 2010. Scenarios for global biodiversity in the 21st century. Science 330, 1496–1502.

Pérez-Mellado, V., García-Díez, T., Hernández-Estévez, J.A., Tavecchia, G., 2015. Behavioural processes, ephemeral resources and spring population dynamics of an insular lizard, *Podarcis lilfordi* (Squamata: Lacertidae). Ital. J. Zool. 82, 556–564. https://doi.org/10.1080/11250003.2015.1093035

Petchey, O.L., Gaston, K.J., 2006. Functional diversity: back to basics and looking forward. Ecol. Lett. 9, 741–758. https://doi.org/10.1111/j.1461-0248.2006.00924.x

Pilastro, A., Tavecchia, G., Marin, G., 2003. Long living and reproduction skipping in the fat dormouse. Ecology 84, 1784–1792. https://doi.org/10.1890/0012-9658(2003)084[1784:LLARSI]2.0.CO;2

Polo, I., 2015. Dinámica poblacional, taxonomía y conservación de algunas especies del género *Iberus*. Madrid, PhD thesis, Complutense University of Madrid, Spain.

Prieto, C.E., 1986. Estudio sistemático y biogeográfico de los Helicidae *sensu* Zilch, 1959-60 (Gastropoda: Pulmonata: Stylommatophora) del País Vasco y regiones adyacentes. Leioa, PhD thesis, University of the Basque Country (EHU/UPV), Spain.

Puente, A.I., 1994. Estudio taxonómico y biogeográfico de la superfamilia Helicoidea Rafinesque, 1815 (Gastropoda: Pulmonata: Stylommatophora) de la Península Ibérica e Islas Baleares. Leioa, PhD thesis, University of the Basque Country (EHU/UPV), Spain.

Reed, D.H., O'Grady, J.J., Brook, B.W., Ballou, J.D., Frankham, R., 2003. Estimates of minimum viable population sizes for vertebrates and factors influencing those estimates. Biol. Conserv. 113, 23–34. https://doi.org/10.1016/S0006-3207(02)00346-4

Selwood, K.E., Mcgeoch, M.A., Mac Nally, R., 2015. The effects of climate change and land-use change on demographic rates and population viability. Biol. Rev. 90, 837–853. https://doi.org/10.1111/brv.12136

Sergio, F., Tavecchia, G., Blas, J., López, L., Tanferna, A., Hiraldo, F., 2011. Variation in age-structured vital rates of a long-lived raptor: implications for population growth. Basic Appl. Ecol. 12, 107–115. https://doi.org/10.1016/j.baae.2010.11.004

Stearns, S.C., 1976. Life-history tactics: a review of the ideas. Q. Rev. Biol. 51, 3–47.

Tavecchia, G., Baccetti, N., Serra, L., 2005. Colony specific variation in the use of a moulting site in the migratory little tern *Sterna albifrons*. J. Avian Biol. 36, 501–509. https://doi.org/10.1111/j.0908-8857.2005.03440.x

Tavecchia, G., Tenan, S., Pradel, R., Igual, J.M., Genovart, M., Oro, D., 2016. Climate-driven vital rates do not always mean climate-driven population. Glob. Chang. Biol. 22, 3960–3966. https://doi.org/10.1111/gcb.13330

Tenan, S., Rotger Vallespir, A., Igual, J.M., Moya, Ó., Royle, J.A., Tavecchia, G., 2013. Population abundance, size structure and sex-ratio in an insular lizard. Ecol. Modell. 267, 39–47. https://doi.org/10.1016/j.ecolmodel.2013.07.015

Thomas, C.D., Cameron, A., Green, R.E., Bakkenes, M., Beaumont, L.J., Collingham, Y.C., Erasmus, B.F.N., Ferreira De Siqueira, M., Grainger, A., Hannah, L., Hughes, L., Huntley, B., van Jaarsveld, A.S., Midgley, G.F., Miles, L., Ortega-Huerta, M.A., Townsend Peterson, A., Phillips, O.L., Williams, S.E., 2004. Extinction risk from climate change. Nature 427, 145–148. https://doi.org/10.1038/nature02121

Traill, L.W., Bradshaw, C.J.A., Brook, B.W., 2007. Minimum viable population size: a meta-analysis of 30 years of published estimates. Biol. Conserv. 139, 159–166. https://doi.org/10.1016/j.biocon.2007.06.011

Turbill, C., Bieber, C., Ruf, T., 2011. Hibernation is associated with increased survival and the evolution of slow life histories among mammals. Proc. R. Soc. B Biol. Sci. 278, 3355–3363. https://doi.org/10.1098/rspb.2011.0190

Tyler, E.H.M., Somerfield, P.J., Berghe, E. Vanden, Bremner, J., Jackson, E., Langmead, O., Palomares, M.L.D., Webb, T.J., 2012. Extensive gaps and biases in our knowledge of a well-known fauna: implications for integrating biological traits into macroecology. Glob. Ecol. Biogeogr. 21, 922–934. https://doi.org/10.1111/j.1466-8238.2011.00726.x

Verberk, W.C.E.P., van der Velde, G., Esselink, H., 2010. Explaining abundance-occupancy relationships in specialists and generalists: a case study on aquatic macroinvertebrates in standing waters. J. Anim. Ecol. 79, 589–601. https://doi.org/10.1111/j.1365-2656.2010.01660.x

White, G.C., Burnham, K.P., 1999. Program mark: survival estimation from populations of marked animals. Bird Study 46, S120–S139. https://doi.org/10.1080/00063659909477239

White, G.C., Burnham, K.P., Anderson, D.R., 2001. Advanced features of program Mark. Int. Wildl. Manag. Congr. 368–377.

Wilkinson, G.S., South, J.M., 2002. Life history, ecology and longevity in bats. Aging Cell 1, 124–131. https://doi.org/10.1046/j.1474-9718.2002.00020sm.x

Wolda, H., Kreulen, D.A., 1973. Ecology of some experimental populations of the landsnail *Cepaea nemoralis* (L). 2. Production and survival of eggs and juveniles. Netherlands J. Zool. 23, 168–188.

Yen, A.L., Butcher R., 1997. An overview of the conservation of non-marine invertebrates in Australia. Endangered Species Program, Environment Australia.

Zamora, R., Pérez-Luque, A.J., Bonet, F.J., 2017. Monitoring global change in high mountains, in: Catalan, J., Ninot, J.M., Aniz, M.M. (Eds.), High mountain conservation in a changing world. Springer Nature, Cham, Switzerland, pp. 385–413.

# Supplementary material

**Supplementary material 1.** Visits' schedule and amount of captures for each visit.

| **2013** | | **2014** | | **2015** | | **2016** | |
|---|---|---|---|---|---|---|---|
| Spring | | Spring | | Spring | | Spring | |
| *Date* | *Captures* | *Date* | *Captures* | *Date* | *Captures* | *Date* | *Captures* |
| 12-June | 231 | 16-May | 230 | 11-May | 240 | 23-May | 184 |
| 17-July | 129 | 18-June | 216 | 23-June | 259 | 28-June | 195 |
| | | | | | | 21-July | 157 |
| Autumn | | Autumn | | Autumn | | Autumn | |
| *Date* | *Captures* | *Date* | *Captures* | *Date* | *Captures* | *Date* | *Captures* |
| 02-August | 183 | 12-August | 249 | 10-September | 161 | 12-September | 199 |
| 01-September | 215 | 13-September | 266 | | | 27-October | 213 |

**Supplementary material 2.** Snail marking method.

On adult snails marks were placed on the umbilical side near the peristome, whereas on juveniles they were situated on the superior side just behind the last growing line to prevent loosing marks when the snail grew. Shells were first dried with absorbent paper where the mark would be placed to get it properly fixed. Then, we gave a brushstroke of correction fluid (brush Tipp-Ex, BIC) (Figure S1) and wait until it dried to write the corresponding number with a black ink pen (G-TEC-C4, Pilot) (Figure S2). Finally, when the ink was dry, we secured the mark with a coat of glue (brush Loctite SUPER GLUE-3, Henkel). After ensuring it was completely dried we left the individuals in the same sector where they were captured. Figure S3 shows employed material.

**Figure S1.** Correction fluid brushstroke placement on adults (left) and on juveniles (right).


**Figure S2.** Marked individuals with their corresponding numbers: adult in the left and juvenile in the right.

**Figure S3.** Material employed during marking.

**Supplementary material 3.** Goodness-of-fit (GOF) values for seasonal and annual matrices of the total population, the adult age class and the young age class. Statistically significant p-levels are highlighted in light grey.

| | | SEASONAL | | | | | | | ANNUAL | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Transient | | Trap dependence | | Global Test | | | Transient | | Trap dependence | | Global Test | |
| | | 3.SR | 3.SM | 2.CT | 2.CL | | | | 3.SR | 3.SM | 2.CT | 2.CL | | |
| **Total population** | *Statistic* | 2.798 | | -2.704 | | $\chi^2$ | **47.405** | *Statistic* | 0.274 | | -1.688 | | $\chi^2$ | **6.716** |
| | *p two sided* | 0.002 | | 0.007 | | **df** | **25** | *p two sided* | 0.784 | | 0.091 | k < 5 | **df** | **4** |
| | *p one sided* | 0.001 | 0.248 | | 0.026 | **ĉ-hat** | **1.896** | *p one sided* | 0.392 | 0.801 | | | **ĉ-hat** | **1.679** |
| **Adult age class** | *Statistic* | -0.024 | | -0.182 | | $\chi^2$ | **25.046** | *Statistic* | -1.227 | | 0.909 | | $\chi^2$ | **8.561** |
| | *p two sided* | 0.981 | | 0.855 | | **df** | **23** | *p two sided* | 0.220 | | 0.363 | k < 5 | **df** | **4** |
| | *p one sided* | 0.510 | 0.451 | | 0.505 | **ĉ-hat** | **1.089** | *p one sided* | 0.891 | 0.801 | | | **ĉ-hat** | **2.140** |
| **Young age class** | *Statistic* | -0.569 | | -0.754 | | $\chi^2$ | **20.932** | *Statistic* | -0.779 | | -0.344 | | $\chi^2$ | **1.992** |
| | *p two sided* | 0.569 | | 0.451 | | **df** | **22** | *p two sided* | 0.436 | | 0.731 | k < 5 | **df** | **4** |
| | *p one sided* | 0.715 | 0.605 | | 0.755 | **ĉ-hat** | **0.952** | *p one sided* | 0.782 | 0.801 | | | **ĉ-hat** | **0.498** |

**Supplementary material 4.** Parameter estimates for adult age class: (a) seasonal values and (b) annual values.

| | | | 95% Confidence Interval | | |
|---|---|---|---|---|---|
| **Parameter** | **Estimate** | **Standard Error** | **Lower** | **Upper** | **Model Variation** |
| *a) Seasonal values* | | | | | |
| $\phi$ | 0.83 | 0.011 | 0.81 | 0.85 | |
| *p* 2013 autumn | 0.72 | 0.035 | 0.65 | 0.78 | |
| *p* 2014 spring | 0.71 | 0.032 | 0.65 | 0.77 | |
| *p* 2014 autumn | 0.73 | 0.030 | 0.66 | 0.78 | |
| *p* 2015 spring | 0.75 | 0.031 | 0.69 | 0.81 | |
| *p* 2015 autumn | 0.37 | 0.036 | 0.30 | 0.44 | |
| *p* 2016 spring | 0.79 | 0.043 | 0.69 | 0.86 | |
| *p* 2016 autumn | 0.61 | 0.053 | 0.50 | 0.71 | |
| *b) Annual values* | | | | | |
| $\phi$ | 0.72 | 0.027 | 0.66 | 0.77 | 5.82% |
| *p* 2014 | 0.91 | 0.033 | 0.81 | 0.96 | 20.35% |
| *p* 2015 | 0.80 | 0.045 | 0.69 | 0.88 | 14.19% |
| *p* 2016 | 0.84 | 0.040 | 0.70 | 0.92 | 44.38% |

**Supplementary material 5.** Ranking of seasonal models performed for young age class including external covariates for recapture probabilities and showing the model structure for survival ($\phi$), recapture probability ($p$), number of parameters (np), deviance (QDEV), AIC values, QAICc-Weight ($\omega_i$) and model likelihood (Model L).

| Model | $\phi$ | $p$ | np | QDEV | QAICc | ΔQAICc | $\omega_i$ | Model L |
|---|---|---|---|---|---|---|---|---|
| 1 | (.) | (t) | 8 | 172.124 | 1570.822 | 0.000 | 0.436 | 1.000 |
| 2 | (2t) | (t) | 10 | 169.067 | 1571.864 | 1.041 | 0.259 | 0.594 |
| 3 | (t) | (t) | 14 | 161.375 | 1572.430 | 1.608 | 0.195 | 0.448 |
| 4 | (.) | (.esf12b) | 3 | 185.052 | 1573.598 | 2.775 | 0.109 | 0.250 |
| 5 | (.) | (.esf123) | 3 | 205.245 | 1593.790 | 22.968 | 0.000 | 0.000 |
| 6 | (.) | (.tmin) | 3 | 211.725 | 1600.270 | 29.448 | 0.000 | 0.000 |
| 7 | (.) | (.pre) | 3 | 213.135 | 1601.680 | 30.858 | 0.000 | 0.000 |
| 8 | (.) | (.hum) | 3 | 219.235 | 1607.780 | 36.958 | 0.000 | 0.000 |

**Supplementary material 6.** Parameter estimates for young age class: a) seasonal values and b) annual values.

| | | | 95% Confidence Interval | | |
|---|---|---|---|---|---|
| **Parameter** | **Estimate** | **Standard Error** | **Lower** | **Upper** | **Model Variation** |
| *a) Seasonal values* | | | | | |
| $\phi$ 2013 summer | 0.73 | 0.032 | 0.62 | 0.81 | 54.45% |
| $\phi$ 13-14 winter | 0.62 | 0.042 | 0.46 | 0.75 | 70.50% |
| $\phi$ 2014 summer | 0.74 | 0.029 | 0.66 | 0.80 | 37.55% |
| $\phi$ 14-15 winter | 0.69 | 0.028 | 0.59 | 0.78 | 66.60% |
| $\phi$ 2015 summer | 0.74 | 0.031 | 0.65 | 0.81 | 37.49% |
| $\phi$ 15-16 winter | 0.67 | 0.030 | 0.58 | 0.74 | 46.28% |
| $\phi$ 2016 summer | 0.73 | 0.028 | 0.66 | 0.80 | 38.05% |
| *p* 2013 autumn | 0.70 | 0.081 | 0.51 | 0.84 | 8.04% |
| *p* 2014 spring | 0.56 | 0.072 | 0.41 | 0.70 | 11.57% |
| *p* 2014 autumn | 0.74 | 0.052 | 0.62 | 0.83 | 4.98% |
| *p* 2015 spring | 0.76 | 0.048 | 0.65 | 0.85 | 8.59% |
| *p* 2015 autumn | 0.38 | 0.044 | 0.29 | 0.47 | 2.84% |
| *p* 2016 spring | 0.82 | 0.049 | 0.70 | 0.90 | 2.31% |
| *p* 2016 autumn | 0.69 | 0.059 | 0.56 | 0.80 | 11.05% |
| *b) Annual values* | | | | | |
| $\phi$ 2013-2014 | 0.44 | 0.043 | 0.34 | 0.55 | 35.97% |
| $\phi$ 2014-2015 | 0.58 | 0.036 | 0.49 | 0.67 | 38.14% |
| $\phi$ 2015-2016 | 0.57 | 0.033 | 0.46 | 0.68 | 67.16% |
| *p* | 0.86 | 0.038 | 0.76 | 0.93 | 22.00% |

**Supplementary material 7.** Individuals captured as juveniles that reached adulthood during this study sorted into three size groups: a) 7–9 mm, b) 9–11 mm, and c) > 11 mm. For each individual, diameter size when captured the first time as well as when reached adulthood and the days needed for the transition are provided. For each group, mean and standard deviation (s) of the time needed to reach adulthood is also provided.

| **a) 7–9 mm** | | | | **b) 9–11 mm** | | | | **c) > 11 mm** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Specimen code | Juvenile Ø (mm) | Passed days | Adult Ø (mm) | Specimen code | Juvenile Ø (mm) | Passed days | Adult Ø (mm) | Specimen code | Juvenile Ø (mm) | Passed days | Adult Ø (mm) |
| *76* | 8.30 | 1434 | 15.75 | *15* | 9.90 | *77* | 13.00 | *155* | 11.10 | 1077 | 16.68 |
| *98* | 8.50 | 821 | 15.61 | *293* | 10.00 | 779 | 15.38 | *166* | 12.00 | 339 | 16.50 |
| *119* | 8.00 | 1077 | 15.01 | *319* | 9.90 | 438 | 15.50 | *177* | 11.10 | 427 | 14.56 |
| *120* | 8.70 | 1136 | 14.82 | *332* | 9.10 | 691 | 14.87 | *178* | 11.20 | 339 | 14.00 |
| *159* | 7.90 | 742 | 16.41 | *384* | 10.50 | 966 | 14.88 | *189* | 12.70 | 1077 | 15.99 |
| *188* | 9.00 | 489 | 15.50 | *390* | 10.00 | 588 | 14.33 | *207* | 12.50 | 112 | 14.00 |
| *275* | 7.50 | 1399 | 16.78 | *452* | 9.50 | 1133 | 14.54 | *230* | 11.30 | 427 | 15.18 |
| *330* | 9.00 | 1422 | 15.86 | *474* | 10.70 | 896 | 16.08 | *402* | 13.00 | 1002 | 15.06 |
| *345* | 8.00 | 1138 | 15.46 | *621* | 9.34 | 818 | 15.86 | *471* | 12.10 | 89 | 14.28 |
| *443* | 7.60 | 775 | 15.41 | *626* | 10.72 | 763 | 15.02 | *493* | 13.30 | 483 | 16.40 |
| *487* | 8.60 | 851 | 16.90 | *627* | 10.10 | 763 | 15.71 | *508* | 13.70 | 896 | 15.60 |
| *560* | 8.03 | 818 | 15.60 | *633* | 9.32 | 1045 | 15.75 | *582* | 12.70 | 706 | 15.42 |
| *568* | 8.80 | 863 | 16.13 | *658* | 9.22 | 763 | 14.14 | *594* | 12.56 | 651 | 16.10 |
| *592* | 8.19 | 1063 | 15.11 | *661* | 9.33 | 763 | 15.75 | *644* | 11.79 | 1008 | 15.37 |
| *602* | 8.77 | 1045 | 14.47 | *682* | 10.50 | 946 | 15.26 | *645* | 11.45 | 763 | 15.46 |
| *616* | 8.63 | 1008 | 14.71 | *689* | 10.00 | 983 | 14.80 | *690* | 12.00 | 746 | 16.36 |
| *638* | 8.10 | 763 | 15.48 | *697* | 10.00 | 701 | 15.79 | *700* | 13.00 | 589 | 16.38 |
| | | | | *701* | 10.50 | 589 | 15.85 | *717* | 13.08 | 736 | 15.88 |
| | | | | *706* | 11.00 | 589 | 15.15 | *730* | 13.46 | 379 | 14.46 |
| | | | | *735* | 10.96 | 415 | 14.32 | *742* | 11.23 | 491 | 15.18 |
| | | | | *759* | 9.45 | 736 | 14.86 | *758* | 11.41 | 736 | 16.12 |
| | | | | *766* | 10.30 | 491 | 14.99 | *776* | 11.03 | 730 | 14.69 |
| | | | | *801* | 9.82 | 448 | 14.74 | *784* | 13.13 | 730 | 16.54 |
| | | | | *821* | 10.33 | 651 | 15.67 | *802* | 12.93 | 448 | 14.50 |
| *Passed time* | | *Mean* | *s* | *Passed time* | | *Mean* | *s* | *Passed time* | | *Mean* | *s* |
| *Days* | | 991 | 264 | *Days* | | 710 | 236 | *Days* | | 624 | 278 |
| *Years* | | **2.72** | 0.72 | *Years* | | **1.95** | 0.65 | *Years* | | **1.71** | 0.76 |

# IV. ARTIKULUA

## *Pyrenaearia carascalensis* (Gastropoda: Hygromiidae) espeziearen populazio jarraipena Gorbeiako Parke Naturalean

- **Laburpena**
- **Eztabaida**
- **Ondorioak**

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*In preparation*

## *Pyrenaearia carascalensis* (Gastropoda: Hygromiidae) espeziearen populazio jarraipena Gorbeiako Parke Naturalean

# Laburpena

Mendialdeak nahiko ondo kontserbatu dira, baita dentsitate handiko inguruetan ere, baina kutsatzaileen urrutirako garraioak eta batez ere aldaketa klimatikoak inguru hauek kaltetzen hasi dira, mendietako dibertsitatearen kontserbazioan desafio berriak sortuz. Egoera honetan, inguru hauetan biodibertsitate galera eragiten ari diren prozesuen ezagutza ezinbestekoa da kudeaketa eta kontserbazio neurri egokiak martxan jartzeko, mendietara mugatutako taxon gakoen epe luzerako jarraipenaren bidez bakarrik lortu daitekeena. Ikerketa honetan, harrapaketa-berrarrapaketa tekniken bidez, mendi inguruetara mugatutako *Pyrenaearia carascalensis* lur barraskilo espeziearen jarraipena hasi genuen Gorbeiako Parke Naturalean espeziearen populazio demografia eta oinarrizko ezaugarri biologikoak aztertzeko. Hemen lau urtetan zehar bildutako datuekin lortutako emaitzak aurkezten ditugu. Lan honetan, heldu eta gazte klaseen biziraupen eta berrarrapaketa probabilitatearen bariazioa denboran zehar aztertu zen eta, bestalde, aldagai klimatikoen eta laginketa esfortzuaren eragina bai biziraupenean eta bai berrarrapaketan ikertu ziren. Heldu eta gazteen biziraupena ezberdina izan zen; helduen biziraupena altua eta konstantea izan zen eta gazteena, berriz, udazkeneko izozte egun kopuruak gora egitean murrizten zen. Espeziea luze bizi dela eta heltzeko denbora tarte luzea behar duela aurkitu genuen eta baita segur aski kumeak handiak direla ere. Espezie alpinoetan arruntak diren bizitza-historia motelen ezaugarri ohikoak dira horiek. Berrarrapaketa probabilitatea denboran aldatzen zen eta, udaberrian zein udazkenean gutxienez birritan lagintzearen garrantzia frogatu bagenuen ere, ez ginen haren bariazioa azaltzeko gai izan. Ikerketa eremuan 434 alez osatutako populazio tamaina kalkulatu genuen eta norbanakoen lekualdatze baxua frogatu genuen. Hala, mendi inguruetan gertatzen ari diren aldaketen berri eman dezakeen espezie baten epe luzerako jarraipena jarri genuen martxan, mendietako dibertsitatea babesteko neurri eraginkorren diseinuan lagunduko duena.

# Gako-hitzak

populazio jarraipena; *Pyrenaearia carascalensis;* harrapaketa-berrarrapaketa; populazio demografia; espezie zelataria; Gorbeia

# 4. Eztabaida

## 4.1. Bizitza-historiaren ezaugarriak

Ikerketa honetan *P. carascalensis* lur barraskilo espeziearen populazio dinamika eta oinarrizko ezaugarri biologikoak aztertu dira lehenengo aldiz harrapaketa-berrarrapaketa teknikaren bidez. Hemen burututako modelizazioari esker, heldu eta gazte klaseak bereizita analizatuz, beraien biziraupen probabilitatea era egokian kalkulatu ahal izan genituen; izan ere, bi talde hauek jokabide ezberdina zutela ikusi zen, bi adin klaseak elkartzen zituen matrizean GOF testek antzeman zuten ale iragankorren efektuak ohartarazi zuenez. Jokabide ezberdintasun hau dela eta, adin klase bakoitzarentzat biziraupen probabilitate ezberdina kalkulatu zen. Barraskilo helduen biziraupena altua eta konstantea izan zen (urtaroko $\phi$ = 0,83; urteko $\phi$ = 0,72), gazteena ordea askoz baxuagoa izan zen eta udazkeneko izozte egun kopuruen eraginpean: zenbat eta izozte egun gehiago orduan eta biziraupen baxuagoa (urtaroko $\phi$ = 0,61–0,73; urteko $\phi$ = 0,44–0,58). Adin klaseen arteko biziraupen ezberdintasuna hautespen naturalak kalitate baxuko fenotipoak kentzearen ondorio izan daiteke, kalitate handiko norbanakoen proportzioa handituz adin klaseetan zehar eta, ondorioz, positiboki aldatuz adinarekin (hautespen hipotesia; Forslund eta Pärt, 1995). Hala ere, adinaren araberako biziraupena, adinari lotutako lehiakortasun hobekuntzaren ondorio ere izan daiteke (hertsatze hipotesia; Pärt, 2001), norbanako helduek eta esperientzia handiagokoek gazteek baino tasa altuagoetan biziraun dezaketelako ingurumen baldintza berdinetan (Sergio et al., 2011). Ezin izan genuen zehaztu hauetako zein hipotesik eragiten dion *P. carascalensis*-i, dena den, honek esan nahi du bai populazioaren aldaketak aztertzean eta bai kudeaketa neurriak proposatzean, bizi indizeetan *age-by-time* interakzio efektu bat kontuan hartu behar dela (Sergio et al., 2011).

Askotan frogatu izan da negua urtaro erabakigarria dela latitude handi eta epeletan eta neguko heriotza-tasa inguru hauetako populazio dinamiken mugatzaile nagusietakoa dela (Carboneras et al., 2013). Bestalde, urteko denboraldi beroena *P. carascalensis*-entzat erabakigarria izan zitekeela hipotetizatu genuen eremu menditsuetara eta inguru laiotzetara lotuta dagoelako. Hala ere, ale helduetan neguko eta udarako biziraupenen artean ez genuen desberdintasunik aurkitu. Are gehiago, helduen biziraupena urteetan zehar konstantea izan zen, populazio heldu egonkorra iradokitzen duena. Hala ere, populazioa lau urtez bakarrik jarraituta, joerak deskribatzerakoan tentuz ibili behar da (Tenan et al., 2013). Gazteen kasuan, ordea, neguko biziraupen probabilitatea udakoa baino baxuagoa izan zen eta negatiboki aldatzen zen udazkeneko izozte egun kopurua handitzen zen heinean.

Udazkeneko izozte egunek gazteengan duten ondorio kaltegarria muturreko gertaera honetan babesteko esperientzia ezarekin erlazionatuta egon daiteke, izoztu eta hiltzen direlako. Udazkenean izozte egun asko egotea baliabide eskasia eragiten duten udazken gogorrekin ere egon daiteke erlazionatuta; honek negurako prestakuntza oztopatu dezake eta horrela ez dute energia nahikoa lortzen udaberrira arte bizirauteko (Pilastro et al., 2003). Jarduten duen mekanismoa jarduten duela, gure emaitzen arabera badirudi udazkeneko aldakortasunak neguko aldakortasunak eta gogortasunak baino eragin handiagoa daukala *P. carascalensis* gazteen neguko biziraupenean. Hibernatzen duten sabel-horiko marmotetan (*Marmota flaviventris*), Lenihan eta Van Vuren-ek (1996) ere ez zuten neguaren gogortasuna identifikatu neguko hilkortasunaren erantzule nagusi gisa, baizik eta hibernatzen hasi aurreko masa. Hala ere, udazkeneko izozte egun kopuruaren eta gazteen biziraupenaren artean korrelazio bat aurkitu arren, urteko modelizazioan, era zehaztugabean denborarekiko menpekoa zen modeloa, $\phi(t)p(.)$, estatistikoki baliokidea izan zen. Beraz, beste faktore batzuek ere eragina izan behar dute gazteen biziraupenean, baina urte gehiagotan zehar bildutako datuak beharko dira biziraupen dinamikak guztiz eta era egokian doitzeko. Edonola ere, gure emaitzen arabera gazteak helduak baino sentikorragoak dira ingurunearen aldaketa estokastikoekiko. Mediterraneoko *Iberus* barraskilo lur generoarentzat, Polo-k (2015) ere gazteek ingurunearen aldaketa estokastikoekiko, helduek baino sentikortasun altuagoa zeukatela adierazi zuen, baina kasu honetan, intsolazioari eta lehorketari lotutako aldagaiekiko.

Helduen biziraupena gazteena baino altuagoa izatea bizitza luzeko espezieen bizitza-historiaren ezaugarri ohikoa da (Tavecchia et al., 2005; Tavecchia et al., 2016). Bizitza luzeko muxar grisean eta Edmondson pinguinoan adibidez, Pilastro et al.-ek (2003) eta Ballerini et al.-ek (2009), hurrenez hurren, bizitzako lehen urteko hilkortasuna hurrengo urtekoena baino nabarmenki altuagoa zela aurkitu zuten. Zinez, *P. carascalensis* bizitza luzeko espeziea dela aurkitu genuen, izan ere, gutxienez 8–9 urte bizi dela kalkulatu genuen. Lurreko barraskiloen bizi itxaropena hilabete batzuetatik 19 urte bitartekoa izan daiteke eta, nahiz eta momentu honetan dauden erregistroen %50a baino gehiago bizitza laburreko espezieenak diren, gastropodo lurtar asko bizitza luzekoak direla uste da (Heller, 2001). Era berean, espezie honek heldutasunera iristeko denbora tarte luzea behar duela erregistratu dugu, batez beste 2,72 urte behar dituelarik heltzeko. Bai bizitza luzea eta bai heldutasun berantiarra bizi-ziklo motelen bizitza-historiaren ezaugarri ohikoa da. Bizitza-historia motelek ugaltze tasa motela eta guraso zaintza inbertsio handia izaten dute, kume gutxi eta handiak ekoiztuz errekrutatze aukerak handitzeko. Oraindik ez daukagu *P. carascalensis*-en ugaltze tasen informaziorik baina, jarraipenean zehar, 2–2.5 mm-ko 5 arrautzez osatutako norbanako baten errute baten lekuko

izan ginen. Arrautzen tamaina, azken finean kumeen tamaina erabakitzen duena, izugarri aldatzen da gastropodo espezie ezberdinen artean eta animaliaren tamainarekin erlazionatuta dago (Heller, 2001). Hala ere, *P. carascalensis*-en arrautzaren tamainaren eta animaliaren tamainaren arteko ratioa espezie honekin estuki erlazionatuta dauden beste espezieen arrautzen diametroarekin konparatuz (Hygromiidae eta Geomitridae) (Heller-en lanean bilduta, 2001), *P. carascalensis*-en ratioa altuenetarikoa da. Beraz, animaliaren tamaina kontuan hartuz, badirudi *P. carascalensis*-ek kume handienetarikoenak ekoizten dituela bere ahaide gertuenen artean. Arrautza kopuruari dagokionez, lurreko barraskiloetan aldakortasun handia aurkitu da, baita espezie beraren barruan ere, eta gurasoen ezaugarriekin zein ingurumen faktoreekin lotu izan da (adibidez Baur eta Raboud, 1988; Wolda eta Kreulen, 1973). Datu bakar batekin beraz, ezin da inolako inferentziarik egin. Ezaugarri honen nondik norakoak zehazteko, ikerketa espezifiko bat burutu beharko litzateke lagin tamaina egoki batekin eta ingurumen aldagaiak kontrolatuz.

Bizi itxaropen maximoaren aldaketa azaltzeko, hibernazioa faktore esanguratsu gisa identifikatu izan da eta bizi luzeko espezieen eragile izan daitekeela proposatu da (Pilastro et al., 2003; Wilkinson eta South, 2002). Orain dela gutxi, Turbill et al.-ek (2011) filogenetikoki informaturiko Karratu Minimo Orokortuen modelizazioa burutu zuten hibernazioaren eragina aztertzeko ugaztunen bizitza-historiaren zenbait atributu garrantzitsutan, besteak beste, urteko biziraupenean, bizi itxaropen maximoan, urteko ugalketa arrakastan, heldutasunera iristeko adinean eta belaunaldi denboran. Aztertutako bizitza-historiaren ezaugarri guztietan hibernazioak eragin esanguratsua daukala aurkitu zuten, baina are garrantzitsuagoa dena, teoria ebolutiboek aurresan bezala, haien emaitzek proposatu zuten hibernatzen duten espezieen biziraupen handipena bizitza-historia motelen koeboluzioarekin erlazionatuta dagoela. Beraz, *P. carascalensis*-ek hibernatzea eta guk aurkitutako bizitza-historia motela adierazten duten ezaugarriak lotuta egon daitezke. Bizitza-historiaren teoriaren arabera, bizi-ziklo motelek inguru aldakorretan abantaila daukate, estrategia honek ugalketa esfortzua mugatzen duelako urte txarretan asko inbertitzea saihestuz, eta bizi itxaropena handitzen duelako, ugalketa urte ugaritan burutzea ahalbidetuz; horrela, inguruneko gertaera estokastikoen ondorio kaltegarriak txikitu egiten dira eta perturbazioak arindu (Stearns, 1976). Inguru menditsuetan, altuera gradienteak eragin handia dauka bizitza-historietan eta, honek, ingurumen erronka berdinei aurre egiten dieten organismoen bizi-zikloen ezaugarriek bat-egitea dakar (Laiolo eta Obeso, 2017). Gradiente honetan, eskualde alpinoak dira aldakorrenak eta gogorrenak eta, ondorioz, populazio alpinoek normalean estrategia motelak izaten dituzte, salbuespenen jatorria ondare ebolutiboan edo historia biogeografikoan errotuta dagoelarik (Laiolo

eta Obeso, 2017). Beraz, nahiz eta hemen ikertutako *P. carascalensis* populazioa estai menditarrean kokatuta dagoen, bere bizitza-historiaren ezaugarriak espezie alpetar batenak dira. Izan ere, *P. carascalensis*-en banaketa altitudinala 460 m-tatik 2640 m-tara doa (1100 m-tatik 2640 m-tara nagusiki, 460 m-tan dagoen populazio bakarra kenduz gero), estai menditar, subalpetarra eta alpetarra hartuz. Aurkikuntza honek *P. carascalensis* bero-tolerantzia gradu bat daukan hotzera egokitutako taxon bat dela konfirmatzen du eta, honi esker, espeziea muga subalpetarretik behera dauden arroka azaleratzeetan bizi daiteke (Caro et al., 2019).

## 4.2. Berrarrapaketa probabilitatea

Berrarrapaketa probabilitateari dagokionez, bai helduen eta bai gazteen matrizeetan burututako modelizazio emaitzek aldagaia denborarekiko menpekoa dela berreskuratu zuten. Hala ere, hemen erabilitako kanpo kobarianteak ez ziren gai izan bere aldakortasuna azaltzeko. Modeloak egokitzeko eta aukeratzeko arazoak izatea eta aldagaien portaera zehazteko zailtasunak izatea, laginketa gutxi egitearen ondoriozko datu-base txiki eta eskasen analisiarekin egon daiteke lotuta (Tenan et al., 2013), baina baita azaltzeko kanpo kobariante desegokiak aukeratzearekin ere. Beraz, berrarrapaketa probabilitatea aurreko hiru egunen baldintza meteorologikoekin ezin azaltzearen arrazoia, egun gehiagoren klima joerak edo aldagaien arteko elkarrekintza konplexuek norbanakoen jarduera, eta ondorioz antzemateko aukera, baldintzatzen dutela izan daiteke. Arazo hauek izan arren, laginketa egun bakar bat egiteak helduen urtaroko modeloan eragin negatiboa zuela zehaztu genuen. Emaitza hau ikusita, populazioa era egokian jarraitzeko urtaroko bi laginketa egitea (bi udaberrian eta bi udazkenean) beharrezkoa eta nahikoa dela determinatu genuen.

## 4.3. Populazio tamaina

Populazio dinamika ikertzeko, populazio baten propietate intrintsekoak (parametro demografikoak) eta haien aldaketa denboran zehar analizatzea metodo sendoagoa da populazioaren tamaina aldaketa jarraitzea baino (Clobert et al., 1988). Hala ere, populazioen tamaina erabakigarria da iraungitze arriskurako (Reed et al., 2003) eta, ondorioz, ikertutako *P. carascalensis* populazioak zenbat norbanakoz osatuta dagoen ere jakin nahi genuen. Nahiz eta populazio tamainaren kalkulu sendoak lortzea zaila den, arroken arrakaletan babestu daitekeen *P. carascalensis*-en antzera saiheskorrak diren populazio itxietan, harrapaketa-berrarrapaketa metodoak nahikoa zehatzak dira populazio tamaina kalkulatzeko (Tenan et al., 2013). Gure kalkuluen arabera,

populazioa 434 norbanakok osatzen zuten 2016an (95% CI: 405–474). Kopuru hau askoz baxuagoa da Traill et al.-ek (2007) 30 urtetan zehar gaiaren inguruan argitaratutako lanen meta-analisiarekin kalkulatu zuten 4169 (95% CI: 3577–5129) norbanakoko populazio tamaina bideragarri minimoa baino. Dena den, egile hauek populazio bideragarri minimoa testuinguruaren araberakoa dela ere ondorioztatu zuten eta, aztertutako 212 espezieetatik, bat ere ez zen gasteropodo lurtarra eta ez zegoen antzeko ezaugarriak zituen beste espezierik ere. Ikerketa eremuak daukan zabalera txikiarekin ez da batere probablea bere gain hartu ahal izatea autore hauek proposatzen duten kopuru handia. Ostera, lur-eremuaren karga-gaitasuna kontuan hartuz, gure populazioarentzat kalkulatutako tamainarekin populazioa iraungitzeko berehalako arriskuan ez dagoela dirudi, gertakari katastrofikorik ezean behintzat. Bestalde, bisita bakoitzean aurkitutako ale kopurua 129-266 bitartekoa izan dela ikusita, argi dago norbanakoen lurperatzeko eta arrakaletan ezkutatzeko gaitasuna oso handia dela eta horrek izugarri murrizten du atzemateko aukera. Gainera, populazio tamainaren modelizazio honek argi utzi zuen, berriz ere, harrapaketa/berrarrapaketa probabilitatea aldatu egiten dela denborarekin $[f_0, p_0(t) = c(t)]$ bere jatorria ezezaguna den arren.

## 4.4. Hazkuntza eta desplazamendua

Espero bezala, gazteak udaberri eta udazken bitartean hazten dira, aktibo dudenean. Hibernatzen duten espezieetan ohikoa da hibernazio garaian ez haztea. Adibidez, *Cornu aspersum* lur barraskiloaren hazkuntza tasa gelditu egiten da hibernazioan eta pisua galtzen dutela ere ikusi izan da (Dupont-Nivet et al., 1997). Zoritxarrez, norbanakoak antzematerakoan dagoen aldakortasuna dela eta, ez genituen gazte nahikoren neurketa jarraiak lortu hazkuntza tasa kalkulatzeko eta ingurumen aldagaiekin korrelazionatzeko. Ezaugarri hau era egokian analizatzeko, berariazko ikerketa esperimental bat beharko litzateke.

Azkenik, lau urte hauetan zehar ikusitako desplazamendu eskasek lur barraskilo hauek dispertsio aktibo txikia daukatela berresten dute; izan ere, arroka berberaren barruan ere, norbanakoak metro karratu bat baino area txikiagoan mantentzen ziren normalean. Dispertsio aktibo txiki hau gizaki, animalia, haize eta ur bidezko dispertsio pasibo potentzial handi batekin konpentsatu daiteke (Aubry et al., 2006). Horrela, *P. carascalensis*-en dispertsioak eta, segur aski *Pyrenaearia* generoko beste espezieen dispertsioak ere, dispertsio pasiboarekiko menpekotasun handia izan dezake, beste lan batzuetan ere iradoki den bezala (Caro et al., 2019; Elejalde et al., 2009).

# 5. Ondorioak

Harrapaketa-berrarrapaketa ikerketa baten bidez, *P. carascalensis* lur barraskiloaren populazio demografiaren eta oinarrizko ezaugarri biologikoen inguruko informazioa bildu dugu hemen lehenengo aldiz. Heldu eta gazte adin klaseentzat biziraupen desberdinak lortu genituen, gazteak ingurunearen aldaketa estokastikoekiko sentikorrago agertu zirelarik, bereziki sentikorrak izanik udazkeneko aldakortasunarekiko eta, zehazki, udazkeneko izozte egunekiko. Espezieak estrategia motelen ezaugarriak aurkezten ditu, besteak beste bizitza luzea, heldutasun berantiarra eta kume handien ekoizpena, espezie alpetarren berezko bizitza-historia ezaugarriak direnak. Ezin izan genuen zehaztu zein faktorek zeukaten eragina berrarrapaketa probabilitatean; hala ere, populazioa era egokian jarraitzeko urtaro bakoitzean bi bisita egitea beharrezkoa dela zehaztu genuen. Populazioaren tamaina 434 norbanakokoa zela kalkulatu genuen, segur aski nahikoa dena berehalako iraungitze arriskuan ez egoteko. Horrela, mendi inguruetan gertatzen ari diren aldaketen berri eman dezakeen espezie baten epe luzerako jarraipena martxan jarri dugu, mendietako dibertsitatea babesteko neurri eraginkorren diseinuan lagunduko duena.

# Esker onak

Lehenik eta behin Gorbeiako Parke Naturaleko basozainen laguntza eskertu nahi dugu, bereziki Ikerri. Eskerrik asko laginketa ugarietan laguntzera Gorbeiara igo diren lagun eta lankideei ere. Lan hau Eusko Jaurlaritzak "*Systematics, Biogeography and Population Dynamics*" Ikerketa Taldeari emandako diru-laguntzarekin (IT575-13) finantzatua izan da partzialki. A. Caro-k Eusko Jaurlaritzaren Hezkuntza, Unibertsitate eta Ikerketa Sailaren doktoretza bekaren babesarekin burutu zuen lana (Ref. PRE_2015_2_0191).

# CHAPTER 5

## Morphological integration of the shell

# 5. KAPITULUA

## Oskolaren integrazio morfologikoa

PAPER V

# Morphological integration in helicoid shell: insights from *Pyrenaearia* (Gastropoda: Hygromiidae)

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*In preparation*

# Morphological integration in helicoid shell: insights from *Pyrenaearia* (Gastropoda: Hygromiidae)


## Abstract

Morphological integration is an important determinant of organisms' shape and, therefore, assessing its extent and, more importantly, the processes behind the integration are crucial to understand morphological evolution. Despite gastropod shell evolution is been extensively studied, few works have focused on its integration. Here, we applied a multilevel approach using geometric morphometrics to investigate different aspects of morphological integration in the shell of the land snail genus *Pyrenaearia*. We found that the main shape changes between species correspond with the main shape changes within-taxa and that those changes involve relative shift of landmark configuration throughout the entire shell indicating integration. Furthermore, we recovered a high (0.67) and significant RV coefficient for the overall association between the suture and the peristome as well as a clear correspondence between the first PLS axes characterizing covariation between these two parts and the PCs of overall shell variation. Our results further support that this morphological integration may be limiting possible shell shapes and constraining also evolutionary change in few directions of phenotypic space.



## Keywords

morphological integration; gastropod shell; geometric morphometrics; multilevel approach; PLS; *Pyrenaearia*

# 1. Introduction

Despite their geometrically simple form, gastropod shells display a great diversity of forms. Due to their simplicity and yet huge amount of diversity, the morphological evolution of gastropod shells has attracted a lot of attention, giving rise to numerous works in which shell form is analysed both empirically, quantifying shell form variation, and theoretically, simulating shell formation (Liew and Schilthuizen, 2016). Yet, morphological integration of gastropod shells has not been investigated in depth.

Morphological integration is the tendency of different traits to vary conjointly (Klingenberg, 2014) and can arise due to many genetic, functional, developmental and ecological factors (e.g. Klingenberg, 2014, 2008; Klingenberg et al., 2012; Kulemeyer et al., 2009; Parsons et al., 2011). Integration exists in multiple level of variation, from individual level to macroevolutionary level (Klingenberg and Marugán-Lobón, 2013). The extent of integration in a biological structure is an important determinant of final shape and, therefore, assessing its extent and, more importantly, the processes behind the integration are crucial to understand morphological evolution.

Geometric morphometrics is a powerful technique to address questions about shape evolution and development because it is able to characterise the patterns of morphological variation in great detail while maintaining the original geometry of the shape (Evin et al., 2008; Mitteroecker and Gunz, 2009). In particular, this technique offers many advantages for investigating morphological integration (Klingenberg, 2014). On the one hand, geometric morphometrics can be used to thoroughly analyse patterns of inter-specific and intra-specific shape variation and then to perform a statistical comparison of the variation at these different levels. Thus, this approach provides information about whether there are patterns of morphological integration in each of the levels and the correspondence between them and, hence, whether integration is acting as a constraint determining evolutionary diversification (Klingenberg et al., 2012). On the other hand, geometric morphometrics allows establishing sub-blocks or modules of landmarks within a complete landmark configuration, each representing a portion of the whole structure, and performing a two-block partial least-squares analysis (PLS) to assess the covariation between the delimited modules (Rohlf and Corti, 2000). Consequently, PLS has been extensively used to characterised patterns of morphological integration (e.g. Chamero et al., 2013; Curth et al., 2017; Neaux et al., 2013; Senck and Coquerelle, 2015; Singh et al., 2012).

Here we apply geometric morphometrics methods to investigate different aspects of morphological integration in the shell of the land snail genus *Pyrenaearia* by

combining shape pattern variation at inter- and intra-specific levels and PLS analysis. This genus is interesting to examine morphological integration because it has been shown that there are morphological differences between species' shells despite they are very subtle (Caro et al., 2019). The analyses were based on 3D landmark configurations capturing shell shape and including all *Pyrenaearia* species sampled to harbour the maximum intra-specific morphological variability. Throughout this multilevel approach we aim to determine if integration is an important factor shaping shell morphology evolution.

# 2. Materials and methods

## 2.1. Morphometric data

In this work we used the same morphometric data set employed by Caro et al. (2019) based on 133 adult shells 3D digitized using computed tomography and representing all *Pyrenaearia* species. The data set consists on three homologous landmarks placed on the starting point of the suture in the apex and on the intersection of the peristome with the parietal and columellar areas, and on equally spaced semilandmarks distributed throughout two homologous curves: the suture (80 semilandmarks) and the peristome outline (22 semilandmarks). Shape information was extracted from these landmark and semilandmark coordinates with a full Procrustes fit as implemented in the R package Morpho v.2.6 (Schlager, 2016) allowing the semilandmarks to slide during superimposition along their tangent directions minimizing bending energy.

## 2.2. Patterns of variation in shape changes: inter-specific vs intra-specific variation

Since principal components (PCs) are axes that maximize the variation for which they account while being constrained to be orthogonal to the preceding PC, principal component analysis (PCA) was used to examine the main shape variation patterns. PCA was carried out at two levels using Morpho. First, the analysis was conducted on the mean shapes calculated for each species (as delimited by Caro et al., 2019) so that it corresponds to inter-specific variation. Then, we performed a PCA on the pooled within-taxon covariance matrix which is a common estimation of shape variation within each group and, therefore, accounts for intra-specific variation.

To determine possible correspondence between the main shape changes of each level, the shape change direction of the two first PCs was visualized by warping the scanned surface of a shell to the extremes of each PC axis. Further, correspondence

between shape changes was also assessed by calculating the angles formed between the shape changes vectors corresponding to the direction of each PC of inter-specific level versus the PC vectors of intra-specific level. Angle calculation was restricted to range between 0º to 90º because the direction of PC vectors is arbitrary (Klingenberg and Marugán-Lobón, 2013) and the statistical significance of the angles was estimated following Li (2011).

## 2.3. Covariation between suture and peristome outline

The covariation pattern between the suture and the peristome was investigated for the whole genus using two-block partial least-squares analysis (PLS) (Rohlf and Corti, 2000) as implemented in Morpho. PLS analysis extracts pairs of PLS axes for each set of variables (sub-blocks of landmark configurations) that have maximum covariance with each other (Rohlf and Corti, 2000), i.e. PLS axes identify shape features with maximal covariation. The PLS analysis was carried out on the coordinate data set resulting from the simultaneous Procrustes fit of the entire landmark configuration so that it considered both the covariation originating from coordinated variation in shape of the parts and covariation arising from variation in relative size and positioning of the parts (Klingenberg and Marugán-Lobón, 2013). The overall association between the suture and the peristome was assessed using the RV coefficient that ranges between 0 for complete independence between sub-blocks to 1 for total interdependence, being the variation of one sub-block of landmarks configuration completely predictable from the variation in the other sub-block; its significance was addressed via permutation test (250 permutations) (Klingenberg, 2009).

The shape change associated to the two first PLS axes was calculated in Morpho (plsCoVar function) and then it was visualized by warping the scanned surface of a shell according to the inferred change. This allowed us to visually compare the shape changes of maximal covariation with inter-specific and intra-specific shape variation. We further compared the shape changes corresponding to PLS axes with inter and intra-specific PC vectors by angular comparison. Since each pair of PLS axes for each sub-block of landmark configurations is computed as separate vector, the two vectors must be combined to compare them with other analyses' vectors (Klingenberg and Marugán-Lobón, 2013). However, for doing so the coefficients of the two PLS axes in each pair must be scaled relative to each other for which we implement the algorithm of Mitteroecker and Bookstein (2008) (in **Supplementary material 1** we provide the script developed to implement this algorithm from the results of the PLS analysis performed in Morpho because the package do not implement it). As in the comparison between PC vectors, angle calculation was restricted to range between 0º to 90º and its statistical significance was estimated following Li (2011).

# 3. Results

## 3.1. Patterns of variation in shape changes: inter-specific vs intra-specific variation

In the PCA of the variation among species means, PC1 accounts for 64% of the shape variation, being clearly the dominant pattern of shell diversification (**Figure 1a**). PC2 and PC3 explained 23% and 7% of the variation respectively and, therefore, the first three PCs accounted for 94% of the total shape variation. Shape changes associated with PC1 and PC2 are shown in **Figure 1a.** PC1 shape variation is related to whorl expansion throughout coiling, ranging from constant increment to logarithmic, which affects also shell contour and aperture contour. PC2, in contrast, is associated with aperture shape, specifically, with the direction of the maximum width with respect to the coiling axis which in turn affects shell's high.

For the PCA conducted on the pooled within-taxon covariance matrix to determine intra-specific variation, PC1 accounts for 34% of shape variation (**Figure 1b**), while PC2 and PC3 explained 15% and 10% respectively and, hence, the first three PCs accounted for 59% of the total variation. **Figure 1b** displays shape changes related to intra-specific PC1 and PC2. By visual comparison we found some resemblances between intra-specific PC2 and inter-specific PC1 and between intra-specific PC1 and inter-specific PC2. Indeed, angular comparisons support correspondence between these vectors: angle between intra-specific PC2 and inter-specific PC1 55º ($p << 0.001$) and angle between intra-specific PC1 and inter-specific PC2 51º ($p << 0.001$).

## 3.2. Covariation between suture and peristome outline

The RV coefficient recovered for the PLS analysis was 0.67 ($p << 0.001$), supporting a strong covariation between the suture and the peristome outline. The first three PLS axes account for 97% of the total squared covariation between suture and peristome, 61% for PLS1, 33% for PLS2 and 3% for PLS3 (**Figure 2**). The shape variation patterns of covariation associated to PLS1 and PLS2 axes are displayed in **Figure 2.** These shape changes showed a clear and strong agreement with the inter-specific PCA shape changes, which were supported by angular comparisons: PLS1 axes were associated with inter-specific shape change of PC1 (angle 12º, $p = 0$) and PLS2 resembled inter-specific shape change of PC2 (angle 20º, $p = 0$). The association with the intra-specific PCA shape changes was less strong, but still important and supported by angular comparisons: PLS1 axes were associated with intra-specific shape change of PC2 (angle 43º, $p << 0.001$) and PLS2 resembled intra-specific shape change of PC1 (angle 56º, $p << 0.001$).

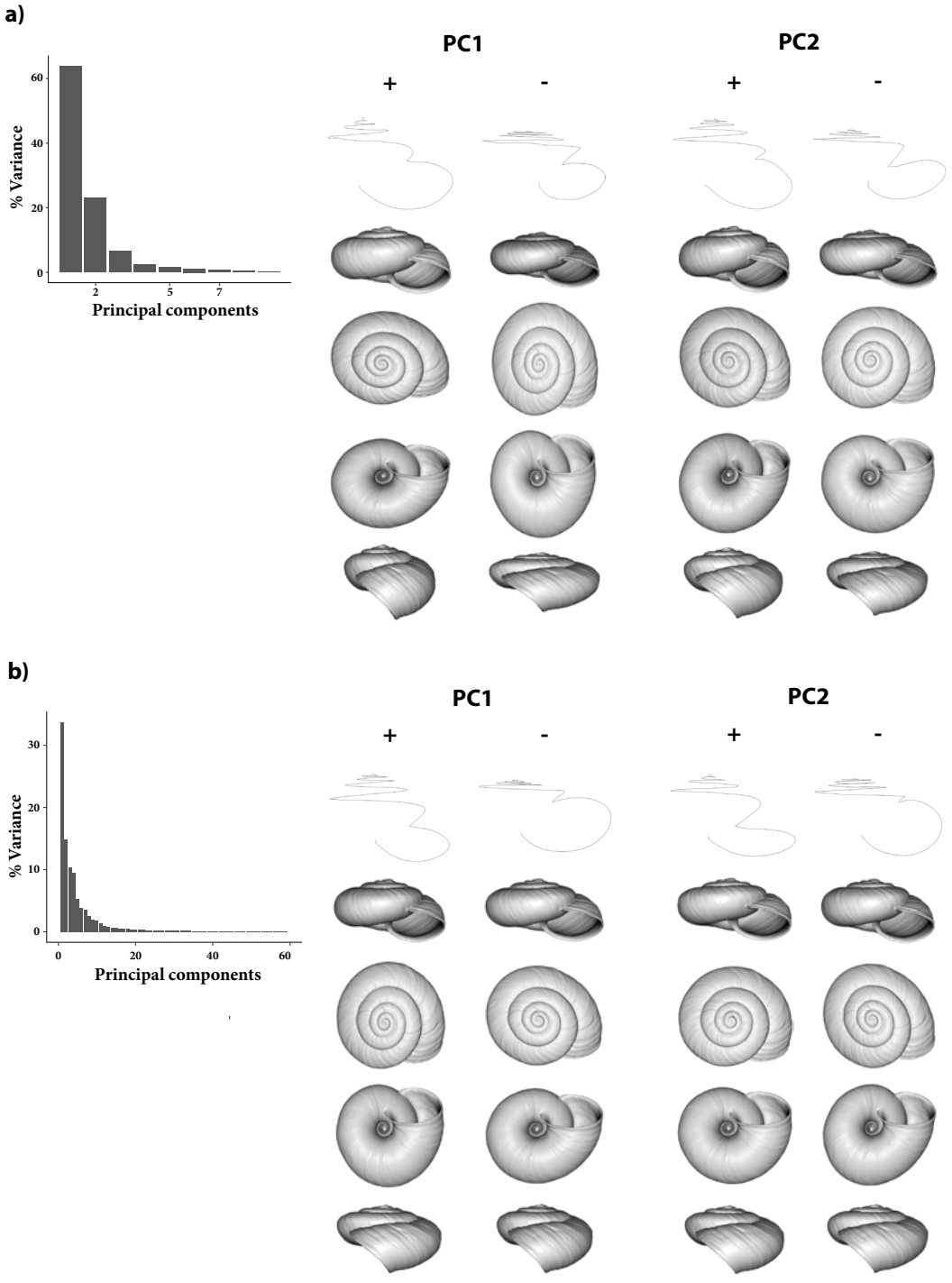


**Figure 1.** Principal component analysis of (a) the variation among species means (inter-specific variation) and (b) the pooled within-taxon covariance matrix (intra-specific variation). Bar charts indicate the percentages of the total variance for with the PCs account and shape changes associated to the first two PCs are shown by means of warped shell mesh and line diagram.

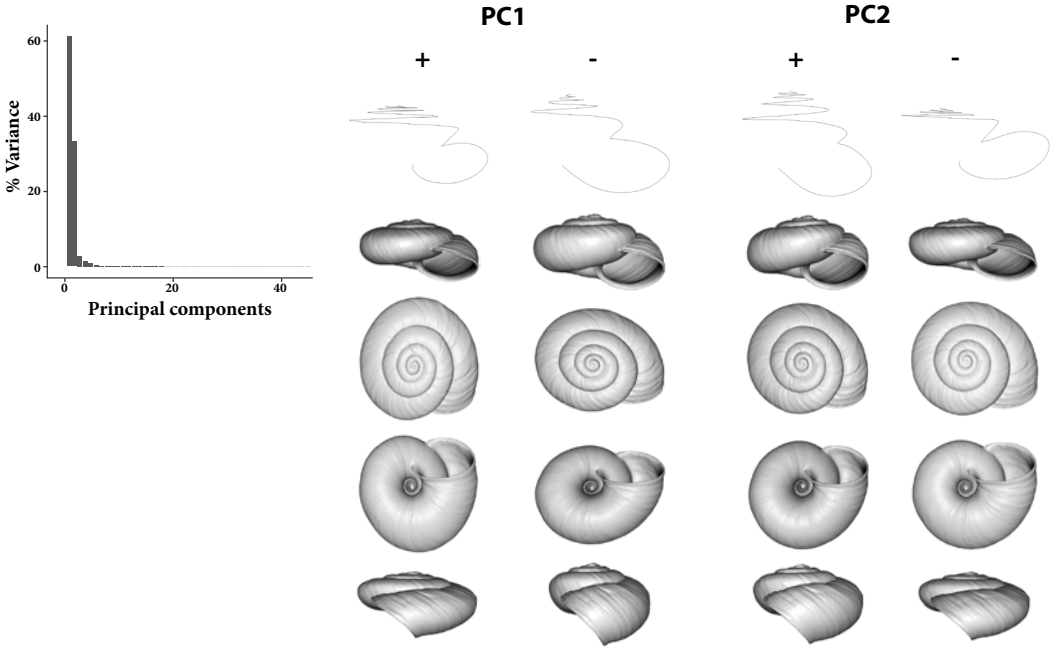


**Figure 2.** PLS analysis between suture and peristome outline. Bar chart indicates the percentages for which each PLS axis account and shape changes associated to the first two PLS axes are shown by means of warped shell mesh and line diagram.

# 4. Discussion

This study has used geometric morphometrics with a multilevel approach to explore the variation of shell shape in the land snail genus *Pyrenaearia* and to investigate shell integration and evolution.

First, we analysed patterns of shape variation at inter- and intra-specific levels. The amount of shape variation for which the first PCs accounted at each level differs clearly. In the case of inter-specific variation, the first two PCs accounted for almost 90% of the variation, being PC1 alone responsible for 64% of the total variation. Instead, in intra-specific variation, the first two PCs only accounted for the half of the variation. Therefore, while inter-specific shape variation is highly concentrated in two directions with a clear dominance of PC1, within taxa shape variation is more distributed in different directions. On the other hand, we found that the shape changes associated with PC1 and PC2, that is the main shape changes, are remarkably consistent between the two levels, although inversely. Thus, the main shape change of evolutionary divergence (inter-specific PC1) resembles the second most important shape change of intra-specific variation (intra-specific PC2) and the second most noticeable change of inter-specific variation (inter-specific PC2) is consistent with the main shape change within species (intra-specific PC1). The origin of shape variation at the two levels arise from different sources: shape differences among species result from evolution by selection and drift; on the contrary, shape variation within taxa is due to genetic variation and phenotypic plasticity (Klingenberg et

al., 2012). The consistency of patterns across the two levels, however, suggests that a common process may be channelling variation at the two levels mainly in two direction of phenotypic space. One of the consequences of morphological integration is that variation is concentrated mostly in some directions in shape space while other directions have less variation (Klingenberg, 2014). Therefore, this resemblance may be associated to shape variation being integrated throughout the shell, which may be constraining possible shell shapes and may also be acting as a constraint in the process of evolutionary diversification of the shell (Hunt, 2007; Klingenberg, 2010; Klingenberg et al., 2012). Indeed, the pattern of shape variation related to the PCs revealed that shape changes involve relative shift of landmark configuration throughout the entire shell indicating integration (**Figure 1**).

Furthermore, we recovered a very high (0.67) and significant RV coefficient for the overall association between the suture and the peristome. This is a very high value comparing with those obtained in other works studying integration (e.g. Gómez-Robles et al., 2011; Klingenberg and Marugán-Lobón, 2013; Laffont et al., 2009; Parsons et al., 2011) and a clear evidence that the shell in *Pyrenaearia* is a highly integrated structure. In addition, that shape variation is integrated throughout the shell is also supported by the clear correspondence between the first PLS axes characterizing covariation between the suture and the peristome and the PCs of overall shell variation. Here, PLS axes are computed solely from information about covariation between the suture and peristome while the first PCs represent the features of the shell shape with the most overall variation. Thus, the resemblance between PCs and PLS axes indicated that the features of integration between suture and peristome are among the dominant features of shell shape variation (Klingenberg and Marugán-Lobón, 2013). In consequence, this correspondence of PCs and PLS axes is proof of overall integration in the whole structure (Klingenberg and Zaklan, 2000). Indeed, in morphological structures of other various organisms with strong integration a clear resemblance between PCs and PLS axes has been found too (Klingenberg et al., 2003, 2001; Klingenberg and Marugán-Lobón, 2013; Klingenberg and Zaklan, 2000; Kulemeyer et al., 2009; Monteiro et al., 2005).

All in all, our results support that shape variation is integrated throughout the shell in *Pyrenaearia* which may be limiting possible shell shapes and constraining also evolutionary change in few directions of phenotypic space.

## Acknowledgements

We would like to thank E. Ibiriku who kindly helped us developing the script for scaling the coefficients of the two PLS axes in each pair. This work was partially funded by the Basque Government through the Research group on "Systematics, Biogeography and Population Dynamics" (IT575-13). A. Caro was supported by a PhD fellowship awarded by the Dept. of Education, Universities and Research of the Basque Government (Ref. PRE_2015_2_0191).

# References


Caro, A., Gómez-Moliner, B.J., Madeira, M.J., 2019. Integrating multilocus DNA data and 3D geometric morphometrics to elucidate species boundaries in the case of *Pyrenaearia* (Pulmonata: Hygromiidae). Mol. Phylogenet. Evol. 132, 194–206. https://doi.org/10.1016/j.ympev.2018.12.007

Chamero, B., Buscalioni, Á.D., Marugán-Lobón, J., 2013. Pectoral girdle and forelimb variation in extant Crocodylia: the coracoid – humerus pair as an evolutionary module. Biol. J. Linn. Soc. 108, 600–618.

Curth, S., Fischer, M.S., Kupczik, K., 2017. Patterns of integration in the canine skull: an inside view into the relationship of the skull modules of domestic dogs and wolves. Zoology 125, 1–9. https://doi.org/10.1016/j.zool.2017.06.002

Evin, A., Baylac, M., Ruedi, M., Mucedda, M., Pons, J.M., 2008. Taxonomy, skull diversity and evolution in a species complex of *Myotis* (Chiroptera: Vespertilionidae): a geometric morphometric appraisal. Biol. J. Linn. Soc. 95, 529–538.

Gómez-Robles, A., Martinón-Torres, M., Bermúdez de Castro, J.M., Prado-Simón, L., Arsuaga, J.L., 2011. A geometric morphometric analysis of hominin upper premolars. Shape variation and morphological integration. J. Hum. Evol. 61, 688–702. https://doi.org/10.1016/j.jhevol.2011.09.004

Hunt, G., 2007. Evolutionary divergence in directions of high phenotypic variance in the ostracode genus *Poseidonamicus*. Evolution 61, 1560–1576. https://doi.org/10.1111/j.1558-5646.2007.00129.x

Klingenberg, C.P., 2014. Studying morphological integration and modularity at multiple levels: concepts and analysis. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 369, 2010249. https://doi.org/10.1098/rstb.2013.0249

Klingenberg, C.P., 2010. Evolution and development of shape: integrating quantitative approaches. Nat. Rev. Genet. 11, 623–635. https://doi.org/10.1038/nrg2829

Klingenberg, C.P., 2009. Morphometric integration and modularity in configurations of landmarks: tools for evaluating a priori hypotheses. Evol. Dev. 11, 405–421. https://doi.org/10.1111/j.1525-142X.2009.00347.x

Klingenberg, C.P., 2008. Morphological Integration and Developmental Modularity. Annu. Rev. Ecol. Evol. Syst. 39, 115–132. https://doi.org/10.1146/annurev.ecolsys.37.091305.110054

Klingenberg, C.P., Badyaev, A. V, Sowry, S.M., Beckwith, N.J., 2001. Inferring Developmental Modularity from Morphological Integration: Analysis of Individual Variation and Asymmetry in Bumblebee Wings. Am. Nat. 157, 11–23.

Klingenberg, C.P., Duttke, S., Whelan, S., Kim, M., 2012. Developmental plasticity, morphological variation and evolvability: a multilevel analysis of morphometric integration in the shape of compound leaves. J. Evol. Biol. 25, 115–129. https://doi.org/10.1111/j.1420-9101.2011.02410.x

Klingenberg, C.P., Marugán-Lobón, J., 2013. Evolutionary covariation in geometric morphometric data: Analyzing integration, modularity, and allometry in a phylogenetic context. Syst. Biol. 62, 591–610. https://doi.org/10.1093/sysbio/syt025

Klingenberg, C.P., Mebus, K., Auffray, J.C., 2003. Developmental integration in a complex morphological structure: how distinct are the modules in the mouse mandible? Evol. Dev. 5, 522–531.

Klingenberg, C.P., Zaklan, S.D., 2000. Morphological integration between developmental compartments in the Drosophila wing. Evolution 54, 1273–1285.

Kulemeyer, C., Asbahr, K., Gunz, P., Frahnert, S., Bairlein, F., 2009. Functional morphology and integration of corvid skulls - a 3D geometric morphometric approach. Front. Zool. 6. https://doi.org/10.1186/1742-9994-6-2

Laffont, R., Renvoisé, E., Navarro, N., Alibert, P., Montuire, S., 2009. Morphological modularity and assessment of developmental processes within the vole dental row (*Microtus arvalis*, Arvicolinae, Rodentia). Evol. Dev. 11, 302–311. https://doi.org/10.1111/j.1525-142X.2009.00332.x

Li, S., 2011. Concise Formulas for the Area and Volume of a Hyperspherical Cap. Asian J. Math. Stat. 4, 66–70.

Liew, T.S., Schilthuizen, M., 2016. A Method for Quantifying, Visualising, and Analysing Gastropod Shell Form. PLoS One 11, e0157069. https://doi.org/10.1371/journal.pone.0157069

Mitteroecker, P., Bookstein, F., 2008. The evolutionary role of modularity and integration in the hominoid cranium. Evolution 62, 943–958. https://doi.org/10.1111/j.1558-5646.2008.00321.x

Mitteroecker, P., Gunz, P., 2009. Advances in Geometric Morphometrics. Evol. Biol. 36, 235–247. https://doi.org/10.1007/s11692-009-9055-x

Monteiro, L.R., Bonato, V., dos Reis, S.F., 2005. Evolutionary integration and morphological diversification in complex morphological structures: mandible shape divergence in spiny rats (Rodentia, Echimyidae). Evol. Dev. 7, 429–439.

Neaux, D., Guy, F., Gilissen, E., Coudyzer, W., Vignaud, P., Ducrocq, S., 2013. Facial Orientation and Facial Shape in Extant Great Apes: A Geometric Morphometric Analysis of Covariation. PLoS One 8, e57026. https://doi.org/10.1371/journal.pone.0057026

Parsons, T.E., Schmidt, E.J., Boughner, J.C., Jamniczky, H.A., Marcucio, R.S., Hallgrimsson, B., 2011. Epigenetic integration of the developing brain and face. Dev. Dyn. 240, 2233–2244. https://doi.org/10.1002/dvdy.22729

Rohlf, F.J., Corti, M., 2000. Use of Two-Block Partial Least-Squares to Study Covariation in Shape. Syst. Biol. 49, 740–753.

Schlager, S., 2016. Package 'Morpho: calculations and visualisations related to geometric morphometrics' version 2.6. Available from https://cran.r-project.org/web/packages/Morpho/index.html

Senck, S., Coquerelle, M., 2015. Morphological Integration and Variation in Facial Orientation in *Pongo pygmaeus pygmaeus*: A Geometric Morphometric Approach via Partial Least Squares. Int. J. Primatol. 36, 489–512. https://doi.org/10.1007/s10764-015-9837-1

Singh, N., Harvati, K., Hublin, J.J., Klingenberg, C.P., 2012. Morphological evolution through integration: A quantitative study of cranial integration in *Homo*, *Pan*, *Gorilla* and *Pongo*. J. Hum. Evol. 62, 155–164. https://doi.org/10.1016/j.jhevol.2011.11.006

# Supplementary material

**Supplementary material 1.** Script developed to implement the algorithm of Mitteroecker and Bookstein (2008) for scaling the coefficients of the two PLS axes in each pair from the results of the PLS analysis performed in Morpho.

```
>bindedPLS1 = rbind(pls$Xscores[,1], pls$Yscores[,1])
>bindedPLS1 = t(bindedPLS1)
>bindedPLS1 = t(bindedPLS1) %*% bindedPLS1
>eigenvectorPLS1 = eigen(bindedPLS1)
>e11.1 = eigenvectorPLS1$vectors[1,1]
>e12.1 = eigenvectorPLS1$vectors[2,1]
>fu1 = e11.1 * pls$svd$u[,1]
>fv1 = e12.1 * pls$svd$v[,1]
>scaledPLS1 = c(fu1[1:24], fv1[1:81], fu1[25:48], fv1[82:162], fu1[49:72], fv1[163:243])
```

In grey: parameters that must be changed according to the PLS axis under study.

In black: parameters to be changed depending on the number of landmarks of each sub-block.

# V. ARTIKULUA

## Integrazio morfologikoa helicoideoen oskoletan: *Pyrenaearia*-rengandik (Gastropoda: Hygromiidae) ikasitakoa

- **Laburpena**
- **Eztabaida**

**Amaia Caro,** Benjamín J. Gómez-Moliner and María José Madeira

*In preparation*

# Integrazio morfologikoa helicoideoen oskoletan: *Pyrenaearia*-rengandik (Gastropoda: Hygromiidae) ikasitakoa

## Laburpena

Integrazio morfologikoa organismoen formaren determinatzaile garrantzitsua da eta, ondorioz, bere hedadura ebaluatzea eta, batez ere, integrazioaren atzean dauden prozesuak zehaztea garrantzitsua da eboluzio morfologikoa ulertzeko. Gasteropodoen oskolaren eboluzioa askotan ikertu izan den arren, lan gutxik jarri dute arreta bere integrazioan. Hemen, morfometria geometrikoarekin maila-anitzeko hurbiltzea aplikatu dugu *Pyrenaearia* lur barraskilo generoaren oskolak duen integrazio morfologikoa ikertzeko. Espezieen arteko forma aldaketa nagusiek espezie barruko forma aldaketa nagusiekin bat egiten dutela aurkitu genuen eta aldaketa horiek oskol osoan zeharreko landmark konfigurazioaren aldaketa erlatiboa ekartzen dutela, integrazioa adieraziz. Are gehiago, RV koefiziente altua (0,67) eta esanguratsua lortu genuen suturaren eta peristomaren arteko asoziazioarentzat, eta baita antzekotasun handia ere bi parte hauen kobariazioa ezaugarritzen zuten lehenengo PLS ardatzen eta oskolaren bariazio osoa ezaugarritzen zuten PC ardatzen artean. Gure emaitzek, gainera, sostengatzen dute integrazio morfologiko honek oskolak hartu ditzakeen formak mugatu ditzakeela eta horrek aldaketa ebolutiboa ere espazio fenotipikoan noranzko gutxi batzuetara mugatu dezakeela.

## Gako-hitzak

integrazio morfologikoa; gasteropodo oskola; morfometria geometrikoa; maila-anitzeko hurbiltzea; PLS; *Pyrenaearia*

# 4. Eztabaida

Lan honetan morfometria geometrikoarekin maila-anitzeko hurbiltzea erabili dugu *Pyrenaearia* lur barraskilo generoaren oskolaren bariazioa aztertzeko eta oskolaren integrazioa eta eboluzioa ikertzeko.

Lehenbizi forma aldaketaren patroiak analizatu genituen, maila inter-espezifikoan eta intra-espezifikoan. Lehenengo PC-ek azaldutako forma aldaketa kopurua oso ezberdina izan zen maila bakoitzean. Bariazio inter-espezifikoari dagokionez, lehenengo bi PC-ek ia aldaketa osoaren %90a azaldu zuten, lehenengo PC-ak %64a bakarrik azaltzen zuelarik. Bariazio intra-espezifikoan, ordea, lehenengo bi PC-ek aldaketa erdia baino ez zuten azaltzen. Beraz, forma bariazio inter-espezifikoa bi noranzkotan oso kontzentratua dagoen bitartean, taxonen barruko forma aldaketa banatuago dago noranzko ezberdinetan. Bestalde, PC1-ari eta PC2-ari loturiko forma aldaketak, hau da, aldaketa nagusiak, mailen artean koherenteak zirela aurkitu genuen, alderantziz ordea. Beraz, dibergentzia ebolutiboaren aldaketa nagusiak (PC1 inter-espezifikoa) bariazio intra-espezifikoaren bigarren aldaketa nagusiaren antza dauka (PC2 intra-espezifikoa) eta bariazio inter-espezifikoaren bigarren aldaketa nabariena (PC2 inter-espezifikoa) espezie barruko aldaketa nagusiaren antzekoa da (PC1 intra-espezifikoa). Maila bakoitzean formaren aldaketak jatorri ezberdina du: espezieen arteko forma ezberdintasuna selekzio eta jito bidezko eboluziotik dator, taxonen barruko aldaketa, ordea, bariazio genetikotik eta plastizitate fenotipikotik (Klingenberg et al., 2012). Bi mailen arteko antzekotasunak, hala ere, iradokitzen du prozesu komun bat egon daitekeela bariazioa batez ere espazio fenotipikoaren bi noranzkotan bideratzen ari dena. Integrazio morfologikoaren ondorioetako bat da bariazioa batez ere forma-espazioaren noranzko batzuetan kontzentratzen dela, gainontzeko noranzkoek bariazio gutxiago daukaten bitartean (Klingenberg, 2014). Ondorioz, antzekotasun hau erlazionatuta egon daiteke formaren aldakortasuna oskol osoan zehar integratuta egotearekin eta honek agian oskolak hartu ditzakeen formak mugatu ditzake eta baita oskolaren dibertsifikazio ebolutiboa ere (Hunt, 2007; Klingenberg, 2010; Klingenberg et al., 2012). Hain zuzen ere, PC-ei loturiko forma aldaketaren patroiek aldaketa horiek oskol osoan zeharreko landmark konfigurazioaren aldaketa erlatiboa ekartzen dutela erakutsi zuten, integrazioa adieraziz (**1. irudia**).

Are gehiago, RV koefiziente altua (0,67) eta esanguratsua lortu genuen suturaren eta peristomaren arteko asoziazioarentzat. Balio hau oso altua da integrazioa ikertu duten beste lan batzuetan lortutako balioekin konparatuz gero (adibidez, Gómez-Robles et al., 2011; Klingenberg and Marugán-Lobón, 2013; Laffont

et al., 2009; Parsons et al., 2011) eta *Pyrenaearia*-ren oskola integrazio handiko egitura denaren froga argia da. Gainera, suturaren eta peristomaren arteko kobariazioa ezaugarritzen zuten lehenengo PLS ardatzen eta oskolaren bariazio osoa ezaugarritzen zuten PC ardatzen arteko antzekotasun handiak formaren aldakortasuna oskol osoan zehar integratuta dagoela ere sostengatzen du. Hemen, PLS ardatzak suturaren eta peristomaren arteko kobariazioaren informaziotik soilik kalkulatu dira; lehenengo PC-ek, ordea, aldaketa handieneko oskol formaren ezaugarriak erakusten dituzte. Beraz, PC eta PLS ardatzen arteko antzekotasunak adierazten du oskol forma aldaketaren ezaugarri dominanteen artean daudela suturaren eta peristomaren arteko integrazioaren ezaugarriak (Klingenberg and Marugán-Lobón, 2013). Ondorioz, PC eta PLS ardatzen antzekotasun hau egitura osoan integrazioa orokorra denaren froga da (Klingenberg and Zaklan, 2000). Beste organismo batzuen integrazio handiko egitura morfologikoetan ere antzekotasun argia aurkitu izan da PC eta PLS ardatzen artean (Klingenberg et al., 2003, 2001; Klingenberg and Marugán-Lobón, 2013; Klingenberg and Zaklan, 2000; Kulemeyer et al., 2009; Monteiro et al., 2005).

Guztia kontuan hartuta, gure emaitzek *Pyrenaearia*-n formaren aldakortasuna oskol osoan zehar integratuta dagoela sostengatzen dute, honek agian oskolak hartu ditzakeen formak mugatzen dituelarik eta baita aldaketa ebolutiboa espazio fenotipikoan noranzko gutxi batzuetara mugatu ere.

## Esker onak

E. Ibirikuren laguntza eskertu nahi dugu PLS ardatzen koefizienteak eskalatzeko script-aren garapenean. Lan hau Eusko Jaurlaritzak "*Systematics, Biogeography and Population Dynamics*" Ikerketa Taldeari emandako diru-laguntzarekin (IT575-13) finantzatua izan da partzialki. A. Caro-k Eusko Jaurlaritzaren Hezkuntza, Unibertsitate eta Ikerketa Sailaren doktoretza bekaren babesarekin burutu zuen lana (Ref. PRE_2015_2_0191).

# CHAPTER 6

## Concluding remarks

The following main conclusions can be drawn from the studies performed in this PhD thesis:

### Systematics and shell morphology variation and evolution in *Pyrenaearia*

**1.** The integrative species delimitation approach, combining multilocus genetic data and 3D morphological information, supports the existence of 10 species in *Pyrenaearia*. Most of the nominal species are further supported, two are synonymized and a hitherto unrecognised cryptic species has been identified. The new species is named as *Pyrenaearia guillenae* spec. nov. and assessed as Near Threatened according to IUCN criteria. The integration of multiple characters arises as an indispensable tool for delimiting species since some of the genus' species were only recognized once molecular methods had revealed their existence and *P. daanidentata* could not be discerned through the molecular markers used.

**2.** We suggest that *Pyrenaearia* is a cold-adapted taxon with certain heat-tolerance so that the availability of shady rock outcrops emerges as a determining factor shaping the genus' distribution. The high complexity of the rock substrate in the Pyrenees could be promoting the genus diversification and thus be responsible for its greater richness in this mountain range.

**3.** Shell morphology in the genus seems to be the product of both population history and adaptation to local environment and allometry emerged as an important factor affecting shell shape.

**4.** Shape variation is integrated throughout the shell in the genus which may be limiting possible shell shapes and constraining also evolutionary change in few directions of phenotypic space.

### Systematics and biogeography of Leptaxinae

**5.** The multilocus molecular phylogeny of Leptaxinae supports four subfamilies for Hygromiidae by elevating Metafruticicolini to subfamily level (Metafruticicolinae) and restricts Leptaxinae to two tribes, Leptaxini and Cryptosaccini. The Lusitanian genus *Portugala* is moved to Leptaxini, previously containing only the Macaronesian genus *Leptaxis*. Within Cryptosaccini, a new genus strictly confined to the Sierra de la Cabrera (Spain) is described, *Fractanella* gen. nov.

**6.** Leptaxinae originated in the Early Miocene in the Iberian Peninsula from which the Macaronesian Islands were posteriorly colonized. The deep split found between the continental and the Macaronesian lineages poses two colonization bias for Macaronesia: throughout the seamounts located between Macaronesia and the continents, now submerged but at one time exposed, or through the oldest now

emerged islands of Macaronesia. In the Iberian Peninsula, the climatic change of the Miocene from subtropical climate towards the present-day Mediterranean climate had great importance shaping the diversification of Cryptosaccini, as well as the Pleistocene glacial cycles.

### Population demographics and basic biological traits of *P. carascalensis*

**7.** *P. carascalensis* displays characteristics of a slow strategy species including long lifespan, delayed maturity and the production of large offspring, which are the typical life-history traits of alpine species.

**8.** The survival of adult and young age classes in *P. carascalensis* is different. While adult survival is high and constant, juveniles are less buffered against environmental stochasticity and especially affected by autumn variability, specifically, by autumn freezing days. The estimated population size of 434 individuals in the studied population of Gorbeia is probably enough not to be in an immediate risk of extinction.

**9.** The monitoring methodology designed has proven to be effective to study *P. carascalensis* biology and population dynamics and we determined that two visits per season are necessary to correctly monitoring the population. This way, we have laid the foundation of a long-term monitoring on a sentinel species that will allow monitoring the changes that are taking place in mountainous areas and will help launching effective approaches to protect mountain diversity.

6

# 6. KAPITULUA

## Ondorioak

Jarraian doktorego tesi honetan egindako ikerketetatik eratorri daitezkeen ondorio nagusiak aurkezten dira:

### *Pyrenaearia*-ren sistematika eta oskol morfologiaren aldakortasuna eta eboluzioa

**1.** Espezieak zedarritzeko erabilitako hurbiltze integratzaileak, *multilocus* datu genetikoak eta 3D informazio morfologikoa konbinatuz, *Pyrenaearia*-ren barruan 10 espezie daudela sostengatzen du. Espezie nominal gehienak balioztatu dira, bi espezie sinonimizatu eta orain arte aintzat hartua ez zen espezie kriptiko bat identifikatu da. Espezie berria *Pyrenaearia guillenae* spec. nov. gisa izendatu da eta Ia Arriskuan kategoria esleitu zaio NKNB irizpideen arabera. Espezieak zedarritzeko hainbat karaktereren integrazioa ezinbesteko tresna dela erakutsi da. Izan ere, generoko espezie batzuk metodo molekularrek haien existentzia ezagutarazi zutenean bakarrik antzeman ziren eta *P. daanidentata* ezin izan zen bereizi lan honetan erabilitako markatzaile molekularrekin.

**2.** *Pyrenaearia* beroarekiko tolerantzia gradu bat daukan hotzera egokitutako taxon gisa proposatzen dugu, arroka azaleratze laiotzen presentzia generoaren banaketaren faktore erabakigarri gisa aurkezten delarik. Pirinioetako substratu konplexutasun handiak generoaren dibertsifikazioa bultzatzen duela dirudi eta mendilerro honetako aniztasun handiagoaren erantzule izan daiteke.

**3.** Generoaren oskolaren forma bai populazio historiaren bai ingurunerako adaptazioaren emaitza dela dirudi eta alometria oskolaren forman eragin garrantzitsua daukan faktore bat bezala identifikatu da.

**4.** Generoan formaren aldakortasuna integratuta dago oskolean zehar, honek agian oskolak hartu ditzakeen formak mugatzen ditzakeelarik eta baita aldaketa ebolutiboa espazio fenotipikoan noranzko gutxi batzuetara mugatu ere.

### Leptaxinae-ren sistematika eta biogeografia

**5.** Leptaxinae-ren filogenia *multilocus* molekularrak lau subfamilia sostengatzen ditu Hygromiidae-rentzat, Metafruticicolini-ri subfamilia maila esleituta, eta Leptaxinae bi tributara, Leptaxini eta Cryptosaccini-ra, mugatzen du. *Portugala* genero lusitaniarra Leptaxini-ra pasa da, aurretik Macaronesiako *Leptaxis* generoa bakarrik biltzen zuena. Cryptosaccini-ren barruan Sierra de la Cabrera-ra mugatzen den genero berria deskribatu da, *Fractanella* gen. nov.

**6.** Leptaxinae Behe Miozenoan sortu zen Iberiar penintsulan eta bertatik Macaronesia kolonizatu zen. Kontinenteko eta Macaronesiako leinuen artean lortutako antzinako banaketa dela eta, Macaronesia bi bidetatik kolonizatu ahal izan zen: Macaronesiaren eta kontinenteen artean kokatzen diren eta behinola

ur-gainean egon ziren urazpiko-mendien bitartez, edo gaur egun ur-gainean dauden Macaronesiako irla zaharrenen bitartez. Iberiar penintsulan Miozenoan gertatu zen aldaketa klimatikoak, klima subtropikaletik gaur egungo klima mediterraneora igaroz, Cryptosacini-ren dibertsifikazioan garrantzi handia izan zuela dirudi, Pleistozenoko ziklo glaziarrekin batera.

### *P. carascalensis*-en populazio demografia eta oinarrizko ezaugarri biologikoak

**7.** *P. carascalensis*-ek estrategia motela daukaten espezieen ezaugarriak aurkezten ditu, besteak beste bizitza luzea, heldutasun berantiarra eta kume handien ekoizpena, espezie alpetarren berezko bizitza-historia ezaugarriak direnak.

**8.** Biziraupena ezberdina da *P. carascalensis*-en heldu eta gazte adin taldeen artean. Helduen biziraupena altua eta konstantea den bitartean, gazteak ingurunearen aldaketa estokastikoekiko sentikorragoak dira, bereziki sentikorrak izanik udazkeneko aldakortasunarekiko eta, zehazki, udazkeneko izozte egunekiko. Ikertutako Gorbeiako populazioaren tamaina 434 norbanakokoa zela kalkulatu genuen, segur aski nahikoa dena berehalako iraungitze arriskuan ez egoteko.

**9.** Diseinatutako jarraipen metodologia *P. carascalensis*-en biologia eta populazio dinamika aztertzeko eraginkorra dela frogatu da eta populazioa era egokian jarraitzeko urtaro bakoitzean bi bisita egitea beharrezkoa dela zehaztu dugu. Horrela, mendi inguruetan gertatzen ari diren aldaketen berri eman dezakeen espezie zelatari baten epe luzerako jarraipena martxan jarri dugu, mendietako dibertsitatea babesteko neurri eraginkorren diseinuan lagunduko duena.

# Appendix

# APPENDIX

Molecular basis of *Cepaea* polymorphism

# ERANSKINA

*Cepaea*-ren polimorfismoaren oinarri molekularra

# PAPER VI
# VI. ARTIKULUA

## Recombination within the *Cepaea nemoralis* supergene is confounded by incomplete penetrance and epistasis

## Barneratze partzialak eta epistasiak *Cepaea nemoralis*-en supergenearen birkonbinazioa nahasten dute

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# Recombination within the *Cepaea nemoralis* supergene is confounded by incomplete penetrance and epistasis

## Abstract

Although the land snail *Cepaea nemoralis* is one of the most thoroughly investigated colour polymorphic species, there have been few recent studies on the inheritance of the shell traits. Previously, it has been shown that the shell polymorphism is controlled by a series of nine or more loci, of which five make a single 'supergene' containing tightly linked colour and banding loci and more loosely linked pigmentation, spread band and punctate loci. However, one limitation of earlier work was that putative instances of recombination between loci within the supergene were not easily verified. We therefore generated a new set of *C. nemoralis* crosses that segregate for colour, banding and pigmentation, and several other unlinked shell phenotype loci. The snails were genotyped using a set of RAD-seq-derived loci that flank the supergene, and instances of recombination tested by comparing inferred supergene genotype against RAD-marker genotype. We found no evidence that suspected 'recombinant' individuals are recombinant between loci within the supergene. As point estimates of recombination between both colour/banding, and colour/pigmentation loci are zero, incomplete penetrance and epistasis are a better explanation for the apparent 'recombinant' phenotype of some snail shells. Overall, this work, therefore, shows that the architecture of the supergene may not be as previously supposed. It also provides a resource for fine mapping of the supergene and other major shell phenotype loci.

This paper is derived from the PhD formation period, but since it is not directly related to *Pyrenaearia* and it is open access, we only provide the abstract. The full paper can be accessed in this doi: http://dx.doi.org/10.1038/s41437-019-0190-6