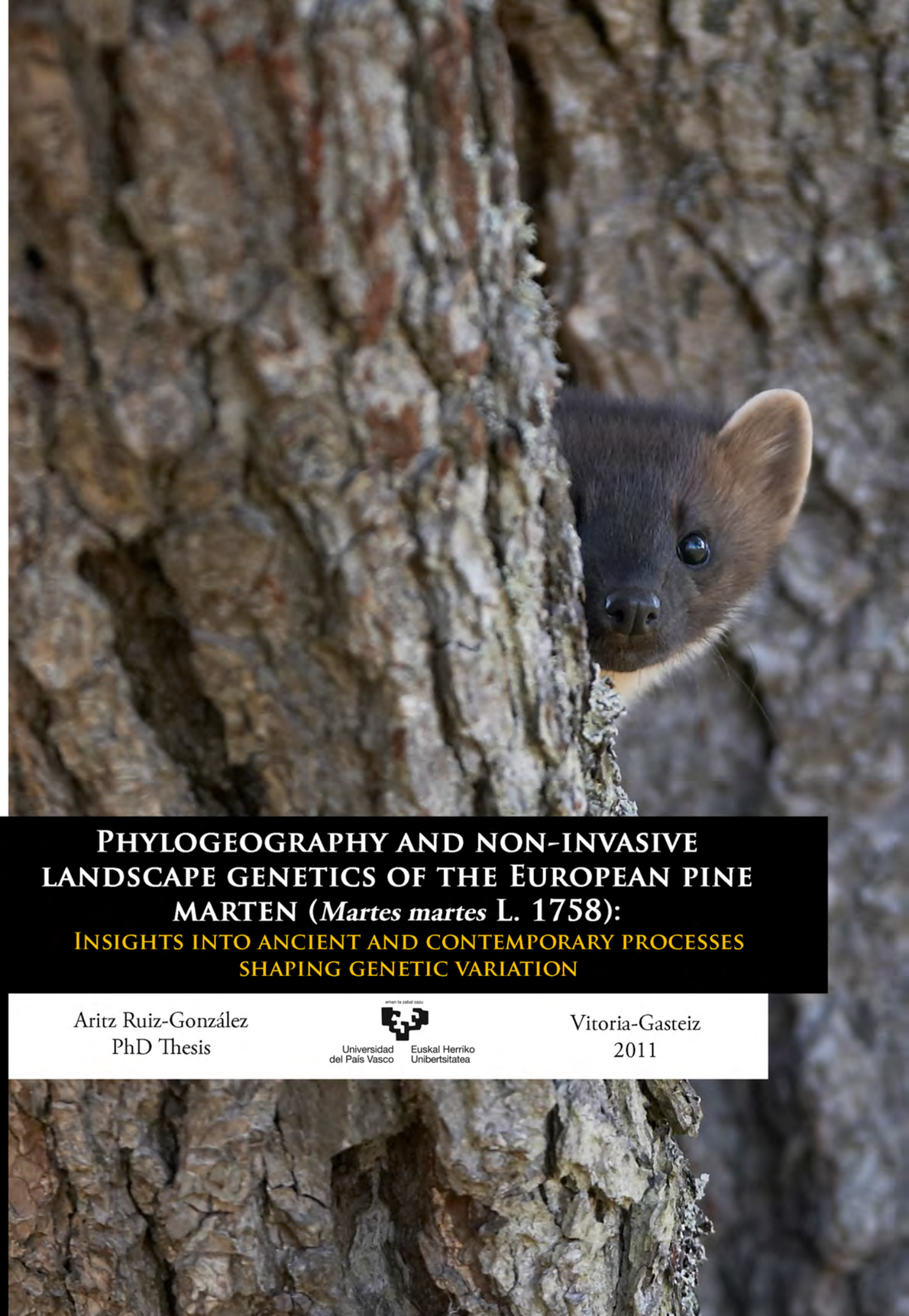
PHYLOGEOGRAPHY AND NON-INVASIVE LANDSCAPE GENETICS OF THE EUROPEAN PINE MARTEN (*Martes martes* L. 1758):
INSIGHTS INTO ANCIENT AND CONTEMPORARY PROCESSES SHAPING GENETIC VARIATION

Aritz Ruiz-González
PhD Thesis 2011



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ACTA DE DEFENSA DE TESIS DOCTORAL

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realizada en el **Departamento Zoología y Biología Celular Animal**

por el Doctorando Don/ña. **Aritz Ruiz-González**

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Tesiaren zuzendariak: Benjamín J. Gómez Moliner jauna eta María José Madeira García andrea.

Jendaurreko defentsa-ekitaldiaren data: 2011-03-18.

Aldez aurreko txostenak honako hauek egin dituzte:

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2. Johan Michaux jaunak

Doktorego-tesia epaitu duen epaimahaiko osakideak:

Mahaiburua: José Luis Telleria Jorge jauna

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Que D. **ARITZ RUIZ GONZALEZ** ha obtenido la mención de **"Doctor Europeus"** por la Universidad del País Vasco tras la elaboración, defensa y aprobación de su Tesis Doctoral, en virtud de la normativa aprobada a tal efecto por la Junta de Gobierno de esta Universidad el 6 de abril de 1993.

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Directores de la Tesis: Don Benjamín J. Gómez Moliner y Doña María José Madeira García.

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Los informes previos han sido elaborados por los Dres:

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Departamento de Zoología y Biología Celular Animal

Facultad de Farmacia

Phylogeography and non-invasive landscape genetics of the European pine marten (*Martes martes* L. 1758):

Insights into ancient and contemporary processes shaping genetic variation

Tesis Doctoral dirigida por:

Dr. Benjamín J. Gómez-Moliner

Dra. Mª José Madeira García

Memoria que para optar al grado de doctor presenta

ARITZ RUIZ GONZÁLEZ

Vitoria-Gasteiz

2011

"La utopía está en el horizonte. Camino dos pasos, ella se aleja dos pasos y el horizonte se corre diez pasos más allá. ¿Entonces para que sirve la utopía? Para eso, sirve para caminar."

Eduardo Galeano

"We only preserve what we love, we only love what we understand, we only understand what we study."

Tibetan Saying

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CONTENTS

ABSTRACT

Understanding the physical and temporal factors that structure populations is essential to their conservation and management. The main objective of this thesis is to identify ancient and contemporary processes shaping genetic variation of the forest dwelling European pine marten (*Martes martes* L. 1758), trough two distinct disciplines: phylogeography (i.e combination of phylogenetics and biogeography) and landscape genetics (i.e combination of population genetics and landscape ecology). Firstly, this thesis reviews the intraspecific phylogenetic, population genetic, and landscape genetic studies conducted on the 8 recognized *Martes* species populations; discusses commonalities found across the species in terms of habitats deemed important for connectivity; and identifies knowledge gaps for understanding movement and substructure. Secondly, we investigated the phylogeographic pattern of the European pine marten, providing new insights into the cryptic northern glacial refugia and postglacial recolonization of Europe as well as into genetic relationships between *M. martes* and *M. zibellina*. Thirdly, in order to understand how landscape and environmental features influence population genetic structure, non-invasive genetic sampling (i.e sampling genetic material without disturbing the target species) of faeces in combination with landscape genetics methods have been used for the study of this rare and elusive species. For the consecution of this objective we initially designed and applied a non-invasive species identification method to asses the distribution of sympatric martens from fecal DNA (*M. martes* and *M. foina*) and thereafter, by means of microsatellite analysis we investigated the spatial genetic structure of the pine marten inhabiting a fragmented landscape. Bayesian spatial and non-spatial models allowed us to detect several populations with genetic discontinuities being associated to human induced landscape transformations (i.e. urban areas, road networks and forest fragmentation processes). Finally, we evaluated the influence of several landscape features on pine marten gene flow within a regional ecological network (Basque country, Spain) through individual-based least-cost distance GIS analysis. Our results confirmed the validity of the designed corridors and the need to keep taking them into account in land use planning. Besides, we identified areas of intensive agriculture and the major road networks as landscape features which limit pine marten gene flow. To the best of our knowledge, this is the first time that a GIS modeled ecological network has been evaluated through a landscape genetics approach.

Keywords: Phylogeography, landscape genetics; Non-invasive genetic sampling; *Martes martes*

RESUMEN

El estudio de los factores físicos y temporales que influyen en los procesos de estructuración poblacional resulta esencial para una adecuada gestión y conservación de las especies. El principal objetivo de esta tesis es identificar los procesos históricos y contemporáneos que han determinado la distribución actual de la variación genética de una especie eminentemente forestal como la marta europea (*Martes martes* L. 1758) a través de dos disciplinas: la filogeografía (i.e. la combinación de la filogenia y la biogeografía) y la genética del paisaje (i.e. la combinación de la genética de poblaciones y la ecología del paisaje). En primer lugar, esta tesis hace una revisión exhaustiva de los diferentes estudios llevados a cabo en las 8 especies del género *Martes* en lo concerniente a las filogenias intra-específicas, la genética de poblaciones y la genética del paisaje; discute los patrones generales detectados en las diferentes especies en relación a los hábitats considerados de especial relevancia para la conectividad; e identifica las principales lagunas de conocimiento en lo que respecta a la comprensión de los patrones dispersivos y la sub-estructuración poblacional. En segundo lugar, investigamos el patrón filogeográfico de la marta europea aportando nuevas evidencias sobre la existencia de refugios crípticos más allá de las Penínsulas mediterráneas; sobre los procesos de recolonización post-glacial de Europa así como acerca de las relaciones genéticas entre *M. martes* y *M. zibellina*. En tercer lugar, la aplicación de técnicas moleculares en muestras no–invasivas (i.e la obtención de material genético sin necesidad de capturar o interferir con la especie objetivo) en combinación con métodos de genética del paisaje nos permitió el estudio de una especie rara y elusiva con el objetivo de comprender los factores ambientales y territoriales que influyen sobre su distribución y sobre la estructuración de sus poblaciones. Para ello, se desarrolló inicialmente un método genético no-invasivo que permitió determinar la distribución de las especies simpáticas del género *Martes* (*M. martes* y *M. foina*) a partir del ADN extraído de muestras fecales y, posteriormente, investigar mediante el uso de marcadores microsatélites la estructuración genética espacial de la marta en un ambiente fragmentado. Los modelos Bayesianos espaciales y no-espaciales nos permitieron detectar diferentes poblaciones cuyas discontinuidades genéticas aparecen asociadas a transformaciones paisajísticas de carácter antropogénico (i.e. áreas urbanas, infraestructuras viarias, y procesos de fragmentación forestal). Por último, evaluamos la influencia de diferentes variables paisajísticas sobre el flujo génico de la marta en el marco de una red ecológica regional (País Vasco, España) mediante la utilización de distancias de mínimo-coste y análisis GIS. Nuestros resultados confirmaron la validez de los corredores diseñados y por tanto, la necesidad de seguir considerándolos como herramientas útiles en la planificación territorial. Además, identificamos que

los elementos paisajísticos que limitan mayoritariamente el flujo génico de la marta son las áreas de agricultura intensiva, las principales infraestructuras viarias así como las zonas urbanizadas. Este trabajo es pionero en la evaluación a través de un estudio basado en genética del pasaje, de una red ecológica modelizada mediante GIS.

Palabras clave: Filogeografía, genética del paisaje, técnicas moleculares no-invasivas, *Martes martes*

LABURPENA

Espezieen kontserbazio eta kudeaketa egokirako, populazioen egituran eragiten duten eragile fisiko eta denborazkoak ikertzea ezinbestekoa da. Tesi honen helburu nagusia, basoko espeziea den lepahoriaren (*Martes martes* L. 1758) gaur egungo aniztasun genetikoaren banaketan eragin duten prozesu historiko eta garaikideak identifikatzea da bi diziplina ezberdinen bidez: filogeografia (i.e. filogenia eta biogeografiaren konbinazioa) eta paisaiaren genetika (i.e. populazioen genetika eta paisaiaren ekologiaren konbinazioa). Lehenik eta behin, tesi honek *Martes* generoko 8 espezieen filogenia intraespezifikoa, populazioen genetika eta paisaiaren genetika arloetan gauzatu diren ikerketak berrikusi; espezie ezberdinetan konektibitaterako garrantzia berezia duten habitatetan antzeman diren patroi orokorrak eztabaidatu eta sakabanatze patroi eta populazioen barne egiturei dagokien ulermen hutsune nagusiak identifikatzen ditu. Bestalde, lepahori europarraren patroi filogeografikoa ikertuz, mediterranear Penintsulakoez gain babesleku kriptikoen existentziaren ebidentziak, Europako glaziazio ondorengo birkolonizazio prozesuak zein *M. martes* eta *M. zibellina* arteko erlazio genetikoei buruzko ekarpenak egin ditugu. Teknika molekular ez inbaditzaileen (i.e. material genetikoa eskuratu espeziea harrapatu edo oztopatu gabe) eta paisaiaren genetika metodoen konbinazioaren erabilera, espezie arraro eta sahieskorren ikerketa egokiak burutzeko eraginkorra da. Teknika hauek, populazioen egituraketan eragiten duten ingurune eta lurralde eragileak ulertzea ahalbidetzen dute. Ondorioz, helburu hau lortzeko lehenik *Martes* generoko (*M. martes* eta *M. foina*) espezie sinpatrikoen banaketa zehazten duen metodo genetiko ez inbaditzailea garatu zen. Ondoren, mikrosatelite markatzaileen bidez, lepahoriak ingurune zatikatuan duen egitura genetiko espaziala ikertu zen. Modelo Bayesiano espazial eta ez espazialek, populazio desberdinetan etenaldi genetikoa izaera antropogenikoa duen paisaiaren eraldaketarekin (i.e. bide azpiegiturak eta basoko zatiketa prozesuak) loturik dagoela antzematea ahalbidetu dute. Azkenik, eskualde mailako sare ekologiko batean (Euskal Autonomia Erkidegoa, Espainia) eta distantzia kostu minimoak eta SIG azterketa erabiliz, lepahoriaren fluxu genikoan aldagai paisajistiko ezberdinek duten eragina aztertu genuen. Gure emaitzek, diseinatutako korridoreen balioa berretsi eta lurralde planifikazioetan tresna baliagarri moduan aintzakotzat hartzen jarraitzea beharrezkoa dela baieztatzen dute. Horretaz gain, nekazaritza intentsiboa, bide azpiegitura nagusiak eta gune urbanizatuak, lepahoriaren fluxu genikoa mugatzen duten elementu paisajistiko nagusiak direla identifikatu genuen. Lan hau, paisaiaren genetikan oinarritutako ikerketa baten bidez SIG bitartez modelizatutako sare ekologiko baten azterketan aitzindaria da.

Gako-Hitzak: Filogeografia, paisaiaren genetika, teknika molekular ez inbaditzaileak, *Martes martes*

LIST OF PAPERS

This thesis is based on the following papers which will be referred to by their Roman numerals:

I Schwartz M, **Ruiz-González A**, Pertoldi C, Masuda, R. *Martes* Conservation Genetics: Using Molecular Genetics to Assess Within Species Movements, Barriers and Corridors. In: Biology and Conservation of Marten, Sables, and Fisher: a new synthesis. Aubry et al. Eds. Cornell University Press. *Submitted Manuscript* *

II **Ruiz-González A**, Madeira MJ, Randi E, Abramov A, Gómez Moliner BJ. New insights into the cryptic northern glacial refugia: Phylogeography of the forest dwelling European pine marten (*Martes martes*). *Manuscript*

III **Ruiz-González A**, Rubines J, Berdión O, Gómez-Moliner BJ (2008) A non-invasive genetic method to identify the sympatric mustelids pine marten (*Martes martes*) and stone marten (*Martes foina*): preliminary distribution survey on the northern Iberian Peninsula. *European Journal of Wildlife Research*, 54 (2): 253-261

IIIa Rosellini S, Osorio E, **Ruiz-González A**, Piñeiro A, Barja I (2008) Monitoring the small-scale distribution of sympatric European pine martens (*Martes martes*) and stone martens (*Martes foina*): a multievidence approach using faecal DNA analysis and camera-traps. *Wildlife Research*, 35: 434–440.*

IIIb Balestrieri A, Remonti L, **Ruiz-González A**, Gómez-Moliner BJ, Vergara M, Prigioni C (2010) Range expansion of the pine marten (*Martes martes*) in an agricultural landscape matrix (NWItaly). *Mammalian Biology*, 75: 412–419*

IIIc Balestrieri A, Remonti L, **Ruiz-González A**, Capelli E, Vergara M, Gómez-Moliner BJ, Prigioni C. Food habits of genetically identified pine marten (*Martes martes*) expanding in agricultural lowlands (NW Italy). *Acta Theriologica*. In press*

IV **Ruiz-González A**, Madeira MJ, Randi E, Gómez-Moliner BJ. Reliable faecal DNA genotyping of sympatric marten species (*Martes martes* and *Martes foina*): The impact of sample collector field experience on species and individual identification success rates. *Manuscript*

V **Ruiz-González A**, Madeira MJ, Randi E, Gómez-Moliner BJ. Non-invasive landscape genetics of the European pine marten (*Martes martes*): assessing spatial genetic structure and distribution in a heterogeneous landscape. *Manuscript*

VI **Ruiz-González A**, Gurrutxaga M, Madeira MJ, Randi E, Gómez-Moliner BJ. Landscape genetics as a tool for the empirical assessment of a regional ecological network: The European pine marten (*Martes martes*) as a target-species. *Manuscript*

***** The introduction of this thesis is based on paper I. Papers IIIa, IIIb and IIIc are derived contributions from the application of the developed species identification method (Paper III) and are included as Appendix.

PREFACE

I subdivide this thesis into six main papers and three additional ones, which address different aspects of the European pine marten (*Martes martes*) concerning the wide research topics of phylogeography, landscape genetics and non-invasive genetics.

The general framework of this thesis is given by the introduction (Paper I), where the intraspecific phylogenetic, population genetic, and landscape genetic studies conducted on *Martes* populations are reviewed; discussed commonalities found across the species in terms of habitats deemed important for connectivity; and identified knowledge gaps for understanding movement and substructure of the 8 species of the genus *Martes*.

In chapter 2 (Phylogeography), we investigate the phylogeographic patterns of the pine marten throughout the current species' European distribution. In this chapter we provide new insights into the cryptic northern glacial refugia and postglacial recolonization of Europe as well as into genetic relationships between *M. martes* and *M. zibellina* (Paper II). We found a complex phylogeographic history for *M. martes* indicating a mixed pattern of recolonization of northern Europe from both Mediterranean and non-Mediterranean refugia. Each of the inferred phylogroups showed a clear correlation to specific biogeographic regions which could probably represent different ecotypes.

Later, we address different aspects of analytical advances with non-invasive genetics for species (Chapter 3) and individual identification (Chapter 4) of sympatric martens (*M. martes* and *M. foina*). In paper III, we describe a reliable non-invasive Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) method for distinguishing between *M. martes* and *M. foina* based on DNA extracted from faeces samples. The efficiency of this technique was evaluated through a preliminary field study across the potential sympatric distribution areas of both marten species in the northern Iberian Peninsula. Moreover, this method has been used to: i) monitor the small-scale distribution of sympatric martens in combination with camera-traps (Paper IIIa); to asses the range expansion of the pine marten in an agricultural landscape matrix (NW-Italy) (Paper IIIb); and to efficiently investigate pine marten food habits (Paper IIIc).

Thereafter, we describe a reliable multiplex panel for faecal DNA genotyping of sympatric marten species (*M. martes* and *M. foina*) (Paper IV). The application of this panel comprising 15 microsatellite markers

facilitated species distinction discarding the presence of putative hybrids between both species. Moreover, we asses the impact of sample collectors field experience on species and individual identification success rates.

These two non-invasive researches (Paper III and Paper IV) set the frame for the following papers, treating the landscape genetic approaches of pine marten in order to understand how landscape and environmental features influence population genetic structure (Chapter 5). In paper V, we analyze the distribution patterns of sympatric martens (*M. martes* and *M. foina*) and the spatial genetic structure of European pine marten on a heterogeneous landscape through non-invasive genetic sampling and Bayesian analyses. Spatial and non-spatial Bayesian models allow us to detect several populations with genetic discontinuities being associated to human induced landscape transformations (i.e. roads, forest fragmentation).

In the final paper (paper VI), we focus on individual-based analyses predicting the genetic differences among all pairs of individuals based on the least cost distances between them as functions of multiple landscape resistance hypotheses: i) the resistance map which was drawn up in the design of the regional Ecological Network in the Basque Country (North Spain) and ii) different binary resistance maps which covered a gradient from greater to lesser preference of the focal species in relation to forest environments.

For each of these papers we provide an abstract and introduction, describe methods and present and discuss the results. The concluding remarks chapter (Chapter 5) summarises the major findings of the previous chapters and points out consequences of these findings for pine marten conservation and management.

AIMS OF THE THESIS

The main objective of this thesis is to apply different molecular tools to study the phylogeography, genetic variability, distribution and spatial genetic structure of the European pine marten (*Martes martes*) in order to identify ancient and contemporary processes shaping genetic variation. More specifically, the aims have been:

1. Review the intraspecific phylogenetic, population genetic, and landscape genetic studies conducted worldwide on the 8 recognized *Martes* species.

2. Use a phylogeographic approach in order to assess the impact of Quaternary glaciations on the current genetic structure of the European pine marten and to identify location of refugia as well as post-glacial recolonization routes after the Last Glacial Maximum.

3. Develop a reliable non-invasive Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) method for distinguishing between *M. martes* and *M. foina* based on mtDNA extracted from faecal samples.

> **3.1** Assess the field suitability of the PCR-RFLP technique, through a preliminary study across the potential distribution area of both marten species in the northern Iberian Peninsula.
>
> **3.2** Apply this protocol for improving our knowledge of different bio-ecological traits of sympatric marten species.

4. Develop a reliable and accurate multiplex panel of 15 microsatellites for individual identification of *M. martes* and *M. foina* from non-invasively collected faecal samples.

> **4.1** Verify possible hybridization events between both marten species in sympatric areas.
>
> **4.2** Assess the impact of sample collector field experience on species and individual identification success rates.

5. Develop a non-invasive landscape genetic approach in order to understand how landscape and environmental features influence population genetic structure.

5.1 Infer the spatial genetic structure of European pine marten using 15 highly variable genetic markers and spatial and non- spatial Bayesian clustering analyses (GENELAND and STRUCTURE).

5.2 Develop an individual-based non-invasive landscape genetic approach in order to evaluate alternative resistance maps relating landscape structure to gene flow. The resistance maps tested were: i) different binary resistance maps which covered a gradient from greater to lesser preference of the focal species in relation to forest environments and ii) The resistance map used to design the regional ecological network of the Basque Country (North Spain).

CHAPTER

1

INTRODUCTION

PAPER I

Martes CONSERVATION GENETICS: USING MOLECULAR GENETICS TO ASSESS WITHIN SPECIES MOVEMENTS BARRIERS AND CORRIDORS

PAPER I

Martes CONSERVATION GENETICS: USING MOLECULAR GENETICS TO ASSESS WITHIN SPECIES MOVEMENTS BARRIERS AND CORRIDORS

ABSTRACT

Understanding the physical and temporal factors that structure *Martes* populations is essential to the conservation and management of the 8 recognized *Martes* species. Recently, advances in three distinct subdisciplines in molecular ecology have provided insight into historical and contemporary environmental factors that have created population substructure and influenced movement patterns of several of the *Martes* species. Intraspecific phylogenetics has allowed us to understand the role of large scale historical events such as the last glacial maxima and their associated refugia on *Martes* populations in Europe, Asia and North America in at least five species (*M. americana*, *M. martes*, *M. melapus*, *M. pennanti*, *M. ziberllina*). In addition, population genetics has examined how connected *Martes* populations are within species across space, and in some cases how this level of connectivity has changed over recent time by examining historical samples in multiple populations. These studies have been conducted on *M. americana*, *M. martes*, and *M. pennanti*. More recently several landscape genetic analyses, including graph theoretic and least cost path approaches, have been used to evaluate the correlation between landscape features and genetic relatedness among individuals (within a species) in a landscape. These new approaches are showing promising results in empirically evaluating multiple habitat features at multiple scales that foster or hinder connectivity. Different forms of this approach have been applied on *M. americana*, *M. martes*, and *M. pennanti* in portions of their range. This paper reviews the intraspecific phylogenetic, population genetic, and landscape genetic studies conducted on *Martes* populations; discusses commonalities found across the species in terms of habitats deemed important for connectivity; and identifies knowledge gaps for understanding movement and substructure of the 8 *Martes* species.

INTRODUCTION

Understanding the biotic and abiotic forces that influence the movement of wildlife has been a central focus of wildlife management for nearly a century. This topic has come to the forefront of wildlife biology in recent years as the perils of habitat fragmentation and climate change are becoming clearer and more pronounced. In many areas lands that were once abundant and connected are now becoming small, degraded or completely isolated. In fact, we can consider these habitat changes on a gradient from changes that completely eliminate movement potentials of animals to those changes that marginally limit the probability of a successful dispersal of an individual. For example, urbanization of a once forested area may act as a complete barrier to movement, isolating populations, whereas various forest thinning treatments may remove the necessary cover for mid-sized and small carnivores to disperse, thus exposing them to predation risks that they did not historically face in unmanaged forests.

Some habitat changes are tied to natural cycles occurring on a temporal scale of centuries or millennia, while others are either functions of short term natural changes or are anthropogenically linked and occur on time scales of one or several generations. Understanding if the contemporary distribution and substructure patterns of animals are a function of long-term influences, such as the last glacial maximum, or more recent landscape uses is critical for managing and conserving wildlife, as this will allow us to infer if putative barriers are historic and out of management control or contemporary and possibly human induced. From these understandings, we will also be able to infer if our management actions, such as corridor protection or habitat improvement will likely increase animal movement and gene flow across their range.

Fortunately, recent advances in molecular ecology are now providing tools that can examine connectivity, and its converse genetic substructure, at multiple time scales. Typically intraspecific phylogenetic methods that examine sequence data (Avise *et al.* 1987) have been used for evaluating events that occur over longer time scales, and population genetic methods that use highly variable nuclear DNA markers have been used for evaluating contemporary patterns. One of the most exciting recent contributions is the use landscape genetic (Manel *et al.* 2003) data, which combines landscape ecology approaches and population genetics data, to disentangle the roles of historical and contemporary forces in structuring existing populations. For example, in recent work on California mule deer (*Odocoileus hemionus*) Pease *et al.* (2009) used landscape genetic analyses in conjunction with environmental niche modeling (ENM) to discern that clustering of California mule deer are largely a function of current ecological factors (topography, temperature, precipitation, seasonality, and plant community) and not from historical factors present since the last

glacial maximum. Armed with this understanding managers can now try to change the environmental and habitat factors that are limiting deer distributions and movement.

In this review, we first provide a basic primer on molecular ecology methods and tools. Subsequently, we discuss recent finding on movement and substructure based on intra-specific phylogenetic information. We do not cover newer interspecific phylogenetic information that provides understandings of the evolutionary history among *Martes* species, as this is well covered in chapter X (Koepfli *et al.* 2008, Koepfli *et al.* This Volume). Next, we review the published population genetic studies from three *Martes* species (*M. americana*, *M. martes*, and *M. pennanti*; Table 1) in various parts of each species geographic distribution. Lastly, we summarize the landscape genetics literature on 3 *Martes* species (*M. americana, M. martes,* and *M. pennanti*). We also discuss the latest methods developed in this field, provide a critique of where we think the major advances will occur (e.g. the use of more complex landscape hypotheses, and more advanced molecular markers and models) which will lead to better understandings on how to manage *Martes* species in light of climate change.

	Intraspecific Phylogenetic	Population Genetic	Landscape Genetic
Martes americana	Carr and Hicks 1997; Stone and Cook 2002, Stone et al. 2002; Slauson et al. 2008, Dawson, 2010	Broquet 2006, Kyle 2000, Kyle and Strobeck 2003, McGowan 1999, Small 2003, Swanson 2006, 2007, Williams 2007	Broquet 2006, Wasserman 2008
Martes flavigula			
Martes foina			
Martes gwatkinsi			
Martes martes	Davison 2001, Pertoldi 2008, Ruiz-González et al. 2009	Kyle 2003, Mergey 2007, Pertoldi 2008	Mergey 2007
Martes melampus	Hosoda et al. 1999, Kurose et al. 1999, Murakami et al. 2004, Sato et al. 2009, Inoue et al. 2010		
Martes pennanti	Williams et al. 2000, Drew 2003, Vinkey 2006, Schwartz 2007, Knaus et al. In Review.	Kyle 2001, Carr 2007, Rella-Hapeman 2006, Schwartz unpublished, Wisely 2004, Williams 1999, Williams 2000	Carr 2007, Garroway 2008,
Martes zibellina	Hosoda et al. 1999, Kurose et al. 1999, Murakami et al. 2004, Malyarchuk et al. 2010, Inoue et al. 2010	Petrovskaya 2007	

Table 1. A list of interaspecific phylogenetic, population genetic or landscape genetic studies conducted on *Martes* populations, many reviewed in this mansucript. There has been no studies conducted to date on *M. flavigula*, *M. foina*, or *M. gwatkinsi*, and no detailed population or landscape genetic studies conducted on *M. melampus* or *M. zibellina*. The table is color coded to denote areas where there has been ample work (green), some work conducted in limited geographic areas (yellow), and no work conducted (red).

THE ROLE OF CONSERVATION GENETICS

Genetic Diversity and Inbreeding

During the last two decades, the role of genetics in conservation biology, and in ecology in general, has been greatly emphasized (see for reviews: Frankham 1995, 2005, Allendorf and Luikart 2007, Pertoldi *et al.* 2007). The conservation genetics field has historically been subject to fierce criticisms from population ecologists arguing that genetic variation is not really relevant when a population faces extinction due to demographic problems. Understanding the consequences of demographic stochasticity in populations

requires detailed knowledge of local fluctuations in population size, extinction probability, colonization potential as well as reproductive success, which can be gained from population dynamics analyses. The difficulties associated with the collection of the demographic data necessary for such analyses make them extremely time consuming. Environmental factors and their changes are mirrored in the genetic composition of affected populations. Therefore, as DNA analyses are increasingly used to estimate and infer the causes of spatio-temporal dynamics, conservation geneticists and ecologists are reconciling. Even small alterations of environmental conditions can affect the genetic composition of the populations, both via demographic and selective responses. In many cases, changes in the genetic composition of populations can be more readily detected than the concomitant demographic changes (Tallmon *et al.* 2010).

Measuring the health of a population is an extremely difficult task, especially in elusive and/or nocturnal species like most of the *Martes* species are, there is therefore a need for a unit of measurement, which can help to quantify, not only in a qualitative way, the *status* of endangerment of a given population, species or group of species. Help comes from conservation genetics, which is likely to play a key role in developing a strategy for both the short and long-term preservation of genetic diversity. The assessment of genetic diversity in endangered animal is now pervasive. This phenomenon is due to acquisitions of powerful methods for DNA analyses, which are being increasingly used to infer the causes of the spatio-temporal dynamics of the populations as well as to estimate the extent of genetic diversity within populations and the organization of genetic diversity between populations (Allendorf and Luikart 2007).

Genetic diversity can be defined in several ways. First genetic diversity can be quantified by molecular genetic methods and be expressed as proportion of polymorphic loci, proportion of heterozygous *loci* and number of alleles in these *loci* and the allelic frequencies. An implicit assumption often made in conservation genetics is a causal relationship between genetic variability and the evolutionary precariousness of a species, both in the short-term and in the long-term (Ouborg *et al.* 2010a,b). Expected heterozygosity (H_E) can provide an indication of the immediate evolutionary potential of the population, although this measure has no deterministic relationship to its future value. Given an initial pool of unrelated founder genes, the potential changes and losses of genetic diversity can be assessed by the increase in relatedness. The initial genetic variability of a population is reduced as relatedness between individuals increases. The decline is proportional to the reduction in heterozygosity or increase of average inbreeding coefficient *(F)* of the parents, caused by inbreeding and genetic drift. The degree of inbreeding within an isolated population can be quantified by a genetic parameter called F_{IS} which vary from -1 to 1 with 0 representing non-inbred population in Hardy Weinberg equilibrium (HWE). F_{IS} equal to 1 is caused when observed heterozygosity (H_O) is smaller than H_E which is the level of heterozygosity that

would be observed if the population is in HWE. Inbreeding depression (the reduction in a fitness trait due to inbreeding) may arise due to mating between close relatives that increases homozygosity, and is most commonly observed in species that normally outcross.

Contrary, increased genetic divergence between the parents may be an advantage increasing heterozygosity of the offspring, which could cause a "heterosis" effect, or "hybrid vigor effect" but only until the genetic distance between the parents reaches a limit. Beyond this limit the divergence and differences in co-adaptation between the parents may reduce fitness in the offspring due to outbreeding depression (Tallmon *et al.* 2005). Therefore, genetic divergence between the parents of an individual can be viewed as a *continuum* with varying fitness consequences for the offspring. Inbreeding is of major interest in conservation genetics, because its increase in small populations is believed to influence the probability of extinction, both in the short-term because of inbreeding depression and the long-term because of loss of variability. Reports on populations which have suffered severe bottlenecks, but nevertheless prosper currently, challenge the assumption that inbreeding depression is a severe threat for the survival of populations. Evidently, this ambiguity could also arise from the difficulties of detecting inbreeding depression effects under natural conditions, from the fact that we are unable to monitor the fate of inbred populations on evolutionary time-scales, or due to the fact that inbreeding depression is only expressed under stressful environmental conditions.

An additional form of genetic variation is quantitative genetic variation, which is correlated to the adaptive potential, or the capacity of a population to adapt in an evolutionary way altering the allelic frequency in their genetic pool. Conservation geneticists try to estimate the quantitative genetic variation by means of neutral markers but the correlation between neutral and quantitative variation is weak and therefore divergence among populations at neutral *loci* is potentially uninformative, as it cannot exclude local adaptations (Lynch 1996).

The impact of habitat fragmentation and connectivity on genetic diversity

Several factors are responsible for population decline; however the principal factors are the anthropogenic ones, such as land development and urbanization that are the primary causes of extinction. These primary anthropogenic factors have ramifying ecological and genetic effects, which contribute to extinction risk. The role of ecological factors, such as metapopulation dynamics, are of fundamental importance for the persistence of populations, bearing in mind that land development causes habitat fragmentation and isolation of small populations, and intensification of metapopulation dynamics. Habitat fragmentation is a major factor limiting the distribution of species in man-altered landscapes and it has always been

considered to have deleterious consequences for the organisms and may be perceived differently by different species, depending on their ability to overcome introduced barriers. For some species, roads have been shown to act as a considerable barrier to dispersal, especially in *Martes* species where the main impact of roads consists in increased disturbance or mortality which can reduce local populations' size and may lead to local extinction. For example, American marten tend to avoid roads (Robitaille and Aubry 2000) and are sensitive to habitat fragmentation. Forsey and Baggs (2001) suggested that tracts of treeless land may act as complete barriers to marten if greater than 5 km in length dispersal (Gibilisco 1994; See also Thompson and Fryxell This Volume).

Many examples exist of species and populations in danger of going extinct or losing significant proportions of their genetic variability due to a restriction of their habitat. Typical cases involve species which were previously abundant and distributed over large geographical areas but are now found in only a few, small isolated populations. Many members of the genus *Martes* and family Mustelidae have experienced such a fate and specific studies conducted on several species will be discussed below.

Generally, small fragmented populations are genetically depauperated. This loss of genetic variability has two potential consequences: (a) Low genetic variability can be a threat in the long-term for adapting and evolving under changing environmental conditions and in disturbed habitats and (b) Small fragmented and isolated populations can suffer from inbreeding depression due to increasing relatedness between individuals (i.e., inbreeding). One of the most common practical conservation strategies to offset concerns regarding inbreeding is to increase the level of connectivity, and thus gene-flow between populations as the combination of small population size and isolation may lower the fitness of a population because of reduced genetic diversity from drift and inbreeding. However, too high levels of gene flow can either reduce or impede the capacity of adaptation to a stressor or they can alternatively introduce essential new genes for future adaptation. In a population, the actual degree of adaptation is the effect of the dynamic interaction between the selective pressure acting on the population and gene flow. Gene flow among populations can be studied using an evolutionary time frame or as current gene flow.

Changes in gene flow can be estimated by comparing 'historical' estimates based on genetic differentiation (using F_{ST} or other related substructure measures which range from 0, or no genetic differentiation between populations, to 1, complete genetic differentiation or no gene-flow) to current estimates based on assignment tests which allows the detection of migrants.

Alternatively, evolutionary questions concerning the role of gene flow for long-term genetic diversity, population differentiation, species identity, and speciation, emphasize the evolutionary time scale and is

usually estimated with phylogenetic analysis of sequence data. One of the main goals of conservation genetics is the identification of evolutionary significant units (ESU) and the preservation of genetic diversity, which should allow the evolutionary processes of natural selection and adaptation to continue in the future. The identification of separate ESU currently requires significant divergence of allele frequencies at nuclear loci and reciprocal monophyly of mtDNA. However this definition of ESU is only based on neutral genetic markers, whereas a broader definition including non-neutral markers would be more appropriate. New statistical methods, which seek to identify the number of 'populations' in a group of samples and/or assign individuals to population of origin, are being widely applied (see for example Carr *et al.* 2007a).

Genetic Monitoring

Recently, there have been many efforts that use molecular markers to monitor wild populations of fish, wildlife and plants (Boulanger *et al.* 2004, Schwartz *et al.* 2007). These methods either use diagnostic molecular markers to infer individuals, population and species and monitor changes in estimated parameters such as abundance using traditional wildlife biology tools, or monitor changes in population genetic metrics (McComb *et al.* 2010). These approaches may be highly useful for the study of *Martes* given the secretive nature of these species. Deciding, the best strategy for monitoring will depend largely on the number of markers available for the species of interest and the power associated with various metrics. Tallmon *et al.* (2010) has recently shown that monitoring the population genetic metrics, such as effective population size, may be as powerful, or potentially more powerful, than monitoring change in abundance for detecting abundance and trends in simple populations.

One common population genetic metric to monitor is the effective population size (N_E). N_E has been considered as the most important and critical surrogate parameter to describe the status of small populations and is based on relatedness within and among individuals and populations. Populations with a N_E smaller than few hundred individuals are nearly independent of the strength of selection and are therefore governed by mutation-drift regimes. With small N_E genetic drift and inbreeding will affect both target traits and neutral alleles, while with large N_E selection will only affect target and linked genes. Hence, small N_E make the populations unable to adapt in an evolutionary way to an environmental change. Populations with large N_E have the potentiality to react to the selective pressures generated by environmental changes if their genetic variability is high enough and if the speed of the environmental changes is not to high (if the rate of adaptive evolution at least matches the rate of environmental change). Therefore, the N_E of a population can predict a population's capacity to survive in changing environment more reliably than the

census size and/or the amount of genetic variability, furthermore it determines the speed at which the genetic variability is lost (Schwartz *et al.* 1999, Luikart *et al.* 2010). The notion of N_E can therefore be viewed as a bridging point between ecology and genetics with, the ecological characteristics, including aspects of life history, social structure and population dynamics being put on the focus for determining N_E and the rate of loss of genetic variation.

To predict the long-term persistence of animal populations, accurate estimates of population size are also necessary, thus abundance is also a commonly monitored metric. Census methods based on direct counts may be inaccurate because individuals are difficult to detect. Furthermore, statistical tools to estimate population size based on trapping methods often depend on unrealistic assumptions. New molecular techniques for the analysis of faeces or hairs typed for diagnostic markers may be of help to count individuals in a population by determining the number of unique multilocus genotypes in the population (Luikart *et al.* 2010).

This possibility has created a relatively new discipline: non-invasive genetics which is a set of field, laboratory and analytical techniques that allow studying the biology of natural populations, without observing or capturing of individuals (Broquet *et al.* 2007, Long and Mackay *et al.* This Volume). Microsatellites are the molecular marker of choice, used to identify species and to detect individual genotypes from non-invasive samples (Broquet *et al.* 2007). However, low amplification rates and genotyping errors due to non-invasive DNA degradation, risk to generate false genotypes, which do not correspond to any extant individual (Bonin *et al.* 2004). To improve the genotyping success and reliability, it is preferable to amplify DNA fragments as short as possible. The use of single nucleotide polymorphisms (SNPs) requires only amplification of very short fragments and this makes them particularly attractive for non-invasive genetic monitoring projects (Seddon *et al.* 2005). Using SNPs requires potentially high-throughput to be compared with microsatellite markers, which makes them particularly promising. The main obstacles in the use of SNPs remains the difficulty of their identification in non-model organisms (Smith *et al.* 2004, Ryynänen *et al.* 2007) and their uncertain performance on non-invasive genetic samples of lower quality.

MOLECULAR MARKERS

Phylogenetics and DNA sequence data

Most accounts of intraspecific phylogenetics trace the roots of mitochondrial DNA (mtDNA) because of its properties such as maternal transmission, extensive intraspecific variation, general lack of interspecific variation, and usual absence of genetic recombination. Intraspecific studies conducted to date on *Martes* sp. thus involved analysis of mtDNA sequences mainly or exclusively (Table 2). mtDNA has a relatively fast rate of nucleotide divergence, well suited to examining events occurred over the last few million years.

Two mitochondrial molecular markers have been used mainly on intraspecific phylogenetic studies of *Martes* species: the control region (D-loop) and the cytochrome *b* (cyt *b).* The sequence length is usually short (300-500 bp). Several studies used both markers to obtain more resolution while others are complemented with RFLPs or with additional mtDNA markers such as the NADH dehydrogenase subunit 2 gene, several tRNA (Thr & Pro) or with internal spacer regions of the nuclear ribosomal DNA (rDNA) (see Table 2 for detailed information). Several studies focusing on the intraspecific phylogenetics have not detected clear structuring (Hosoda *et al.* 1999, 2000; Kurose *et al.* 1999, Davison *et al.* 2001), possibly due to insufficient information, because most of the studies used a small fragment of mtDNA (Cyt *b* or D-loop) which did not provide enough resolution to resolve intraspecific patterns. Appropriate efforts in the conservation of *Martes*, therefore, require that genetic diversity should be analyzed more comprehensively, with different markers and/or with sufficient DNA sequence length, so that conservation units can be defined in terms of geographical distribution of the obtained lineages. Each DNA sequence has its own genealogy and they may evolve at different rates. Furthermore, the various methods of analysis probe different aspects of the molecular and spatial history. Consequently, to reconstruct a species phylogeographic history one would ideally like to use a range of sequences (including nuclear, cytoplasmic, sex linked, autosomal, conserved, neutral, high and low mutation rate DNA fragments) and apply a suite of pertinent data analyses (Sato *et al.* 2009; Balloux 2010). New techniques are becoming quickly available to sequence the entire mitochondrial genome, which promise to provide a multitude of new data and insights to *Martes* intraspecific relationships (Knaus *et al.* In Review).

Neutral and non-neutral markers (Microsatellites, SNPS)

Microsatellites, which are regions of the genome with high mutation rates, have been widely used in conservation genetic investigations (Schwartz and Monfort 2008) and many software programs are available for analysis of these data (Excoffier and Heckel 2006). Microsatellites, which are considered

neutral genetic markers, have been very useful in estimating effective population size, abundance, gene flow, hybridization, and measuring genetic diversity in natural populations. However, focusing on quantitative traits or traits under selection, rather than neutral markers, may be a more direct avenue for a better understanding of the adaptive potential of populations and the consequences of inbreeding and outbreeding depression. Molecular markers like microsatellites cannot identify the likelihood of loss of genetic variance in traits of ecological significance, as the correlation between neutral molecular diversity and quantitative genetic variation is weak and becomes even weaker in expanding or declining populations. Recently there has been a surge of new molecular genetic tools that will allow researchers to examine markers under selection.

One of the most promising new molecular tools is the single nucleotide polymorphisms (SNPs). SNPs represent the most widespread source of sequence variation within genomes (Brumfield *et al.* 2003) and hold the potential to significantly expand the ability to survey both neutral variation as well as genes under selection in natural populations as they have the very favorable characteristic of being detectable in both non-expressed and expressed sequences. SNPs have only emerged recently as valuable genetic markers in conservation genetics as they are biallelic markers. However, for some applications microsatellites will still be the molecular marker of choice as SNPs are inherently less informative compared to the multiallelic microsatellites, thus less informative when used for individual identification, parentage analysis and population genetics.

Species	Intraspecific Phylogenetic Data	Molecular Marker (lenght bp)	References
Martes americana	Yes	Cyt b (441 bp) Cyt *b* (1140 bp); ald C (241 bp) Cyt *b* (441 bp) and complete Cyt *b* (1140 bp) in combination with RFLPs; 1428 bp Cyt- *b* (1140 bp), tRNA-Pro (25 bp) and D-loop (263 bp) mtDNA and 14 Microsatelites	Carr and Hicks, 1997 Stone and Cook, 2002 Stone *et al.* 2002 Slauson *et al.* 2008 Dawson, 2010
Martes flavigula	No	-	-
Martes foina	No	-	-
Martes gwatkinsii	No	-	-
Martes martes	Yes	D-loop (321bp) D-loop (350bp) Cyt *b*, tRNA-Thr , tRNA-Pro, complete D-loop and rDNA 12S (1608 bp)	Davison *et al.* 2001 Pertoldi *et al.* 2008 Ruiz-González *et al.* 2009
Martes melampus	Yes	Cyt *b* (402 bp)and RFLPs of rDNA Cyt *b* (1140 bp); Cyt b , tRNA-Thr, tRNA-Pro , D-loop (521-524 bp) Cyt *b*, D-loop, and NADH-2 (2814 bp) D-loop (535-537 bp)	Hosoda *et al.* 1999 Kurose *et al.* 1999 Murakami *et al.* 2004 Sato *et al.* 2009 Inoue *et al.* 2010
Martes pennanti	Yes	Isoenzymes D-loop (301 bp); D-loop (301 bp) & Cyt-b (428 bp)	Williams *et al.* 2000 Drew *et al.* 2003; Vinkey *et al.* 2006; Schwartz, 2007.
Martes zibellina	Yes – parts of range	Cyt *b* (402 bp)and RFLPs of rDNA Cyt *b* (1140bp); Cyt *b*, tRNA-Thr, tRNA-Pro , D-loop (521-524 bp) Cyt b (702 bp) D-loop (535-537 bp)	Hosoda *et al.* 1999 Kurose *et al.* 1999 Murakami *et al.* 2004 Malyarchuk *et al.* 2010 Inoue *et al.* 2010

Table 2. A list of interaspecific phylogenetic studies conducted on *Martes* species with the type of molecular marker used.

PHYLOGENETIC INFERENCE WITHIN SPECIES

With the development and more routine use of molecular methods, it is now possible to investigate geographical variation using different molecular markers, and to deduce phylogenetic inference within species. Intraspecific genetic variations applied within the framework of phylogeography provides further insight into the history of range expansions and contractions of many *Martes* species (e.g. Stone *et al.* 2002; Davison *et al.* 2001; Ruiz-González *et al.* 2009). DNA sequences have been crucial in identifying lines of descent both at the intra- and inter-population levels (e.g. Pertoldi *et al.* 2008b, Sato *et al.* 2009), identifying the genetic legacy of the species translocations (Drew *et al.* 2003, Vinkey *et al.* 2006, Schwartz 2007), elucidating taxonomic doubts or delineating conservation units (e.g. Stone *et al.* 2002, Slauson *et al.* 2008, Sato *et al.* 2009), reconstructing colonization histories (Davison *et al.* 2001, Ruiz-Gonzalez *et al.* 2009), and even exploring temporal variation with the use of ancient DNA (e.g. Pertoldi *et al.* 2001, Schwartz 2007, Pertoldi *et al.* 2008a). Molecular (and morphological) studies of the extant species, in combination with palaeoecology, may provide opportunities to test hypotheses related to the effect of fluctuations during Pleistocene ice ages on genetic diversity in extant populations. Thus, molecular genetics can describe intraspecific geographical structures by identifying genetic lineages, and, consequently, can reveal postglacial recolonization routes from the glacial maximum refugia, of which locations are known (Davison *et al.* 2001; Stone *et al.* 2002; Ruiz-González *et al.* 2009).

Research on intraspecific genetics of *Martes* species are limited and biased towards some species (see Table 1 and 2). While some robust research on American martens (*Martes americana*) (Stone *et al.* 2002; Slauson *et al.* 2008; Dawson 2010; Dawson and Cook This Volume), fishers (*Martes pennanti*) (Drew *et al.* 2003, Vinkey *et al.* 2006, Schwartz *et al.* 2007) and the Japanesse marten (*Marten melampus*) (Hosoda *et al.* 1999; Kurose *et al.* 1999; Murakami *et al.* 2004; Sato *et al.* 2009; Inoue *et al.* 2010) are available, other species, such as the European pine marten (*Martes martes*) (Davison *et al.* 2001; Pertoldi *et al.* 2008; Ruiz-González *et al.* 2009), and the sable (*Martes zibellina)* (Hosoda *et al.*, 1999; Kurose *et al.*,1999; Murakami *et al.* 2004; Inoue *et al.* 2010; Malyarchuk *et al.* 2010) are only partially or insufficiently studied (Table 1 and 2). The absence of intraspecific phylogenetics studies for the Stone Marten (*Martes foina*), the Nilgiri Marten (*Martes gwatkinsii*) and the Yellow-Throated Marten (*Martes flavigula*) is noticeable.

During the last decade, DNA sequence-based studies on the phylogeny, evolutionary history and taxonomy have begun to change our understanding about the genetic relationships between *Martes* species (Hosoda *et al.* 1997; Hosoda *et al.* 2000; Stone *et al.* 2002, Sato *et al.* 2003, Koepfli *et al.* 2008). These studies using both mitochondrial and nuclear DNA sequences highlight that some phylogenetic

relationships within the genera remain uncertain or unknown. Recently, a nearly complete generic-level phylogeny of the Mustelidae using ~12,000 bp of mitochondrial and nuclear DNA data obtained from 22 gene segments (Koepfli *et al.* 2008) was even unable to resolve some relationships betwen *Martes* species (See Koepfli This Volume). Consequently, in this framework of very closely related species, phylogenetic inference within species is a challenging endeavour. Furthermore, as the species boundaries are sometimes difficult to assess and can be questionable, data on intraspecific variation between closely related species is sometimes difficult to elucidate (Davison *et al.* 2001; Sato *et al* 2009). Therefore, more slowly evolving sequences are required in combination with mtDNA markers to investigate deeper phylogenetic history that could also elucidate hybridization processes between closely related species commonly occurring between many *Marte*s species. On the other hand, research on phylogenetic inference needs to include closely related species to evaluate both, intra- and inter –specific patterns. Below we go through our intraspecific phylogenetic understanding for each of the *Martes* species:

The Pine Marten (Martes martes)

While information about the intraspecific relationships of the pine marten is very limited, there are a few studies concerning its phylogeography (Davison *et al.* 2001, Pertoldi *et al.* 2008b; Ruiz-González *et al.* 2009). The phylogeography of *M. martes* has been investigated using a small fragment (321 bp) of the control region (Davison *et al.* 2001). This work suggests that the present-day *M. martes* in central and northern Europe is the result of colonization from one or several different refugia with subsequent intermixing of expanding populations. However this work gives no clear clues for glacial refuges location and the post-glacial recolonization of central Europe as it was based on a small fragment of DNA that gave poor resolution to resolve the phylogeography of the species. Moreover, a lack of samples from the main Mediterranean refuge areas (only 1 specimen from the Iberian Peninsula, 3 from Italy and 2 from Northern Balkans) limited the support of the recolonization hypothesis. Davison *et al.* (2001) reported evidence for historic introgression of *M. martes* with the sable (*M. zibellina*) in Fennoscandia, along with mtDNA and morphological evidence for introgression with American martens (*M. americana caurina*) in England later confirmed by microsatellite data (Kyle *et al.* 2003).

However, no samples of *M. martes* and *M. zibellina* from Russian populations were included to underline the processes for the presence of a divergent lineage in Fennoscandia. More recently, Ruiz-Gonzalez *et al.* (2009) investigated the unresolved questions posed in previous work (Davison *et al.* 2001), and re-examined the phylogeographyc pattern of the European pine marten throughout the current species' distribution. This study was more comprehensive in terms of number of specimens and length of mtDNA

sequences (1,600 bp). The sampling also covered a broader geographic range than the previous phylogeographic study, sampling individuals and populations from Scandinavia in the North, Russia in the East, and the Iberian Peninsula in the South-West. For clarifying the relationships between *M. martes* and the sable *M. zibellina* in Fennoscandia and Russia, sable samples were also included. Ruiz-Gonzalez *et al.* (2009) revealed the presence of 69 different haplotypes for *M. martes* and 11 for *M. zibellina*, which are split into two major assemblages: European-Mediterranean and Fennoscandian-Russian clades. The first clade, including all *M. martes* samples collected throughout its entire current European distribution, is subdivided into two groups joining haplotypes distributed in central-northern Europe and in the Mediterranean regions. Most interestingly, haplotypes in the Mediterranean clade apparently did not contribute to the postglacial recolonization of most of the Palaearctic range of the species. It seems that Central-Northern Europe was recolonized by a pine marten phylogroup that survived the last glaciations in an undetermined Central European refuge as it has been previously proposed by paleontological data (Sommer and Benecke, 2004). In addition to this complex recolonization of Europe, genetically differentiated populations of pine marten, distributed in Fennoscandia and Russia, are introgressed with mtDNA of *M. zibellina*. In conclusion, the study of Ruiz-Gonzalez *et al.* (2009) indicates a complex phylogeographic history for *M. martes,* a species sufficiently adaptable to survive, facing historical climate changes, both in southern and northern forest habitats.

In a more restrictive study in terms of spatial distribution, Pertoldi *et al.* (2008b) studied the genetic differentiation and changes over time in genetic variability of the pine marten in three isolated geographic regions from northern Europe: Jutland and Sealand (Denmark) and southern Scania (southernmost Sweden), by sequencing the hypervariable domain of the mtDNA D-loop (350 bp). Both recent and museum samples were analysed in order to evaluate any temporal loss of genetic variability. Pertoldi *et al.* (2008) found eight different haplotypes: two haplotypes were shared by individuals from all three regions, but unique haplotypes were also found in all localities. When comparing these data with previous haplotype analysis (Davison *et al.* 2001), they confirmed the presence of the two main distinct haplogroups of central and Northern Europe along this region, with the samples from southern Scania being well differentiated from central Sweden samples**.** The obtained genotypic data for Jutland and Sealand suggest a recent independent evolutionary history for the Danish pine marten.

The American Marten (Martes americana)

The intraspecific phylogeny of the American marten has been debated during several decades, with clear doubts about sub-specific and specific status. Traditionally, 8 subspecies of *M. americana* have been

described (Clark *et al.* 1987) and placed into two morphologically distinct groups, *americana* and *caurina* (Merriam 1890; Clark *et al.* 1987). Although several studies (e.g. Merriam 1890; Anderson 1970; Hall 1981; Clark *et al.* 1987; Carr & Hicks 1997, Stone and Cook 2002, Stone *et al.* 2002, Small *et al.* 2003, Dawson 2010) have corroborated the separation of *M. americana* into these two groups, the level of distinctiveness between them has been largely debated.

Before 1953, these two groups of martens were recognized as distinctive species in North America: *Martes americana* and *Martes caurina* (Merriam 1890). However, Wright (1953) noted that both species intergrade in British Columbia and Montana, and he proposed that they should be combined into one species. Since then, they have been synonymised under *Martes americana*, and the conclusion has been reflected by the current taxonomy (Wilson & Reeder 1993).

However, the dichotomy (*americana* and *caurina*) in North American marten has continued to be acknowledged. Preliminary molecular data corroborate the distinction of *caurina* and *americana* as two monophyletic mitochondrial clades (Carr & Hicks 1997; Stone *et al.* 2002). Carr & Hicks (1997) compared the divergence of the two groups with that of 4 Palearctic species of *Martes*. As the mtDNA divergence levels found between the *americana* and *caurina* clades were similar to those existing among the other four Palearctic species, Carr & Hicks (1997) concluded that *M. americana* and *M. caurina* should be recognized as distinct species. However, subsequent studies gave subspecific status to *caurina* and *americana* clades (Stone and Cook 2002; Stone *et al.* 2002).

These two lineages are largely allopatric. The *americana* clade is widespread from interior Alaska south to Montana and eastward to Newfoundland and New England (i.e. northwestern, north-central and northeastern North America). By contrast, the *caurina* clade occurs in western North America, extending from Admiralty Island in southeastern Alaska south to Oregon and Wyoming (Wright 1953; Hall 1981; Carr and Hicks 1997; Stone *et al.* 2002). Within the *americana* clade, little or no geographical structure was present among populations, while within *caurina*, several haplotypes were confined to single populations (Stone *et al.* 2002). Interbreeding between these two lineages where they come into contact (i.e. Montana and one island in southeastern Alaska) have been demonstrated by DNA studies, (Stone and Cook 2002; Stone *et al.* 2002). According to Stone *et al* (2002), these lineages appear to have diverged due to isolation in distinct southern glacial refugia (*caurina* populations isolated in the west and *americana* populations isolated in the east of the United States, respectively). They hypothesized that the individuals belonging to the *caurina* clade represent an early Holocene colonization northward along the coast as coastal ice receded at the end of the last glaciation, whereas *americana* populations represent a later

colonization from continental source populations that expanded through river corridors traversing the coastal mountains.

Later Small *et al.* (2003) explored the distinctive histories of *caurina* and *americana* populations using 14 nuclear microsatellite markers. Microsatellite studies corroborated the population structure patterns obtained by the DNA sequences of the mitochondrial cytochrome *b* gene fragment (Carr & Hicks 1997; Stone *et al.* 2002), the nuclear aldolase *C* gene fragment (Stone & Cook 2002) as well as earlier morphological works (Merriam 1890; Anderson 1970). Small *et al.* (2003) suggest that these lineages may well represent distinctive species and that further investigations of ecological, behavioural or physiological characteristics should be conducted to elucidate this question.

More recently Dawson (2010) and Dawson and Cook (This Volume) reviewed the previous molecular studies and developed a more detailed view about the genetic differentiation across the whole range of North American marten lineages. In this work this author addressed the question of how many species of marten are extant in North America. Mitochondrial DNA studies identified 2 monophyletic groups within North American *Martes* that corresponded to the 2 morpho-species described. Investigations using nuclear loci were also consistent with species-level differences in North American *Martes*. Based on a series of studies, they concluded that *M. americana* and *M. caurina* are valid species paralleling the original descriptions.

The subspecific status of several American marten populations from Oregon and California has been also investigated by Slauson *et al.* (2008). These authors investigated the subspecific identity of a rediscovered population of American martens within the range of a presumed extinct subspecies (*Martes americana humboldtensis*). They compared mtDNA (1,428 bp) sequence diversity of contemporary specimens within the described range of *M. a. humboldtensis*, close to ranges of *M. a. caurina* and *M. a. sierrae*, and a museum specimen of *M. a. humboldtensis*. The historical Humboldt marten museum sample shared one haplotype with martens from the rediscovered population, coming from coastal regions of Oregon and California. This result suggests that the rediscovered population represents descendants of a relictual population that previously existed in coastal California. They also concluded that subspecific boundary between *M. a. humboldtensis* and *M. a. caurina* may not be valid, because this haplotype was shared with coastal Oregon and coastal California current populations and no known contemporary or historical biogeographic barriers prevent north–south movement. Thus, marten populations currently located in coastal forests of California and Oregon should be managed collectively to preserve the connectivity that these results suggest. Moreover, M. *a. sierrae* differed substantially from both *M. a. humboldtensis* and *M.*

a. caurina, suggesting marten populations were not one large, genetically homogeneous population along the Pacific states and that the divergence may have occurred in separate glacial refugia.

The Fisher (Martes pennanti)

Intraspecific genetic research on the fishers (*Martes pennanti*) has been conducted to examine the genetic consequences of past and future translocations and examine the validity of morphologically based sub-species designations. Translocations to re-establish extirpated populations or to maintain declining ones have historically been carried out without genetic information on source or target populations or adequate consideration of the potential effects of mixing genetic stocks. The first research on this topic was limited to translocations within the eastern range of the fisher, and showed little genetic subdivision among populations (probably because of the low variability of the molecular marker use; Williams *et al.* 2000). Subsequently, Drew *et al.* (2003) considered the conservation status of *Martes pennanti* and evaluated the potential genetic consequences of past and future translocations by examining population variation of D-loop sequences. They sampled populations throughout the fisher range in North America including five populations unaffected by translocations and two western populations that had received long-distance translocations. Populations in Oregon, Montana, and Idaho received several translocations and, as a result, these three populations showed greater similarities to source populations than to adjacent ones. Additional sequences obtained from museum specimens collected prior to any translocation suggested historical gene flow among populations in British Columbia, Washington, Oregon, and California. This study concluded that anthropogenic impacts in that region have greatly reduced and isolated extant populations in Oregon and California. Therefore, British Columbia would be the most appropriate source population for future translocations to recover those of Washington and some localities in Oregon and California. This result was confirmed using the same molecular markers by Warheit (2004) as reported in Lewis and Hayes (2004). Recent work by Knaus *et al.* (In Review) has re-examined some of the Drew *et al.* (2003) results using complete mitochondrial genomes (~16,000 bp). The most striking result was that D-loop region sequences incorrectly identified full genome population structure. For instance, Drew *et al.* (2003) showed that both northern and southern California shared a common haplotype, suggesting gene flow, yet the full genome revealed that these geographic areas each had unique haplotypes, concordant with microsatellite data (Wisely *et al.* 2004) and consistent with long term isolation.

Until recently it was assumed that *Martes pennanti* specimens living in the Rocky Mountains all were descendants from reintroduced stocks. However, a recent study reported that mtDNA (428 bp of Cyt-*b* and 301 bp of D-loop) haplotypes found only in west-central Montana fishers were likely derived from a

relict population that escaped harvests conducted in the early 20th century (Vinkey *et al.* 2006). Schwartz (2007), using the same molecular markers as the study of Vinkey *et al.*(2006) compared fishers in west-central Montana with samples from north-central Idaho and found no differences between these groups. One museum specimen, collected in 1896 in north-central Idaho before any known translocation, had the same haplotype as the ''native Montana haplotype'' discovered in the recent study of Vinkey *et al.* (2006). Thus, fishers in north-central Idaho and west central Montana are the only confirmed native fishers in the Rocky Mountains, and one of a few populations in the West that have maintained native genetic characters. Fishers from Idaho and Montana are not all descendants of translocated individuals, but are also the descendants of fishers that persisted despite early 20th century trapping. Recent data by Knaus *et al.* (In Review) confirm these results.

The Japanese Marten (Martes melampus)

The Japanese marten is an endemic species to Japan, where it is distributed on the main Japanese islands, except Hokkaido Island: Honshu, Shikoku and Kyushu Islands (Masuda *et al.* 2009). By contrast, the sable (*Martes zibellina*) is present only in Hokkaido Island within Japan (Murakami *et al.* 2009). However, *M. melampus* was artificially introduced to Hokkaido from Honshu, and is currently expanding in southern Hokkaido, whereas the native *M. zibellina* is distributed in central and eastern Hokkaido. The contact zone between the two species is in central Hokkaido (Masuda *et al.* 2009; Murakami *et al.* 2009).

The Japanese marten has a complicated taxonomic history. Moreover, the presence of the closely related species, *Martes zibellina* (Hosoda *et al.*, 1997, 2000; Sato *et al.*, 2003, 2006; Koepfli *et al.*, 2008) makes correct intraspecific assignment more difficult. Several studies have focused on the genetic relationships within and between the Japanese marten, and *M. zibellina brachyura* (Temminck, 1844) (Hosoda *et al.* 1999; Kurose *et al.* 1999). Hosoda *et al.* (1999) used the restriction fragment length polymorphism (RFLP) of the nuclear ribosomal DNA (rDNA) spacer and the mitochondria1 cytochrome *b* (402bp) gene fragment sequences, and Kurose *et al.* (1999) sequenced the entire Cyt-*b* (1140 bp), to reveal the extent of intra- and inter-specific variation between the two species. Both studies showed high genetic differences between these two species. However, the clustering of haplotypes in phylogenetic trees did not correspond with the geographically expected relationships between populations of the different Japanese islands. Only the Tsunima island populations (*Martes melampus tsuensis*) showed some genetic differentiation from the other populations of *M. melampus*. The results suggested that mtDNA introgression between local populations of *M. melampus* might have resulted from the incomplete geographic isolation within each

island, and/or that *M. melampus* might have recently expanded to the Japanese islands during a short period.

Based on 521-524 bp fragments of mtDNA including D-loop, Murakami *et al.* (2004) further investigated these questions and indicated that the two *Martes* species in Hokkaido were closely related, in contrast to previous studies (Hosoda *et al.* 1999; Kurose *et al.* 1999) which indicated that *M.melampus* and *M. zibellina* were grouped in two different groups. However, only Hosoda *et al.* (1999) included one individual from Hokkaido were both species coexist. Murakami *et al.* (2004) revealed two distinct clusters, each containing both haplotypes from *M. zibellina* and *M. melampus* providing three possible explanations: past hybridization between both species might have occurred; the two species might have similar heteroplasmy of mtDNA; or these haplotypes might have come from the nuclear genome.

By contrast, Inoue *et al.* (2010) examined 535-537 bp of mtDNA D-loop for Japanese martens from native populations of Honshu and Kyushu and from introduced populations of Hokkaido, together with sables of Hokkaido and Russia, and showed high genetic differentiation between the two species. In addition, Inoue *et al.* (2010) reported that there were neither individuals showing *melampus*-type mtDNA haplotype together with *zibellina* morphology, nor those showing reciprocal characters in specimens from Hokkaido. These results indicated that no hybridization occurred between these two species on this island. The genetic diversity of the introduced populations of the Japanese martens in Hokkaido was lower than that found in the native populations of Honshu and Kyushu. This could be due to founder effects.

One of the most comprehensive studies regarding phylogenetic inference within *M. melampus* populations was recently published by Sato *et al.* (2009). The first studies focused on the molecular phylogeography of the Japanese marten did not detect different genetic units (Hosoda *et al.* 1999, 2000; Kurose *et al.* 1999), possibly due to insufficient information from the molecular markers used. Furthermore, the populations studied were poorly sampled. Previous works showed that genetic diversity of the Japanese marten should be analyzed more comprehensively, so that conservation units could be defined in terms of geographical population. Sato *et al.* (2009) conducted a more reliable research in terms of molecular markers (mtDNA and nDNA), sequence length and sampling, to overcome this lack of information. In this study, they performed molecular phylogenetic analyses of 49 specimens of Japanese martens collected from several areas in Japan and focused on three mtDNA loci (Cyt-*b*, D-loop, and the NADH subunit 2 gene) and one nuclear gene (the growth hormone receptor gene, including the polymorphic intron regions). Evaluating the phylogeny and the genetic variation estimated by mtDNA and nDNA sequences, Sato *et al.* (2009) identified nine intraspecific groups. The grouping was not correlated with winter coat color, but was consistent with geography of the Japanese islands. In particular, they obtained the monophyly of the

Tsushima martens, *M. m. tsuensis*, supporting the view that the Tsushima marten's long history of isolation on small islands is responsible for its genetic distinctiveness and uniformity, validating the Tsushima population as an evolutionarily significant unit.

The Sable (Martes zibellina)

The Sable shows substantial interpopulation variation of morphological characters and a multiplicity of local forms which make the elaboration of unified intraspecific taxonomy complicated (Monakhov, 1976; Pavlinov and Rossolimo, 1976). Moreover, the massive human introductions and re-introductions throughout most of its geographic range in Russia in the 20th century complicate this goal (Monakhov, 2001).

Several studies have been focused on the Hokkaido population, to investigate genetic relationship within and between the sable, which is classified as a separate subspecies *M. zibellina brachyura* (Temminck, 1844), and the closely related Japanese marten (*Martes melampus)*, which was introduced to this island (see above). However, the results are of limiting significance for the intraspecific relationship within *Martes zibellina* after taking into account the wide range of the species over most of Asia.

In the Russian populations, previous analysis of the population genetic polymorphism in sables pointed to the existence of intraspecific heterogeneity (Balmysheva and Solovenchuk, 1999a;b; Petrovskaya *et al.* 2007). Analysis of restriction polymorphism of the mtDNA Cyt-*b* in populations of sables from Siberia and Far East showed the prevalence of three different haplotypes, which probably represented three monophyletic lineages (Balmysheva and Solovenchuk, 1999a;b; Petrovskaya *et al.* 2007). To confirm these findings, fine-scale analysis of mtDNA variation at the level of nucleotide sequences was recently conducted by Malyarchuk *et al.* (2010). This study was focused on the analysis of phylogenetic relationships of the mtDNA Cyt-*b* sequences in 17 sables from Magadan oblast, Kamchatka, and Khabarovsk Krai, and also included previously published data of *Martes zibellina brachyura* from Hokkaido, Japan.

These authors identified two supported phylogenetic groups of sable mtDNA. The first group was predominantly represented by sables from different regions of Northeast Asia, including Kamchatka, Khabarovsk Krai, and the Magadan oblast together with samples from Hokkaido Island (Japan). The other group was composed by haplotypes of sables from Magadan oblast and Khabarovsk Krai, without haplotypes representing populations of Kamchatka or Hokkaido. Interestingly, a pine marten specimen from Sweden was clustered within this group, supporting previously reported evidence for historic

introgression of pine martens with sable (*M. zibellina*) in Fennoscandia (Davison *et al.* 2001, Ruiz-González *et al.* 2009). The high sequence divergence values obtained between both haplogroups imply that the ancestral gene pool of sables was once split into two parts, probably, as a result of glaciation. Later on, during the last deglaciation period, the two gene lineages were reunified in a new contact zone (Malyarchuk *et al.* 2010).

In the populations from Central Kamchatka, all sables examined belonged to the first clade, as well as all examined *Martes zibellina brachyuran* individuals from Hokkaido (n = 10). Hosoda *et al.* (1999) also identified one unique haplotype on Hokkaido which is closely related to one of the two identified haplotypes in Russia. They hypothesized that divergence between Hokkaido and the Russian Far East populations has been a recent process. However the limited number of specimens analyzed limits the support of this hypothesis. Hokkaido is thought to be a refugium for *Martes zibellina* during the last glacial age (Kurose *et al.* 1999). However, Kurose *et al.* (1999) hypothesised that populations of the sable could have expanded in Hokkaido recently. Otherwise, the repeated processes of expansion and reduction of their habitats through the glacial and interglacial ages would have impeded the fixation of haplotypes to local populations. Further studies are required to examine genetic variation within each local population by using more specimens from comprehensive areas including the continent and using more polymorphic DNA markers such as microsatellites. On the other hand, the observed spatial heterogeneity of the sable populations of Magadan oblast with individuals from both clades, could be explained by the introduction of Kamchatka and Khabarovsk sables, starting in the 1950s (Petrovskaya *et al.* 2007; Malyarchuk *et al.* 2010).

It seems likely that morphological differences between sables belonging to each haplogroup still have certain contributions to the contemporary phenotypic diversity of sable populations. This suggestion is supported by morphologic specific differences of Kamchatka sables, which are treated as a separate subspecies *M. z. kamtschadalica* (Pavlinov and Rossolimo, 1976, Anderson, 1970), and characterized by the presence of mtDNA haplotypes of the first group (Malyarchuk *et al.* 2010). It should be noted that haplotypes from both groups are in contact with sable inhabiting Khabarovsk Krai and the Magadan oblast and historically belonging to another subspecies, *M. z. jakutensis*.

Overall, a more reliable study in terms of spatial distribution and number of individuals is necessary to better understand the phylogenetic relationships within *Martes zibellina* populations over the wide range of the species.

Martes POPULATION GENETIC STUDIES

The phylogenetic studies represented above consistently show complex evolutionary history, markedly influenced by large scale events like glacial refugia and subsequent reunitions. These intraspecific phylogenetic studies set the stage for examining more fine scale temporal and spatial relationships among populations. To date there have been population genetic studies on three of the *Martes* species: the American marten, the pine marten and the fisher, many of them examining the impact of habitat fragmentation on the movement of populations within a species.

The pine marten is distributed throughout Europe and has been subject to long term decline in numbers in most regions (Mitchell-Jones *et al.* 1999). The pine marten is a habitat specialist confined to mature deciduous and coniferous forests (Domingo- Roura, 2002), has a limited dispersal ability compared with other mustelids (Kyle *et al.* 2000), and a slow reproduction rate, rendering it particularly vulnerable to habitat changes (Bright, 2000, Webster 2001).

The pine marten populations has been shown to have a higher level of genetic structure (with an overall F_{ST} value of 0.18, range: 0.016–0.330) and lower genetic variation (H_E range excluding the insular populations: 53.8%-63.8%) than their North American sibling species, the American marten (*Martes americana*), sampled throughout Canada (H_E Yukon: 69%; Kyle *et al.* 2003). Even if it is difficult to exclude more ancient processes such as the influence of glaciations as a cause of the differences observed between these two species, it is suggested that the greater level of persecution and habitat fragmentation experienced by the pine marten could be the reason (Kyle *et al.* 2003).

Despite the fact that the continental pine marten populations shows a relatively homogenous levels of genetic variation, with no significant differences among them (Kyle *et al.* 2003), the level of genetic differentiations between the populations appear to be correlated with the geographic distance between the populations. This is true even if the pairwise F_{ST} did not correlate so strongly with distance (r = 0.31, p = 0.11), as other measures of genetic differentiations such as Nei's genetic distance (D_S) and the genotype likelihood ratio (D_{LR}) have shown both highly significant correlations (D_S: r = 0.55, p= 0.007, D_{LR}: r = 0.91, p = 0.00006; Kyle *et al.* 2003).

These results may be related to the relative differences in the level and duration of anthropogenic disturbances in Europe and northern North America. These influences may have resulted in smaller, more isolated populations in Europe where the effects of genetic drift would lead to more genetic structure. Differences in N_E can also be the causes of the observed pattern as smaller N_E is translated in a quicker loss

of genetic variability. For these reasons, it is important to monitor the levels gene flow and of genetic variation between populations to help identify populations where conservation actions may be appropriate.

However, a cautionary approach must be undertaken when trying to interpret differences in the level of genetic structure or variability between species as these differences can also be attributed to other factors. Kyle *et al.* (2003) discussed the possibility that the structure observed in their study could also reflect a greater degree of philopatry in the pine marten compared with the American marten. Additionally they discussed the possibility that more ancient processes still influence the gene frequencies, such as post-glacial founder effects and historical introgression from sable (*Martes zibellina*) in Fennoscandia.

Sometimes, studies conducted on insular populations are more easy to interpret as often there is no "confounding" gene-flow occurring and the populations has typically been isolated for a well known period of time. This allows us to make more accurate predictions about the fate of genetic variability and substructuring. Among island populations of the two siblings species, the Scottish pine marten population revealed a similar level of structure and variation to the Newfoundland martens (*Martes americana atrata*) (H_E Scotland: 42.3%, H_E *Martes Americana atrata:* 44.6%), however Ireland (H_E Ireland: 34%), was more differentiated with less genetic variation (Kyle *et al.* 2003).

Abundance estimates of the Newfoundland martens (which seems to be genetically similar to mainland populations despite their biogeographic separation of nearly 10,000 years; Carr and Hicks 1997; McGowan *et al.* 1999) decreased from 630–875 animals in 1986 to only 300 animals in 1995 (Snyder 1986, Forsey *et al.* 1995). Such a drastic and rapid population decline has raised concern that inbreeding depression could affect the average fitness of this population (Forsey *et al.* 1995). The level of genetic variability of the English pine marten (H_E England: 66.1%), could not be used for comparisons as Kyle *et al.* (2003) provided further evidence for the possibility that a hybridization event between *Martes americana* and *Martes martes* has occurred (see phylogenetic section above).

M*artes americana caurina* is a distinct clade of American marten inhabiting the far western North America (Stone *et al.* 2002), when compared with *Martes americana americana*. In fact the genetic differences found between *Martes americana caurina* and *Martes americana americana* are at a level observed among other *Martes* species, which have lead some to consider this group two distinct species – *Martes caurina* and *Martes americana* (Carr and Hicks 1997, Stone *et al.* 2002, Small *et al.* 2003; Dawson 2010 and Dawson and Cook This Volume) (see phylogenetic section above). Small *et al.* (2003) have shown that northern insular populations of *M. caurina* have higher genetic differences among populations and lower within population genetic diversity compared to northern populations of *M. americana* likely caused by

the longer periods of isolation in coastal forests that were fragmented during the early Holocene period (Small *et al.* 2003). The lack of differences among *M. americana* populations has been attributed to either continued gene flow or a more recent expansion throughout the Pacific Northwest (Small *et al.* 2003)

The comparisons of genetic parameters between species can be quite misleading both because of the different genetic markers utilized but also because of the species different histories of postglacial recolonization and eventual introgression. Hence, a better approach, in order to understand the reasons for the observed genetic pattern seems to be to compare the degree of genetic variability and genetic structure within the same species but comparing populations living in different habitats. An example of such an approach, (using a generous sample size of individuals and markers), has been conducted by Kyle and Strobeck (2003) again on the American marten (1,262 individuals, genotyped at 11 microsatellite loci) which compared the level of genetic variability and of genetic differentiations between populations living in the unfragmented habitat of northern Canada with the genetic variability and structure of the populations living in the more southern Canadian regions which have a more fragmented habitat. As expected, and in agreement with previous studies, little genetic structure was observed in northern regions, where few barriers to marten dispersal are thought to exist. However, contrary to their expectations, no strong breaks in gene-flow were observed between any of the 35 sampled regions with the exception of the insular Newfoundland population. The lack of genetic structure observed may suggest very large N_E of the populations and that, at a larger scale, marten dispersal is not as limited by some landscape features as was previously thought. In Canadian populations of martens (with the exception of the insular Newfoundland population) the lack of genetic structure may be explained by large N_E and relatively continuous habitat, conditions that are not present for the European species.

Hence, despite the fact that life history traits of European pine martens more closely resemble those of the American marten, the demographic trends of this species seem to quite similar to the pattern observed for fisher (*Martes pennanti*) which often live in similar geographic areas as the American marten.

Kyle *et al.* (2001) which investigated the fisher populations sampled from across the Canadian provinces, revealed relatively high levels of genetic structuring (F_{ST} = 0.14; range: 0.028-0.261) compared to the F_{ST} found for the American marten (F_{ST} = 0.020) and a relatively high genetic variability (H_E = 62%) over short geographic distances. The level of structure in fishers could be a reflection of philopatry and the large demographic changes that affected most populations of this species in the early 1900's. Fishers were extirpated from much of their range as a result of anthropogenic influences (fur harvests and logging).

Therefore, mainland populations of European martens may be similarly structured to fishers where smaller N_E has potentially led to more genetic drift between populations. However the fisher showed relatively high levels of genetic variability, despite the recent history of populations decline. The level of genetic variability found for fisher (Kyle *et al.* 2001, Wisely *et al.* 2004) and successively by Carr *et al.* (2007a,b) (H_E range: 0.599–0.679) has been suggested to be due to the partitioning of genetic variation into multiple refugia during the period of population fragmentation, where the presence of multiple refugia has probably maintained relatively higher levels of genetic variation than predicted for a single source. Subsequently, contact among expanding reproductive fronts may have counteracted loss of genetic variation within any one cluster by increasing the gene-flow among clusters (Thompson 2000).

However, an examination of the distribution of genetic structure of fisher at different distances showed higher rates of gene flow than predicted under a strict isolation by distance model at small distances (40 km) within clusters and at larger distances up to 100 km among clusters. Such a pattern has been associated with expanding reproductive fronts and translocations events (of individuals from known sources: Berg 1982) as many of the patches where the fisher disappeared during the population decline have now been recolonized. It should also be mentioned that Wisely *et al.* (2004) found much lower values of H_E for fishers in the fragmented populations along the Pacific coast, ranging from(H_E range: 0.16-0.42) associated with relatively high estimates of F_{IS} values, suggesting inbreeding. Also this pattern of reduced heterozygosity appears to follow a north-south gradient, with fisher populations in the southern part of the Sierra Nevada population showing the lowest levels of genetic variation as compared to sister populations to the North (Wisely *et al.* 2004).

As previously mentioned the comparisons of genetic variability and genetic differentiations (among species or among populations within the same species) can be quite problematic when using different genetic markers in different species, but can be quite problematic also when comparing genetic differentiations among populations collected at different geographical scales. One way to overcome the latter problem is to perform a Mantel test, which consists in plotting the pairwise geographic and genetic distances between populations, and such an approach can also be used to illustrate the difference between the different species. This has been illustrated by Kyle and Strobeck (2003), who plotted a linear regression of the D_S and geographic distances for mainland populations of pine marten, fisher and American marten (Figure 1).

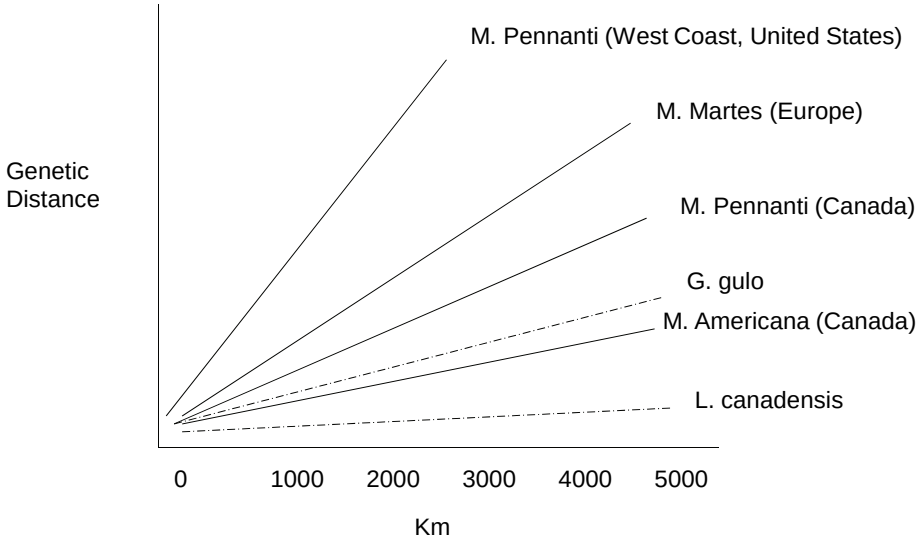

Figure 1. A schematic showing the approximate relationship between genetic distance and Euclidean distance for several *Martes* species. Dotted lines show the approximate relationship for other mid-sized carnivores and is presented as a reference.

The highest level of structure per unit distance (which is directly correlated with the degree of genetic sub-structuring) was found for the pine marten: 0.140/1,000 km, followed by fisher 0.092/1,000 km and American marten 0.057/1,000 km (Figure 1). The Mantel test is a rough test and nowadays there are more sophisticated techniques which have been developed by the emerging field of landscape genetics which allow the detection of subtle sub-structure and of barriers.

In addition to genetic substructure analyses, population genetic analyses have also provided insight into hybridization among *Martes* species. As previously mentioned microsatellite data has confirmed mtDNA data suggesting hybridization between *M. americana* (introduced) and *M. martes* (native) in England (Davison et al. 2001; Kyle *et al.* 2003), although there still remains several native *M. martes* populations in Great Britain that show no sign of genetic introgression. Hybridization has also been identified using microsatellite data between *M. caurina* and *M. americana* in two regions, the Kuiu Island in southeastern Alaska and in Montana, USA (Small *et al.* 2003, see above). To date no studies have examined the extent of the hybrid zone or the fitness of these hybrids relative to the parental populations.

Population genetic data has also been used to evaluate the success of reintroductions of *Martes*. Williams *et al.* (2000) show that older reintroductions of fisher show significant allele frequency differences from their source populations. While some of this difference may be due to initial sampling error, as typical reintroductions use relatively few individuals, the fact that recent introductions do not show significant allele frequency differences form source populations suggests that this is due to genetic drift. Drift can occur rapidly in small populations, especially with species that exhibit polygynous mating. Swanson *et al.* (2006) and Swanson and Kyle (2007) examined reintroduced populations of marten in Michigan, USA and found that the reintroduced populations had high levels of genetic variation. This may be due to the use of multiple source locations or the temporal separation of the reintroductions, which occurred over a 24 year period (Williams and Scribner 2007, Swanson and Kyle 2007).

Landscape Genetics

Population genetic data has been used to effectively delineate substructure, identify isolated populations, and define units of conservation. However, by definition these data rely on group or population statistics. This can be difficult when species appear to be continuously distributed across a landscape and groups are not readily apparent. In addition, while some elements of population genetics, such as measures of between population genetic distance, are inherently spatial, they do not specifically take the landscape into account. The field of landscape genetics is an extension of population genetics that uses either individual or population data, explicit spatial information, and associated covariates (i.e., elevation, forest type, distance to roads, etc) to make inference to environmental variables that influence species movement.

Landscape genetic approaches are relatively new, but since the term was coined in 2003 (Manel *et al.* 2003) there have been over 400 published papers that reference or use these methods. The most common landscape genetics approach used is to compare ecological distances among either individuals or populations to a matrix of genetic distances (or the inverse, genetic relatedness). These ecological distances can be distance among populations or individuals measured in stream distances, distance through forest cover, distances through riparian zones, distances across non-human habitation, distances across savanna or steppe, or any other environmental variable deemed important to the organism's life history, survival and ability to disperse. This approach becomes more complicated when the landscape is a mosaic of habitat patches, and there is not a continuous path within the ecological covariate of choice forcing populations or individuals to move through non-optimal habitats to interact. Here the standard landscape genetics approach has been to impose cost values on habitats of different quality and type, and conduct least cost-path modeling to derive a matrix of least cost paths among individuals or populations. Given

that a specific cost per habitat type is rarely known, often multiple models with different cost penalties are created. These multiple models are then evaluated by comparing the many matrices of least cost paths to the matrices of genetic distances. In more complex models, these resistance values can be an aggregation of costs imposed by multiple variables or can be evaluated using multiple matrix regression modeling, where each covariates influence on genetic relatedness can be evaluated (Balkenhol *et al.* 2009).

In addition to least cost path modeling, there have been several graph theoretic based approaches that have been developed for landscape genetic analyses. These approaches allow identification and prioritization of important locations and populations for maintaining connectivity. The most widely used graph theoretic approach is one based in electrical circuit theory and is incorporated into the program CIRCUTSCAPE (McRae and Beier 2007, McRae and Shah 2009). This model simultaneously considers all possible paths connecting individuals or populations based on resistance distances. This approach is similar to least-cost path modeling, but can provide different results as it can simultaneously evaluate contributions for multiple dispersal pathways which can identify areas where connectivity is most tenuous (i.e., pinch-points, McRae and Shaw 2009).

The field of landscape genetics is relatively new and has not been used with many *Martes* species yet, however, there are some important exceptions. Broquet *et al.* (2006a) tested whether American marten in the boreal forest of Ontario, Canada showed isolation by distance (i.e., genetic distance was positively correlated with geographic distance) and found no significant relationship. However, the samples were collected in 11 different habitat patches that could be categorized as logged (regenerating tree stands of different ages and types) and unlogged old-growth forest (>80 years old). Examining patterns of isolation by distance in unlogged landscapes revealed a significant pattern of isolation by distance (Mantel test P=0.01), whereas isolation by distance was not significant in the logged replicates (Mantel test P=0.42). This suggests that marten dispersal is changed in suboptimal, logged habitats compared to intact environments and is consistent with demographic studies on marten movement (Broquet *et al.* 2006a). The authors subsequently used least-cost path modeling to confirm these results. Interesting in this ensuing work they also showed that this result was partially dependent on the resolution of the maps, with intermediate grid cell sizes showing the strongest relationship (Broquet *et al.* 2006b). The authors explain this result by suggesting that the largest grid cell sizes (coarsest resolution) may miss important landscape features that marten are sensitive to, yet the smallest grid cell sizes (finest resolution) would require more complex parameterization of habitat features (by changing the resistance of particular landscape features such as rivers, or by adjusting resistance to the width, shape and orientation to other landscape elements) to produce more accurate model results (Broquet *et al.* 2006b).

Similar least cost path modelling has also been conducted on American marten in Idaho, U.S.A. and European pine marten in Ardennes, La Bresse, and L'Isere, France (Wasserman 2008, Mergey 2007) both showing the importance forest structure for dispersal across large landscapes. In addition, Wasserman (2008) showed the importance of elevation, which was a proxy for snowpack, with marten avoiding lower elevations and dispersing in mid to high elevation forests characterized by moist cool sites with subalpine fir (*Abies lasiocarpa*) and Engelmann spruce (*Picea engelmannii*).

Several new landscape genetics approaches have been used to evaluate a recolonizing fisher population in southern Ontario, Canada (Carr *et al.* 2007a, b, Garroway 2009). Initial research tested the idea that Algonquin Provincial Park was the source population for this colonization by examining microsatellite profiles of 35 sites (groups of samples or "populations") surrounding the Park (Carr *et al.* 2007a). The authors found that these 35 sites could be clustered into 5 discrete genetic groups first showing multiple origins. These origins were not Algonquin Park as initially predicted, but rather remnant populations within Ontario, Quebec, and New York (U.S.A.). Carr *et al.* (2007a) also showed that these populations were rapidly homogenizing among expansion fronts. Subsequent research used assignment tests to infer the proportion of immigrants into each of the 5 genetic clusters and relate the proportion of immigrants to habitat variables (Carr *et al.* 2007b). Carr *et al.* (2007b) showed a positive relationship between snow depth and the proportion of immigrants, and a negative relationship between the proportion of coniferous forest in the landscape and the proportion of immigrants. The best regression model was one that included both snow depth and proportion of coniferous forest, suggesting that the most suitable landscapes for fishers had low snow and more coniferous forests.

Lastly, this same dataset was used in a graph theoretical framework to examine network structure for evaluating habitat quality, gene flow, and population substructure (Garroway *et al.* 2009). The graph theoretical framework is a new approach in the landscape genetics arena that can be used to evaluate complex systems of connectivity that lead to system-level properties not readily discerned by examining among population relationships. This analytical approach has been adopted in the fields of social network analysis, neurobiology, and transportation efficiency network analysis (Costa *et al.* 2007). Basically every complex network, in this case a network of connectivity among fisher populations (or groups of samples), has very specific topological features that typify its connectedness and how it responds to perturbation (Costa *et al.* 2007). Garroway *et al.* (2009) showed that the fisher network in Ontario displayed high levels of clustering, and short mean path lengths connecting pairs of nodes (populations). Using the graph theoretic approach also allowed exploration of the effect that removal of nodes had on system connectivity and robustness. Garroway's removal analysis suggested that harvest (removal of nodes) is unlikely to affect

genetic connectivity given current conditions. In addition, Garroway *et al.* (2009) was able to show a negative relationship between measures of node connectivity and both the proportion of immigrants into a node and snow depth, confirming the previous results of Carr *et al.* (2007b).

Overall, these landscape genetic approaches are allowing us to test more detailed hypotheses regarding gene flow through different environments. While these approaches are still nascent, they already have confirmed some of our ecological ideas about what hinders and enhances connectivity among the *Martes* species. In addition, once we have gained understandings of gene flow from these landscape genetic models we can create putative corridor maps for target species (Cushman *et al.* 2009, Schwartz *et al.* 2009).

FUTURE DIRECTIONS

It is an exciting time to be working with molecular genetic data. The field of genomics is exploding which is providing unprecedented power to ask detailed questions regarding relationships among populations within a species, and the processes that created these patterns. Furthermore, we can use genomic data to enhance our understanding of the ecological requirements of the *Martes* species. Additionally, the development of new theoretical models and the use of computer simulations will significantly contribute to the conservation biology of *Martes* through, for example, the integration of genetic or genomic data into metapopulation frameworks and the developments of predictive models which incorporate both environmental and genetic data sets (Bouchy *et al.* 2005, Nomura 2005). Using a Bayesian approach, the integration of genetic and non-genetic data is also possible in order to go beyond the simple estimation of parameters and tests of hypotheses about the factors that control demographic and genetic changes. In particular, the development of Bayesian models aimed to infer historical population dynamics and population parameters are extremely promising (Pertoldi and Topping 2004; Bach *et al.* 2006). We portend the further development of genetic models that integrate both spatial variability (i.e., heterogeneous landscapes) and temporal variability (i.e., metapopulation dynamics), to examine how these variations influence the genetic structure of populations and thereby our interpretations of genetic structure.

The combination of individual-based models and genetics is just emerging now, but it will soon be feasible to evaluate the impact of environmental changes on genetic composition of populations. Models should be developed to address the unresolved questions in conservation genetics. The future promise of the development of these theoretical models and the use of computer simulations is to support conservation genetics investigations through: (a) modelling alternative scenarios for the dynamics of genetic diversity

within and among populations exposed to different environmental regimes and evaluation of short and long-term risks; (b) linking the genotype with phenotype, for example, modeling how a given trait (life-history or morphological trait) would develop in a given scenario. If the information obtained can be combined with empirical and molecular data, the models will be a powerful tool for understanding real-world dynamics.

In addition, we can now use landscape genetic approaches, including graph theoretic approaches to evaluate how various environmental elements influence the flow of genes through landscapes. We can then use these understandings to predict corridors under contemporary conditions and under future climate change scenarios. For example, how will fisher populations interact with one another given a reduction in snowpack in some regions and a change in forest types? Or how will existing connectivity change for pine marten in Europe given anthropogenic development and habitat restoration projects?

As previously mentioned in this chapter, one new and very promising tool are SNPs that may avoid some of the problems attached to microsatellites. SNPs hold the potential to significantly expand our ability to survey both neutral variation as well as genes under selection in natural populations (Beja-Pereira *et al.* 2009). Furthermore, fast and inexpensive methods are continuously developed to screen hundreds or thousands of SNPs per sample in populations (Chen and Sullivan 2003, Ellegren 2008, Wang *et al.* 2009). SNP genotypes based on single nucleotide changes, are universally comparable and do not require standardization across detection platforms. In contrast, it is difficult to compare microsatellite data sets produced by different laboratories, do to inconsistencies in allele size calling (Vignal *et al.* 2003). This enhanced ability to collaborate between laboratories in different countries or continents will aide in our ability to understand *Martes* population dynamics.

Additionally, there will be in the near future the possibility to create for every *Martes* species of interest, a subset of very informative SNPs (48 or 96 SNPs) that could be applied relatively cheap. The cost of SNP microchip beads application is gradually decreasing, making its application available to a wider range of users. The planned cost of a panel of 50 loci using VeraCode SNPs, SNPlex or Fluidigm EP1 system would e.g. cost less than $10, which is 1/20 the amount of the application of 12 microsatellites with 4 PCR and genotyping repetitions. Given the developments in modeling, the new power provided by cheaper and more molecular markers, we anticipate rapid advances in our understandings of the various *Martes* species by the time of the next *Martes* synthesis.

CONCLUSIONS

There are few generalities that can be made across the studies of *Martes*, as each species has evolved a unique niche. However, we can see a few general patterns. For instance, it is clear that the complex glacial histories of Europe and North America left refugia populations that are only recently coming back into contact. Detailed phylogenetic studies have provided an understanding of how *Martes* have responded to previous climate changes, and these studies are absolutely essential for distinguishing short-term anthropogenic changes versus longer term climatic changes that have structured *Martes* populations.

In the field of landscape genetics, which examines the finer-scale movements of *Martes* we are confirming field data that shows the importance of intact old forests with ample structure for several species. Furthermore, for species such as fisher we can use both population genetic and landscape genetic data to further elucidate understandings regarding their avoidance of areas with heavy snow. It will be critical to predict how these areas will change given general circulation models, which often predict the disappearance of snow pack.

Overall, we still have an incomplete taxonomic and evolutionary framework for a significant portion of the *Martes* complex. The current knowledge about intraspecific genetics of genus *Martes* is limited and devoted to only a few species (Table 1). However, the advance in DNA technology is producing a wealth of data for intraspecific phylogenetic studies of *Martes* and there are concomitant developments in analytical methods to deduce demographic history and evolutionary relationships and to test their significance. In this case, whole-genome analyses will provide unprecedented phylogenetic resolution and the power to distinguish even extremely closely related groups. For genera that have emerged relatively recently, such as the *Martes* genus (Koepfli *et al.* 2008), whole genomes will provide fine-scale differentiation. With next-generation sequencing technologies making sequencing cheaper and faster, whole-genome intraspecific phylogenies will soon become a reality for a growing number of marten species.

New scientific studies concerning several species and several geographic regions not studied, is needed to improve our understanding about intraspecific genetics of *Martes* worldwide (Table 1). We hope that knowledge gaps identified in this review will be addressed by research organizations in near future so that, with the development of new techniques in this field, a more precise and more reliable data of all *Martes* species are expected to be fulfilled during the next decade.

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CHAPTER

2

Phylogeography

PAPER II

New insights into the cryptic northern glacial refugia: Phylogeography of the forest dwelling European pine marten (*Martes martes*)

PAPER II

NEW INSIGHTS INTO THE CRYPTIC NORTHERN GLACIAL REFUGIA: PHYLOGEOGRAPHY OF THE FOREST DWELLING EUROPEAN PINE MARTEN (*Martes martes*)

ABSTRACT

The role of southern European peninsulas as glacial refugia for temperate species has been widely established, but the role of cryptic northern refugia is being only recently addressed. Here we describe the phylogeographic pattern of the forest dwelling European pine marten (*Martes martes*) in order to assess the impact of Quaternary glaciations on the genetic structure and to identify location of refugia as well as post-glacial recolonization routes. We used mtDNA sequences 1600bp long generated from 287 samples, which were collected from 21 countries throughout the pine marten distribution. Aiming at clarifying the relationships between *M. martes* and the sable *M. zibellina* in Fennoscandia and Russia, 10 sable samples were also included. Our results reveal the presence of 69 different haplotypes for *M. martes* and 10 for *M. zibellina*, which are split into three major assemblages: Mediterrenean, central-northern European and Fennoscandian-Russian clades, each of them related to specific biogeographic regions which could probably represent different ecotypes. The Mediterranean phylogroup apparently did not contribute to the postglacial recolonization of most of the Palaearctic range of the species, suggesting continuous gene flow across southern Europe during the late Pleistocene. It seems that most of Europe was rather colonized by the central-northern European phylogroup probably surviving the last glaciations in northern cryptic refugia, as it has been previously proposed by paleontological data. A highly divergent phylogroup has been discovered in Fennoscandia-Rusia, wich comprises specimens from both *M. martes* and *M. zibellina* morphospecies. All divergences estimates fall within the Pleistocene suggesting that glacial-interglacial cycles played an important initiating phylogeographic differentiation as well as sculpting this pre-existing phylogeographic variety into today's sister species *M. martes* and *M. zibellina*. Overall, our study indicates a complex phylogeographic history for *M. martes* indicating a mixed pattern of recolonization of northern Europe from both Mediterranean and non-Mediterranean refugia.

Keywords: Phylogeography, cryptic refugia, *Martes martes*, glacial refugia, mitochondrial DNA, Quaternary glaciations

INTRODUCTION

The dramatic climate changes during the Quaternary have had strong impacts on the range dynamics of species within the Palaearctic region (Webb & Bartlein 1992; Hewitt 2000; Hewitt 2004). It has long been recognized that the species living in temperate forest habitats, were forced to shift their distribution range, so that they would have survived mainly in the glacial maxima in Mediterranean refuge areas (Taberlet et al. 1998; Hewitt 2001; Randi 2007). Interglacial and postglacial recolonizations of central and northern Europe could therefore have arisen from these Mediterranean glacial refugia (Taberlet et al. 1998; Hewitt 2001). Although different species may have had different refugia and colonisation routes according to their ecological and biogegraphical traits (Bhagwat & Willis 2008; Stewart et al. 2010), this pattern of glacial survival in the South, followed by post-glacial recolonization of northern regions, seems to be a general pattern among a variety of temperate taxa (Taberlet et al. 1998; Hewitt 2001). More recently, however, in addition to traditional southern refugia for temperate species, cryptic refugia have been proposed in the North during glacials (i.e glacial refugia for temperate taxa situated at higher latitudes than the expected areas of suitable habitat to the South; Stewart & Lister 2001; Stewart et al. 2010). Indeed, the cryptic northern refugium hypothesis (Stewart & Lister 2001) has received significant support since its publication (Willis & van Andel 2004; Bhagwat & Willis 2008; Provan & Bennett 2008; Zoltán 2010), with phylogeographic studies finding evidence for northern refugia in various temperate organisms (Jaarola & Searle 2002; Deffontaine et al. 2005; Kotlik et al. 2006, Saarma et al. 2007; Teacher et al. 2009). Further, evidence in support of this hypothesis has come from the study of mammal fossil records (Sommer & Benecke 2004; Sommer & Nadachowski 2006), fossil pollen data and macrofossil remains (Willis & van Andel 2004) and species distribution modelling (Svenning et al. 2008; Fløjgaard et al. 2009) which suggested relatively widespread distributions for some mammals and tree species to the north of the traditional southern refugia during the Last Glacial Maximum (LGM).

Recently, there have been attempts to reconstruct the glacial refugia of mustelids on the basis of fossil evidence that in addition to the traditional Mediterranean refugia suggest the existence of a cryptic glacial refuge in the Carpathians for the forest-dwelling European pine marten (Sommer & Benecke, 2004, Sommmer & NadachowskI 2006). Moreover, recent studies using both molecular and fossil data for the bank vole (*Myodes glareorus*) evidenced the existence of central European refugia for this species (Deffontaine et al. 2005; Kotlik et al 2006). Taking into account that the bank vole is one of the main prey species of the pine marten (Zalewski et al. 2004), we could expect a similar phylogeographic pattern for the pine marten. Moreover, Bhagwat & Wills (2008) suggest that species that have persisted in northern refugia have shared biogreographical traits that matched with those found in the European pine

marten: a present-day northern distribution, small body size and cold tolerance. Taking into account all these evidences suggesting for the existence of northern cryptic refugia, the time is ripe to ask whether the pine marten have persisted only in southerly refugia or have had populations farther north in Europe during glacials.

The aim of this study is to detail the phylogeographic pattern of the European pine marten (*Martes martes*), a carnivore species closely associated with forest habitats (Proulx et al. 2004; Zalewski & Jędrzejewski 2006). The pine marten (*Martes martes*) occurs throughout much of Europe and northern and central Asia, from northern Portugal to western Siberia (Proulx et al. 2004). The pine marten is generally associated with forest habitats, mainly mature coniferous and mixed forests (Proulx et al. 2004). The phylogeography of the pine marten is poorly known. Davison et al. (2001) have suggested that the *M. martes* populations currently distributed in central and northern Europe originated from several different refugia with subsequent admixture. However, this study gave no clear clues for the post-glacial recolonization of central Europe, because it was based on a small fragment of mtDNA (325 base pairs, bp) that were not enough to resolve the species phylogeography. Moreover they did not analyse enough samples for the main Mediterranean refuge areas (Balkans, Iberian Peninsula and Italy) and from the eastern Russian populations. Here, we present a more comprehensive study in terms of number of specimens (n=287) and length of mtDNA sequence (1600bp). This study enlarges the number of specimens and the range of distribution of previous phylogeographic works, getting to Scandinavia in the North, Russia in the East, and the Iberian Peninsula in the South-West. Sable (*Martes zibellina*) samples were also included to better understand the relationships between *M. martes* and the closely related *M. zibellina* in Fennoscandia and Russia. Indeed, recent studies (Koepfli et al. 2008), support that the subgenus *Martes* diversified during the Plio-Pleistocene and recognized *M. martes* and *M. zibellina* as sister species within this subgenus. Thus, additional analyses, including population genetic-level sampling, between these related species, will be needed to confidently resolve relationships among these recently evolved species (Koepfli et al. 2008). Thus, we aim to 1) identify the main phylogeographic patterns in this species; 2) reconstruct the post-glacial colonization routes of central-Europe and 3) obtain first information on genetic structure of eastern European *Martes* populations with special emphasis on the genetic relationship between *M. martes* and *M. zibellina*

MATERIAL AND METHODS

Samples and Laboratory procedures

Tissue and hair specimens were collected from 287 pine martens from throughout 21 countries that correspond to the main areas of its distribution range (Table 1 and Fig.1). We also added 10 sable samples from Russia. These specimens were obtained from collaborators and museums collections and were used to isolate DNA from *M.martes* and *M.zibellina* (see Supplementary material 1). DNA was isolated from tissues and hairs using the Qiagen DNeasy® Tissue DNA extraction kit according to the manufacturer's instructions.

The mitochondrial DNA region selected in this study includes the final part of the cytochrome b gene, tRNAPro, tRNAThr, the control region (D-loop) and the initial part of rRNA12S used on a previously work about genetic variability of *Mustela putorius* in Europe (Pertoldi et al. 2006). This fragment of c.a. 1600 bp length was amplified using the forward primer LutbF (5′-AGAACACCCATTCATCATTATCG- 3′), and the reverse primer LLU12SH91 (5'-CTAGAGGGATGTAAAGCA CCG- 3′) (Pertoldi et al. 2006). The standard PCR amplifications were conducted in 15 µL reactions containing 1 µL diluted template DNA, 3.2 pmol of each primer, 1.75µM dNTP, 1.33 µM MgCl2, 1.56 µL buffer STR 10 and 0.6U Taq DNA polymerase using the following cycling conditions: an initial denaturing step at 94 ºC for 5min; 35 cycles of denaturing at 94 ºC for 50 s, annealing at 58.5 ºC for 45 s and extending at 72 ºC for 90 s; and a final extending step of 72 ºC for 10 min.

PCR products were purified using EXO-SAP IT (USB, Cleveland, OH, USA) and sequenced using the BigDye Terminator Kit V1.1 (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM Model 3130 Genetic Analyzer (Applied Biosystems). Eelectropherograms were visually inspected using SeqScape 2.5 (Applied Biosystems) and nucleotide sequences were aligned using the default parameters of CLUSTAL X version 2.0 (Larkin et al. 2007) and manually checked in the BIOEDIT sequence editing program v. 5.0.9 (Hall 1999). The minisatellite repetition of the control region [(TACGCACACG)-n] was removed from the phylogenetic analysis to reduce ambiguous sites with the selected outgroups.

Phylogenetic analyses

The data set used for phylogenetic analysis includes the haplotype sequences of the selected mtDNA region that were obtained from 287 pine martens and 10 sables. Three related species were selected as outgroups: *Mustela putorius* (the polecat; AY962040), *Martes flavigula* (the yellow throated marten;

FJ719367) and *Gulo gulo* (wolverine NC_009685). Number of polymorphic sites, transitions and transversions, and haplotype (h) and nucleotide (π) diversities were obtained with ARLEQUIN version 3.0 (Excoffier et al. 2005).

Phylogenetic reconstructions were performed by a distance method using the neighbour-joining algorithm (NJ) (Saitou & Nei 1987) and using maximum-parsimony criterion (MP) (Fitch 1971) algorithm implemented in PAUP version 4.0b10 (Swofford 2002). For distance analyses, the HKY85 model with rate heterogeneity and invariable sites (α= 0.732, *I*= 0.8693) was selected as the best-fit model of nucleotide substitution for the molecular data set by the Akaike information criteria approach using MODELTEST 3.6 (Posada & Crandall 1998). Therefore, we used this model and parameters for inferring distance matrices. MP analysis was conducted with the heuristic search algorithm, tree-bisection–reconnection (TBR) swapping and a maximum number of trees constrained to 1000. Phylogenetic trees were rooted with homologous region for the selected outgroups (*Mustela putorius*, *Martes flavigula* and *Gulo gulo*). The robustness of the trees was assessed by bootstrap resampling (BS) (10000 random replications for NJ analysis and 5000 for MP; Felsenstein 1985).

We also performed a Bayesian phylogeny estimation using MRBAYES 3.0b4 (Huelsenbeck & Ronquist 2001). Metropolis-coupled Markov chain Monte Carlo sampling was performed using four chains run for 2,000,000 iterations and using the most suitable model determined by MODELTEST. Bayesian posterior probabilities(BPP) were picked from the 50% majority rule consensus of trees sampled every 20 generations, after removing trees obtained before the chains reached an apparent plateau ('burn in' determined by empirical checking of likelihood values). The whole procedure was repeated three times starting from different random trees and the tree topologies obtained were the same.

Phylogeographic analyses

The NETWORK software version 4.1.0.6 (Bandelt et al. 1999; http.www. fluxus-engineering.com) was used to construct a median joining (MJ) network. MJ is a powerful method that reconstructs phylogenies based on intraspecific genetic differentiation (Posada & Crandall, 1998; Bandelt et al. 1999). The data matrix included the nucleotide sequences of the mtDNA region from all the martens and sables sequenced.

The genetic structure of populations was examined using an analysis of molecular variance (AMOVA) performed in the ARLEQUIN version 3.0 (Excoffier et al. 2005). AMOVA was conducted at three hierarchical levels of populations subdivision: among geographical groups; (see Table 1 and Fig. 1), among

populations within regional groups and within populations (See Table 1 for population designation). The significance of these parameters were estimated by 10 000 permutations of the distance matrix.

Demographic analysis

Demographic histories of different phylogroups were inferred by a pairwise mismatch distribution analysis between individuals (Rogers & Harpending 1992) computed under a population growth-decline model in DNASP version 5.0 (Librado & Rozas 2009). Multimodal distributions were consistent with demographic stability, while sudden expansion would generate a unimodal pattern (Slatkin & Hudson 1991). Hypotheses of demographic expansion were tested using Fu and Li's *F* (Fu & Li 1993) and Tajima's *D* statistics (Tajima 1989). Significances for *F*S statistics were obtained by means of coalescent simulations of a panmictic population of constant size conditioning on the number of segregating sites. For each case, 1000 simulations were run in DNASP and the number of trees with values of interest statistic equal or more extreme than the observed value was recorded.

Estimation of divergence times

We estimated divergence times of splits using the Bayesian relaxed phylogenetic approach implemented in BEAST v1.4.6 (Drummond & Rambaut 2007). Analyses were performed using the HKY model of nucleotide substitution (Hasegawa et al 1985). Rate variation among sites was modelled using a gamma distribution with four rate categories. The uncorrelated lognormal relaxed molecular clock model was used to estimate substitution rates for all nodes in the tree, with uniform priors on the mean (0, 100) and standard deviation (0, 10) of this clock model. We employed the expansion growth as the coalescent prior and, with the ingroup assumed to be monophyletic with respect to the outgroup.

Molecular dating was derived using as calibration point the age of the fossil record of the extinct *M. vetus*, which has been considered ancestral to both *M. martes* and *M. zibellina* (Anderson 1994). This date ranges from almost the beginning of the Pleistocene to about 400 kya (kilo years ago) (Wolsan 1993). A lognormal distribution suitable for modelling fossil data (Ho 2007) was used as prior with parameter values of 300 kya as the minimum age (lower bound parameter), 400 kya as the mean and the standard deviation of the distribution was chosen to 1. Moreover, we set the mean of the normal distribution of the root height prior to 4.8 Mya (million years ago), assuming this time as the time of divergence between *M. flavigula* and the subgenus *Martes* (Koepfli et al. 2008) with a standard deviation of 1.0 Mya. Three independent MCMC runs of 20,000,000 steps were performed. Samples from the three chains, which yielded similar results, were combined to estimate the posterior distribution of the substitution model and

tree model parameters, as well as node ages. Analyses of these parameters in TRACER 1.4 (Rambaut & Drummond 2007) suggested that the number of MCMC steps was more than adequate, with effective sample sizes of all parameters often exceeding 100 and Tracer plots showing strong equilibrium after discarding burn-in.

To compare the genetic findings with subfossil records, spatial and temporal information on the distribution of the pine marten during the Pleistocene was obtained from Sommer & Bennecke (2004) and Sommer & Nadachowski (2006).

RESULTS

Pattern of sequence variation

We identified a total of 69 haplotypes among the 287 pine marten specimens analyzed. The 10 analyzed *Martes zibellina* samples showed 10 different haplotypes. We included two genebank sequences that correspond to a new sable haplotype (Mz11). In the alignment comprising both species (1566 bp), there were 95 variable sites of which 59 were parsimony informative. The average transitions/transversion ratio was = 18.4. When considering *M. martes* haplotypes only (excluding *M. martes* haplotypes belonging to Fennoscandia-Rusian phylogroups; see below), there were 47 variable sites of which 25 were parsimony informative.

Phylogenetic and phylogeographic analyses

The geographic distribution and frequency of the 69 and 11 mtDNA haplotypes found in the 287 European pine martens and the 10 sables is shown in Table 1. The haplotype distribution clearly differentiated sequences from three main geographic regions: (i) Southern Europe (i.e Mediterranean peninsulas), (ii) Central-Northern Europe, and (iii) Fennoscandia-Russia. The first group (Haplotypes: Mm1 - Mm27) included unique haplotypes discovered only in the three main Mediterranean peninsulas (Mm1 – Mm8, Mm10 - 17, Mm19), some shared haplotypes between southern Europe and Central-Northern Europe (Mm9 and Mm18) and some closely related haplotypes that have been discovered only in Central-Northern Europe (Mm21 – Mm27) and Ireland (Mm20). The second group (Mm28-Mm55) included haplotypes mainly distributed in Central-Northern Europe, some of them shared across a wide geographic range of this region (Mm29 and Mm31), Fennoscandia (Mm31, Mm45-Mm46), Russia (Mm49-Mm55) and Scotland (Mm28). The northern parts of Fennoscandia and Russia include unique haplotypes not found in any other regions where both *M. martes* and *M. zibellina* haplotypes were mixed

together (Mm51-59 and Mz1-8). Twenty-nine out of the 69 haplotypes detected in *M. martes* specimens were restricted to a single individual.

The NJ reconstruction of phylogenetic relationships between haplotypes is shown in Fig. 2, the MP, and Bayesian trees showing identical topologies. The pine martens split into two major groups: a Fennoscandian-Russian (FNR) clade (BS: ≈96%, BPP: 1.0 including haplotypes: Mm56-Mm65 & Mz1-Mz11) and a large clade grouping all the other pine martens haplotypes (Mm1-Mm55) (BS: 100%, BPP: 1.00). This last group, including pine martens from all the current European distribution of the species, is separated into two different phylogroups: the Mediterranean (MED) and Central-Northern European (CNE) phylogroups (Fig 1).

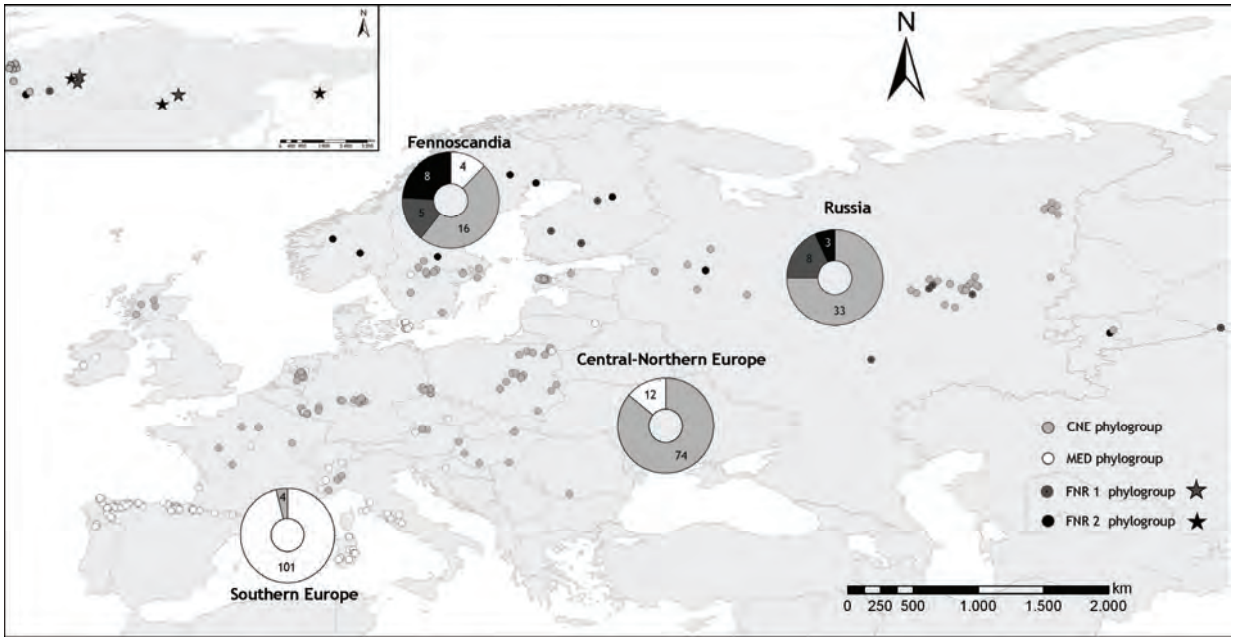


Fig. 1 Geographic distribution of the pine marten (*Martes martes*) (n=287) and sable *(Martes zibeliina*) (n=10) samples represented as dots and stars, respectively. The correspondence of each sample with the discovered phylogroups is also shown. Mediterranean (MED), Central-Northern European (CNE), Fennoscandian-Russian 1 (FNR1) and Fennoscandian-Russian 2 (FNR2) are represented in white, light grey, black grey and black respectively.

The Mediterranean phylogroup (BS: ≈63%, BPP:0.86) is made up predominantly of animals from the main three Mediterranean peninsulas (corresponding to 101 out of 123 samples in this group i.e. 82.11%; Spain: Mm1 - Mm9, *n* = 59; Portugal: Mm9, *n* = 4; Italy-Sardinia: Mm9 - Mm19 *n* = 34; Croatia: Mm19 *n* = 4) and some individuals (*n* = 12) from central Europe (France n = 2; Austria n = 1; Luxembourg n = 1

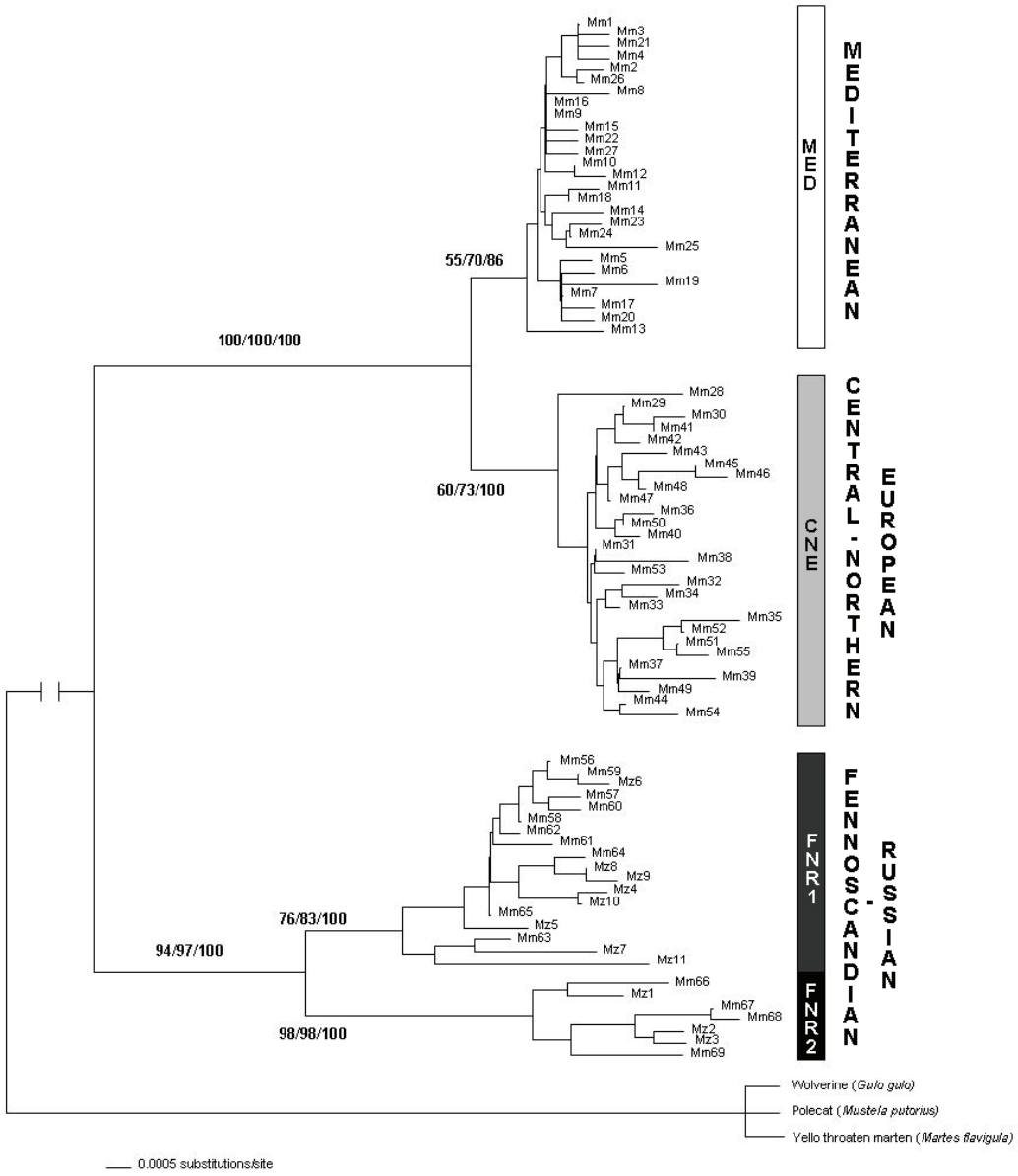
Germany n = 4; Netherlands n = 1; Latvia n = 1; Czech Republic n = 1 Southern Sweden n = 4) and Ireland (n = 6) (see Fig 1 and Table1). The Central-Northern group (BS: ≈67%, BPP: 1.00) is widely distributed throughout Europe with the exceptions of the Mediterranean region and the northern area of Fennoscandia (see Figs 1 and 2 and Table 1). The Fennoscandian-Russian phylogroup includes pine martens from northern Sweden (Mm67 and Mm69) Norway (Mm68), Finland (Mm58, Mm59, Mm69) and Russia (Mm56, Mm57 Mm60-66, Mm69) which are grouped together with *Martes zibellina* haplotypes (Mz1-Mz11). This phylogroup is subdivided into two mayor haplogroups: the first one (FNR1) (BS: ≈80%, BPP: 1.00) is composed of *Martes zibellina* specimens from Russia (Alexandrovskoe; South Transbaikalia; Kamtchatka) and *Martes martes* from Russia (Chelyabinck Province, Leningrad province), North Sweden and Norway; the second haplogroup (FNR2) (BS: ≈98%, BPP: 1.00) is composed of *Martes zibellina* specimens from Russia (Alexandrovskoe; North Transbaikalia) and *Martes martes* from Finland and Russia (Penza Province, Kirov region, Tver province) (Figs 1 and 2 and Table 1).

Table. 1 Geographic distribution and frequency of the 69 mtDNA haplotypes found in the 287 *M.martes* (Mm) and 11 haplotypes found in 10 *M.zibellina* (Mz) samples and two EMBL/Genbank sequences. The correspondence of haplotypes with the discovered phylogroups is also shown. Mediterranean, Central-Northern European, Fennoscandian-Russian 1 and Fennoscandian-Russian 2 are shown in white, light grey, black grey and black respectively. EMBL/GenBank database with Accession numnbers are indicated for each haplotype.

		Southern Europe									Central-Northern Europe										Fennoscandia			Russia	
GenBank Acc	Haplotype	SP_NE	SP_NW	POR	IT_CS	IT_IS	IT_N	CRO	IR	SC	FR	LUX	AU	NTH	DE	CR	PL	LT	EST	HU	RO	SW	FIN	NW	RUS
HM025990	Mm1	19																							
HM025991	Mm2	2																							
HM025992	Mm3	1																							
HM025993	Mm4	1																							
HM025994	Mm5		2																						
HM025995	Mm6		3																						
HM025996	Mm7		1																						
HM025997	Mm8		1																						
HM025998	Mm9		29	4	1	5									2	1									
HM025999	Mm10				6																				
HM026000	Mm11				2																				
HM026001	Mm12				1																				
HM026002	Mm13				2																				
HM026003	Mm14					4																			
HM026004	Mm15					5																			
HM026005	Mm16						2																		
HM026006	Mm17						2																		
HM026007	Mm18						4							1	1							4			
HM026008	Mm19							4																	
HM026009	Mm20								6																
HM026010	Mm21										1														
HM026011	Mm22										1														
HM026012	Mm23																	1							
HM026013	Mm24																1								
HM026014	Mm25												1												
HM026015	Mm26											1													
HM026016	Mm27															1									
HM026017	Mm28									12															
HM026018	Mm29										1	1		6	5					1					
HM026019	Mm30													3											
HM026020	Mm31										4	3			5		1					3			
HM026021	Mm32												2		1										
HM026022	Mm33														2		3		8						
HM026023	Mm34														2		7								
HM026024	Mm35																2								
HM026025	Mm36																1								
HM026026	Mm37														1										
HM026027	Mm38																			1					
HM026028	Mm39																				1				
HM026029	Mm40																		5						
HM026030	Mm41														1										
HM026031	Mm42											1													
HM026032	Mm43																			2					
HM026033	Mm44																1			1					
HM026034	Mm45																					11			
HM026035	Mm46																					2			
HM026036	Mm47						2																		
HM026037	Mm48						2																		
HM026038	Mm49																								1
HM026039	Mm50																		2						8
HM026040	Mm51																								13
HM026041	Mm52																								2
HM026042	Mm53																								7
HM026043	Mm54																								1
HM026044	Mm55																								1
HM026045	Mm56																								2
HM026046	Mm57																								1
HM026047	Mm58																						4		
HM026048	Mm59																						1		
HM026049	Mm60																								1
HM026050	Mm61																								1
HM026051	Mm62																								1
HM026052	Mm63																								1
HM026053	Mm64																								1
HM026054	Mm65																								1
HM026055	Mm66																								2
HM026056	Mm67																					1			
HM026057	Mm68																							3	
HM026058	Mm69																					2	2		1
HM026059	Mz1																								1
HM026060	Mz2																								1
HM026061	Mz3																								1
HM026062	Mz4																								1
HM026063	Mz5																								1
HM026064	Mz6																								1
HM026065	Mz7																								1
HM026066	Mz8																								1
HM026067	Mz9																								1
HM026068	Mz10																								1
FJ429093	Mz11																								2
	n	23	36	4	12	14	12	4	6	12	7	6	3	10	21	1	16	1	15	5	1	23	7	3	57	299
		SP_NE	SP_NW	POR	IT_CS	IT_IS	IT_N	CRO	IR	SC	FR	LUX	AU	NTH	DE	CR	PL	LT	EST	HU	RO	SW	FIN	NW	RUS	TOTAL
		Southern Europe									Central-Northern Europe											Fennoscandia			Russia	

Geographical groups are indicated: Southern Europe, Central-Northern Europe, Fennoscandia and Russia. Abbreviations of populations: Sp_NE, Spain northeast; Sp_NW, Spain northwest; Por, Portugal; It_S, south Italy; ; It_IS, Italy, Island of Sardina; ; It_N, North Italy; Cr, Croatia; IR, Ireland; SC, Scotland; FR, France; LU, Luxembourg; NTH, The Netherlands; DE, Germany; PL, Poland; LT, Lithuania; EST, Estonia; HU, Hungary; CR, Czeck Republic; AU, Austria; RO, Romania; FIN, Finland; SW, Sweden; NW, Norway; RUS; Russia.

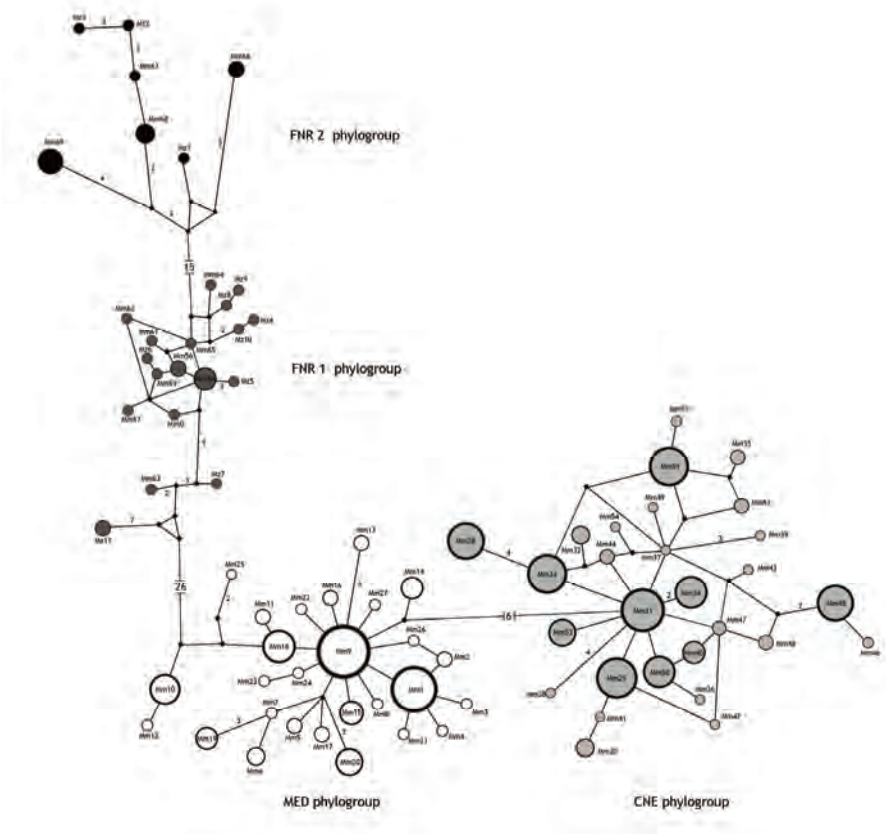
Fig. 2 Neighbour-joining tree of the 69 *Martes martes* and 11 *Martes zibellina* mtDNA haplotypes. Bootstrap values (%) obtained by the NJ and MP as well as Bayesian posterior probabilities are shown. See Table 1 for the haplotype designations and distribution.



From the geographic distribution of each phylogroup (Fig. 1) some suture zones are evidenced between them. A suture zone between MED and CNE phylogroups located in the Pyrenees and in the Alps; and two suture zones between CNE and FNR phylogroups located in central Sweden and in the Ural Mountains, respectively.

The median-joining network gave complementary information and confirmed the existence of these four marten phylogroups (Fig. 3). The distinction among phylogroups was strongly supported respectively by 6 and > 30 mutations which separated the Mediterranean and the Central-Northern European, and the Fennoscandian-Russian phylogroups. In contrast, sequence divergence within each of theses three haplogroups was low. Central-Northern phylogroup is organised around a dominant haplotype (Mm35). This group shows star-like topology, suggesting exponential growth of populations from a small numbers of individuals. Also the Mediterranean phylogroup, is organised around a dominant haplotype (Mm9) with star-like topology. The Fennoscandian-Russian haplogroups is also subdivided into two different subgrroups (FNR1&FNR2), separated by 15 mutations.

Fig. 3 Median-joining network of the pine marten (*Martes martes*) and sable (*Martes zibellina*) mtDNA haplotypes. Numbers of mutations (greater than one) between haplotypes are indicated on branches. See Table 1 for the haplotype designations.



In spite of the presence of two well differentiated subgroups within Fennoscandia-Russia clade, we consider it as one unique phylogroup for remaining analysis taking into account the low number of samples and the presence of admixture of *M. martes* and *M. zibellina* haplotypes within both subgroups.

The mismatch distribution (Fig. 4) and Tajima's *D* and Fu and Li's *F* statistics (Table 2) also suggested varied demographic histories for the marten phylogroups. The negative and statistically significant values of Fu's statistic (Table 2) and the bellshaped mismatch distributions are indicative of population expansions in the past within the MED and the CNE phylogroups (Fig. 4). The FNR (FNR1 & FNR2) phylogroup showed a multimodal mismatch distribution (Fig. 4) that could indicate the admixture of two expanding populations as also suggested by the positive result of Tajima test. However, non significant result of Fu's and Tajima test could indicate also a long-term stability and the reduced sample numbers could also be responsible for not detecting significance; thus, a larger number of samples is needed to clarify the demographic history of this group.

Fig. 4 Mismatch distribution analysis for the three major phylogroups.

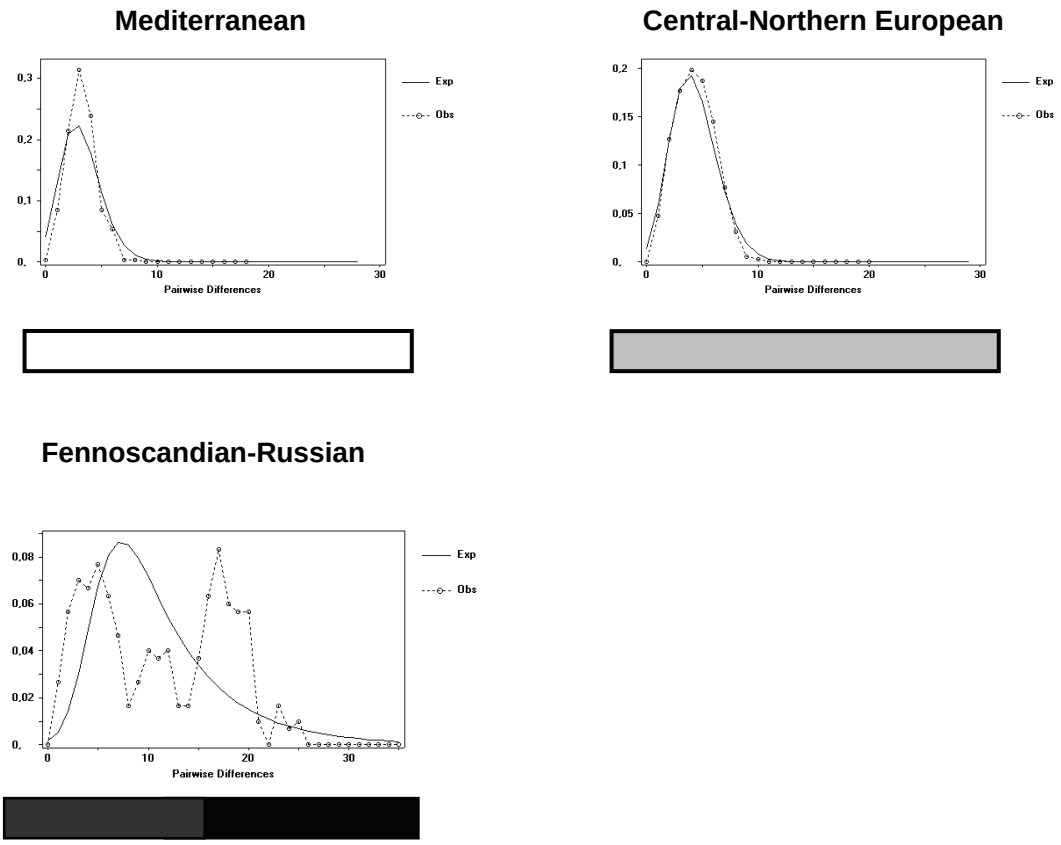


Table 2 Genetic variability observed within the main genetic phylogroups and Tajima's *D* and Fu and Li's *F* statistics test.

	n	Number of haplotypes	Nucleotide diversity π (±SD percentage)	Haplotype diversity (h ±SD)	Tajima'sD	Fu and Li's *F*
Total	299	79	0.647±0.048	0.955±0.006	-	-
MED	123	26	0.120±0.010	0.837±0.027	-2.1672 (P<0.05)	-3.340 (P<0.01)
CNE	139	28	0.234±0.012	0.936±0.006	-1.68615 (P>0.05)	-2.42733 (P<0.05)
FNR	37	14	0.707±0.054	0.930±0.030	0.03059 (P>0.05)	-0.09011 (P>0.05)

Population structure and genetic diversities statistics

AMOVA results were also consistent with the regional subdivision of samples in three main groups as suggested by the median-joining network and the phylogenetic trees. Most probable phylogeographic structures were those with maximum and statistically significant percentages of variation explained by differences among groups (Table 3). The AMOVA showed that the majority of the total mtDNA variation (%73.11) was distributed among geographical groups whereas a low percentage (%12.83) was observed among populations within the groups. Moreover, the φ statistic suggests a low level of gene flow between populations ($\varphi CT = 0.859$, $P < 0.001$).

Table 3 Analyses of molecular variance based on mtDNA data from the main geographical groups.

Source of variation	Variance components	Percentage of variation	P value	φ statistics
Among groups	6.841	73.11	< 0.001	$\varphi CT = 0.859$
Among populations/ groups	1.201	12.83	< 0.001	$\varphi SC = 0.477$
Within populations	1.315	14.06	< 0.001	$\varphi ST = 0.731$

Intra- and inter-group genetic distances were very low: between 0.001-0.008 and 0.006-0.021, respectively (Table 4). The genetic distance between CNE and MED phylogroups was 0.006. The genetic distance between the FNR and CNE and MED phylogroups were slightly higher: 0.021 and 0.020, respectively (Table 4).

The highest value of nucleotide diversity was found in the FNR phylogroup, and the lowest in the MED phylogroup (Table 2). On the other hand, haplotype diversity was similar in CN and FNR phylogroups, being higher than in the MED phylogroup (Table 2).

Table 4 Matrix of distances between phylogeographic groups inferred from the data (below diagonal) assuming a HKY85 model with rate heterogeneity and invariable sites (α = 0.732, I= 0.8693). Values within regions and are shown in bold.

	MED	CNE	FNR
MED	**0.001**		
CNE	0.060	**0.001**	
FNR	0.020	0.021	**0.008**

MED: Mediterrenean phylogroup; CNE: Central-Northern European phylogroup; FNR: Fennoscandian-Rusian phylogroup (FNR1&FNR2).

Divergence times

Assuming the fossil record of *M. vetus* as calibration for the divergence point between *M. martes-M. zibellina* complex, different periods of diversification can be recognized for *Martes* populations, falling all of them within the Pleistocene period: (i) 0.29 Mya (95% HPD: 0.12-0.48) for the TMRCA of the Fenoscandia-Russian phylogroup which began to differentiate before the two European phylogroups, (ii) 0.15 Mya (95% HPD: 0.06-0.26) and 0.13 (95% HPD: 0.04-0.22) for the timing of FNR1 and FNR2 groups respectively, (iii) 0.16 Mya (95% HPD: 0.064-0.28) for the separation time between the two major European phylogroups (i.e. MED and CNE), (iv) 0.092 Mya (95%HPD:0.03-0.15) and 0.081 Mya (95% HPD: 0.03-0.14) as the divergence times for the CNE and MED phylogropus respectively

DISCUSSION

The role of Pleistocene glaciations on phylogeographic patterns and species diversification

There are several previous studies focused on phylogeny, evolutionary history and taxonomy of *Martes* genera species which confirmed that true martens (subgenus *Martes*) are a monophyletic group (Hosoda et al. 2000; Stone & Cook 2002; Sato et al. 2003; Marmi et al. 2004; Sato et al. 2006; Koepfli et al. 2008). Within this subgenus, *Martes foina* has generally been recognized as basal to a clade containing the

remaining present-day species (*M. americana, M melampus, M. zibellina* and *M. martes*) (Anderson 1970; Carr & Hicks 1997; Hosoda et al. 2000; Sato et al. 2003; Sato et al. 2006; Koepfli et al. 2008). However, previous studies were not able to confidently resolve the phylogenetic relationships of the remaining four members of the subgenus *Martes* (Koepfli et al. 2008). Indeed, this four species have been described as a superspecies complex of closely related, yet largely allopatrically distributed taxa (Hagmeier, 1961; Anderson, 1970). In the most recent and complete study published up to now, Koepfli et al. (2008), using 22 gene segments (~12,000 base pairs), were also uneven to fully resolve the phylogenetic relationships within this group and found different phylogenetic placements of *M. americana* and *M. melampus* relative to the clade which comprises *M. martes* and *M. zibellina* as sister species. Koepfli et al. (2008) suggested a relatively recent speciation event within this group and calculated that diversification of this forest dwelling species, coincides with expansion of this type of habitat across the Holarctic region during the Plio-Pleistocene. This finding is consistent with fossil records that indicate that extinct ancestral species to true martens primarily evolved in forested habitats (Anderson 1970; Anderson 1994; Koepfli et al. 2008). The early Miocene *Martes laevidens* has been proposed as the oldest known member of the subgenus *Martes* (Anderson 1994). However, the basicranial anatomy indicates that this species is not congeneric with current living martens (Sato et al. 2003). Thus, the earliest known true marten appears to be *M. wenzensis* (Anderson, 1970, 1994) from the early Villafranchian (4Mya). *M. wezensis* may have been ancestral to *M. vetus* (Anderson 1994). The fossil record of the extinct *M. vetus*, which has been considered ancestral to both *M. martes* and *M. zibellina* (Anderson 1994), ranges from almost the beginning of the Pleistocene to about 400 kya (Wolsan 1993). Thus, using the most recent fossil record of *M. vetus* as calibration of the divergence point of the *M. martes-M. zibellina* complex, our divergence analysis estimates that the separation time between the two major European phylogroups (i.e MED and CNE) of pine marten took place during the middle-late Pleistocene c.a 160.000 BP [0.16Mya (95% HPD: 0.064-0.28)], probably during the late Riss or early Würm glaciations. However, the two different lineages of the FNR phylogroup, which comprises specimens of both morphospecies *(Martes martes* and *Martes zibellina)*, begin to differentiate before the European phylogroups, c.a 290.000 BP, but also associated to the glacial periods of the middle-late Pleistocene [0.29 Mya (95% HPD: 0.12-0.48)]. All divergences estimates fall within the Pleistocene, which is typical of mammalian intraspecific phylogroups (Avise 2000). The earliest record of *M .martes* is from the Riss-Wurm interglacial (ca. 120kya) in central Europe (Anderson 1994). However, the earliest known records of *Martes zibellina* is much more posterior, from late Pleistocene in age (Anderson 1994). However, the absence of fossil record data from Asia, limits the information regarding the latter species and probably more ancient records of sable could exist in this region. Overall,

our results suggest that Pleistocene conditions played an important initiating phylogeographic differentiation as well as sculpting pre-existing phylogeographic variety into today's consider sister species' *M. martes* and *M. zibellina*. A combination of evidences from the fossil record and the divergence times ontained in the present study indicates that, the populations of the common ancestor to both species (i.e *M. vetus*) became reproductively and genetically isolated and started to diverge into two different lineages giving first to the origin of the FNR phylogroup, where all the specimens morphologically identified as *M. zibellina* are included, intermixed with some phenotypically *M. martes* indivuals, and latter to the pine marten European phylogroups. We therefore propose that isolation of the FNR phylogroup in a refugium presumably located in Eastern Asia (see below), may have played an important role in the origin of the current *M. zibellina* morphospecies which occurs from East of Ural across all the Siberian coniferous taiga forests.

The mtDNA groups inferred in this study shows a strict phylogeographic pattern throughout the species range. Altogether three major mtDNA phylogenetic lineages have been discovered with a high correlation between the currently recognized biogeographic regions in Europe (EEA 2008) and the distribution of each phylogroup. Although some overlap occurs between specimens of different groups, overall a spatial segregation pattern is evidenced. The MED phylogroup is closely associated with the Mediterrenean peninsulas but mainly linked to the Atlantic and Alpine areas where the temperate mixed forests are predominat. The CNE phylogroup, which covers most of the pine marten distribution, is clearly distributed across the Continental biogeographic region. Finally, the FNR phylogroup is strongly related to the Boreal region. These results suggest that each of the inferred groups could be adapted to specific environmental conditions; and could represent different ecotypes. Indeed, there is a clear pattern of latitudinal variation in pine marten body size, decreasing from south to north (Reig 1992) but also in diet composition, food niche breadth, and prey size for martens in Europe (Zalewski 2004). However, in all the pine marten distribution range the bank vole is the main prey species suggesting a close relationship between both forest species (see below). Probably geographic variation in habitat and diet plays an important role in shaping pine marten evolutionary adaptations, life-history strategies and ecological roles. Additionally, similar phylogeographical patterns have been described in other forest species (Deffontaine et al. 2005) suggesting that similar processes of coevolution can occur in species related to this specific habitat.

Gene flow across southern European glacial refugia during the LGM

The role of southern European peninsulas (Iberia, Italy and the Balkans) as glacial refuge for temperate species has been widely established (Taberlet et al. 1998; Hewit 2000; Weiss & Ferrand 2006). Sommer & Nadachowski (2006) provide information about pine marten fossil remains from different archaeological sites from the Younger Palaeolithic, which are precisely dated to the Last Glacial Maximum (23,000–16,000 BP). They confirmed the existence of the pine marten during the LGM in the well-known refugium of the Mediterranean peninsulas. In spite of they found records only in the Italian and the Balkan Peninsulas, the few records of pine marten reported, probably reflect only single records of its former distributions in potential refuge areas, rather than a complete range of distribution (Sommer & Nadachowski 2006). Indeed, in a previous work focused on the late and post-glacial colonization of mustelids, Sommer & Bennecke (2004) suggested that pine marten could also be present into the Iberian Peninsula during the LGM taking into account that subfossil records from the Late Glacial until the Middle Holocene were continuously present in the Iberian Peninsula. According to fossil data (Sommer & Bennecke 2004; Sommer & Nadachowski 2006), and the fact that our phylogeographic analyses reveal a mtDNA phylogroup joining all the pine martens populations from these three regions (Figs 1 and 2), there is strong evidence for the widely recognized refuge of the Mediterranean peninsulas for the pine marten. Moreover, these results suggest a large Mediterranean population during the late Pleistocene, where gene flow between populations was possible. This pattern of continuous geneflow across southern Europe has been also reported in Brown bear populations (Valdiosera et al. 2007).

For many temperate species southern refugial areas currently exhibit high genetic diversity (Hewit 2004). However, in this study the Mediterranean phylogoup shows the lowest genetic variability of the three identified phylogroups. The low nucleotide and haplotype diversities characterizing this phylogroup could be associated with population fragmentation followed by severe population bottlenecks during the Quaternary glaciations.

Central-Northern phylogroup: New insights into the cryptic northern glacial refugia

The low proportion of pine martens from the Mediterranean lineage (18 out of 123 i.e 14.6%) identified in central-northern Europe strongly suggests that this lineage has not been the source of major postglacial recolonizations of this region. Moreover, no haplotypes from CNE phylogroup was found in any of the know southern refuges of Europe (except 2 samples near the contact zone in the Italian Alps, that may come from a recent recolonization from CNE lineage populations in the Alps; Balestrieri et al. 2010), suggesting that central-northern Europe was rather recolonized by a pine marten phylogroup surviving the

last glaciations in a central European glacial refugia as it has been previously proposed by paleontological data (Sommer & Benecke, 2004; Sommer & Nadachowski 2006). Indeed, the CNE phylogroup presently covers most of the pine marten distribution range in the Paleartic region (Fig. 1) and was subjected to a recent population expansion (Figs 3 and 4). Additionally, this phylogroup is characterized by a high haplotype and nucleotide diversities in comparison to the MED phylogroup, suggesting that this populations have been affected by less severe population bottlenecks. Moreover, fossil records of pine marten were found during the LGM in the East of the Carpathians, in Moldova (Markova et al. 1995; Sommer & Benecke, 2004) and in Deszczowa and Mamutova Cavesin in southern Poland (Sommer & Nadachowski 2006). Consequently, we can reasonably assume that this region acted as glacial refugium for the CNE pine marten phylogroup.

The existence of a non Medtirrenean refugium would also be consistent with past and present ecological traits of pine martens. The current range distribution of the pine marten includes coniferous forests and cold environments. As a cold tolerant species, the pine martens would have been able to survive at northern latitudes even at former boundary between woodland and tundra. Indeed, the northernmost subfossil record from the Late-Glacial was situated in Denmark assigned to the younger Dryas (14 kya). Moreover, Bhagwat & Wills (2008) suggest that the persistence of species in northerly glacial refugia is closely related to some biological and biogeographical traits, such as small body size, a present-day northerly distribution and cold-tolerance, which in fact strongly matches with those traits found on the pine marten.

There is mounting evidence that some European temperate species did not respond to the last glaciation by simply shifting their distributions to the Mediterranean region but also survived at higher latitudes previously considered inhospitable (Willis & van Andel 2004; Bhagwat & Willis 2008; Provan & Bennett 2008; Stewart et al. 2010). Evidences in support to this model came from phylogeographic studies (Jaarola & Searle 2002; Deffontaine et al. 2005; Kotlik et al. 2006, Saarma et al. 2007; Teacher et al. 2009) mammal fossil records (Sommer & Benecke 2004; Sommer & Nadachowski 2006), fossil pollen data and macrofossil remains of tree species (Willis & van Andel 2004) as well as from species distribution modeling (Svenning et al. 2008; Fløjgaard et al. 2009).

The present genetic study, as well as fossil remains (Sommer & Benecke, 2004; Sommer & Nadachowski 2006), indicates that a temperate forest species such as the pine marten was also able to survive in central European regions during glacial periods, providing new insights into the existence of cryptic northern glacial refugia.

The Fennoscandian-Russian group

In our analysis, we identified the existence of a third continental phylogroup that joined pine martens form Norway, Northern Sweden, Finland and several regions of Russia together with *Martes zibellina* specimens collected from a wide geographic area East of Urals (from Urals to Katnchatka). Moreover this phylogroup is subdivided into two diferent subgroups sugessting two different mtDNA lineages present in marten populations of Fennoscandia-Rusia (Fig 2). This group is characterized by very different demographic history, genetic diversity and genetic divergence as compared to the other European phylogroups (Figs 2, 3; Tables 2 and 5). However, we have not found correspondence between these two subgroups, neither with their geographic distribution, nor with the two morphospecies considered. Thus, in this phylogroup (i.e FNR) the morphological species concept does not correspond to the phylogenetic species concept (De Queiroz et al. 2007). Our results suggest that, Pleistocene conditions played an important initiating phylogeographic differentiation as well as sculpting this pre-existing phylogeographic variety into today's sister species' pine marten and sable. It seems likely that this ancient phylogeographic varieties (i.e FNR1&FNR2) could be therefore evolved given to the origin of the sable east of Urals with a secondary contact between pine marten and sable giving rise to a process of genetic hybridization of both morphospecies in Fennoscandia and Russia region. Previous studies have found that *M. martes* and *M. zibellina* formed a monophyletic group with *M. martes* paraphyletic with respect to *M. zibellina* (Stone & Cook 2002, Marmi et al. 2004). Although incomplete lineage sorting can result in paraphyletic relationships (Davison et al. 1999), these two species are not reproductively isolated and successful hybridization between them is possible (Grakov 1994; Rozhnov et al. 2010). Indeed, Davison et al. (2001) detected some individuals on Fennoscandia with mtDNA of *M. zibellina* and explained the existence of those specimens as the past introgression of the sable mtDNA into pine martens. More recently, Rozhnov et al. (2010) found a high degree of reciprocal mtDNA introgression in sympatric populations of these species in the sympatric zone in the northern Urals. Hybridization or mtDNA introgression between other species of the subgenus *Martes* has been previously documented (*M. martes* x *M. americana*, Kyle et al. 2003; *M. zibellina* x *M. melampus*, Murakami et al. 2004) confirming that interspecific hybridization is a common event within this subgenus. The four species of the subgenus *Martes* have been considered as a superspecies by some authors, suggesting close phylogenetic relationships (Hagmeier1961; Anderson, 1970) and defined as a monophyletic group of allopatric species that are morphologically too different to be included in a single species. Although some species begin to separate during the Pleistocene, in most cases it took more than two million years to reach full speciation (Avise et al. 1999). This consideration is concordant with the results obtained for the FNR phylogroup. Probably, the origin of Post-Glacial

recolonization during the Holocene lies in one or more Asian glacial refugia (Sommer & Bennecke 2004) that could be located in southern or eastern Urals or even in the Caucasus. However, this phylogroup should be further investigated by the use of nuclear markers and a more extensive sampling from these regions. Moreover, a comparison with the phylogeographic patterns of the sable (currently underway) will probably provide valuable information concerning the evolutionary history of this different marten phylogroup and whether refugial isolation could have lead to speciation between these species.

Postglacial re-colonization of pine marten populations

The impact of Quaternary glaciations and the identification of the main post-glacial colonisation routes from glacial refugia have been widely studied in different mammal species across Eurasia (Taberlet et al. 1998; Hewitt 2004: Stewart et al. 2010).

In this study, the evidence of population structuring, into three different phylogroups, found within European pine marten populations is the clear sign of a postglacial recolonization from different refuge areas with posterior intermixing. The high levels of haplotype diversity and low levels of nucleotide diversity found (Table 2) may suggest rapid demographic expansions from small effective population sizes, multiple refuges and secondary contact of populations from different refuges. The geographic distribution of some shared haplotypes and phylogeographic groups (Table 2, Fig. 1) agrees with this last possibility. The study by Davison et al. (2001), found a similar population structure in Europe with the existence of the same three groups inferred in this study. However the low support values obtained for each of the detected groups, due to the small mtDNA fragment used (325bp) and the limited sampling from Mediterranean and from the eastern Russian populations, did not allow providing clear clues for the post-glacial recolonization of central Europe.

Our data suggest that during the LGM, the two lineages retreated in separate refugia (southern European peninsulas for the MED lineage and the Carpathians or more eastern refugium for the CNE lineage). Populations of MED lineage probably went through a bottleneck during the last glaciation, and after the LGM, the population expansion from Mediterranean peninsulas was likely associated with a haplotype diversification, as suggested by the star-like phylogeny (Fig. 3) and the low nucleotide and high haplotype diversities (Table 2). The MED lineage expanded to the north up to southern Sweden. However, the low proportion of pine martens from the MED lineage discovered in Central-Northern Europe strongly suggests that this lineage has not been the source of major postglacial recolonizations of this region.

The persistence of CNE pine marten populations in Central European refugia must have significantly reduced the times by which recolonizing animals reached the northern parts of Europe after the LGM (Sommer & Nadachoswki, 2006). Indeed, while the MED phylogroup is more restricted to the southern European areas, the CNE phylogroup arrives up to northen Urals. The rapid recolonization of Central Europe by CNE populations which survived in central European refugia, could be the reason for the presence of pine marten in regions like Denmark and Czech Republic during Younger Dryas and the Magdalennian: (c.a 17.00-9.000 BP), respectively. Probably, the CNE phylogroup was the first to colonise central Europe limiting the expansion processes of the MED phylogroup. Consequently, the low presence of MED lineage on central Europe could be interpreted as a minor recolonization process from the Mediterrenean penisulas to this area.

According to our results, it is noticeable that the current island populations of Britain and Ireland are represented by the two main European phylogroups: CNE and MED, respectively. However, the recent discovery of an haplotype representative of the Iberian peninsula martens in museum specimens from Wales, indicates that both phylogroups were present in Britain (C. O'Reilly, unpublished data; Mullins et al. 2009).

There are two main hypotheses explaining the post- glacial re-establishment of the Brithish islands martens according to this data, which are not mutually exclusive: a natural post glacial re-colonization of the MED group from the Iberian Peninsula, tracking the coast line for both Ireland and Britain; and a natural colonization of CNE phylogroup from continental Europe to Britain. However, we cannot discard anthropic origin of these populations, which is also congruent with the early trade routes that have been established between south-west Europe and Ireland since Mesolithic, as has been previously proposed (Searle 2008).

The presence of the three major phylogroups in Fennoscandia suggests that the pine marten recolonized this area from the northeast, by the FNR phylogroup, and from the south, by the MED and CNE phylogroups. Similar north–south phylogeographical patterns with a suture zone in central Fennoscandia have been described for several other mammals strongly corroborating this recolonization model (e.g. Taberlet et al. 1995; Jaarola et al. 1999, Brunhoff et al. 2003).

At present, it is impossible to know exactly neither how hybridization occurred between martens in Fennoscandia-Rusia, nor whether is the exact location of the FNR populations refugia. However, as *M. zibellina* is an eastern species that is better cold adapted than *M. martes*, it may have been the first to colonize the northeast of Fennoscandia from and undetermined Eastern refugia in Asia (Sommer &

Bennecke 2004). As the climate ameliorated, *M. martes* could have replaced *M. zibellina*, with mitochondrial introgression as the dwindling *M. zibellina* population mated with *M. martes* (Grakov 1994; Rozhnov et al. 2010). An alternative scenario is suggested by Davison et al. (2001). *M. zibellina* is generally limited by the Ural mountains to the west, but during the "little ice age" (c.a.1550-1850 BP) there is evidence that the sable penetrated deep into Europe providing a suitable scenario for mating with the pine marten. As the temperature increased again, the range of *M. zibellina* was restricted once again to the Urals. Actually, while the FNR phylogroups arrives up to central Sweden, the CNE phylogroups is restricted Eastward up to the Ural Mountains.

Prey-predator relationships: a linked phylogeographic pattern between the bank vole and the pine marten

Coevolved relationships may lead to high congruence of distributional history that could be strong between predators and their potential prey species (Abrams 2000). The bank vole is one of the main prey species of the pine marten across its entire distribution area (Zalewski et al. 2004). Consequently, a linked phylogeogrphic pattern could be expected for prey-predators which inhabit the same forest habitats. Indeed, recent phylogeophic studies conducted in the bank vole (*Myodes glareolus*) have found a close pattern to that found in the pine marten (Deffontaine et al. 2005; Kotlik et al. 2006). Interestingly, in southern Europe the bank vole has three different Mediterranean lineages belonging to each of the Mediterranean peninsulas (Deffontaine et al. 2005), while we found a unique Mediterranean lineage for the pine marten that suggest continuous gene flow across southern Europ for the latter. These traits are congruent with the restricted dispersal capabilities of the bank vole in comparison to a highly mobile mid-sized carnivore such as the pine marten. Moreover, Kotlik et al. (2006) provided the clearest phylogeographic evidence of a northern glacial refugium for temperate species in central Europe. Thus, the same location of a glacial refugium for the main predator of the bank vole gives strong support for central European refugia in temperate forest mammals. Regarding the Fennoscandia and Russia region (west of Urals) an Ural phylogroup was identified also for the bank voles which were closely related to the red-backed vole *Myodes rutilus*, the species found east of Urals. These data is congruent with the FNR phylogroup where pine marten and sable haplotypes are admixed together. Thus, similar patterns between closely related species (*M. martes* and *M. zibellina*; *M. glareolus* and *M. rutylus*) in Fennoscandia-Russia region could therefore highlight the importance of the Ural Mountains as the main barrier for gene flow between lineages, given rise to species diversification processes. Indeed, the Urals has been evidenced as a important suture zone (Hewitt 1996), and probably had an important role on species diversification in the

Paleartic region taking into account that this mountain barrier suppose the distribution limit between a number of related taxa.

CONCLUSIONS

The role of glacial refugia in intraspecific evolution has been widely addressed for many different taxa through phylogeographic analysis (Avise 2009). However, the respond of each species due to climatic changes of the Quaternary depends largely on their adaptations and environmental tolerances (Stewart et al. 2010). The mtDNA groups inferred in this study shows a strict phylogeographic pattern throughout the species range with the presence of three major phylogroups MED, CNE and FNR each of them related to specific biogeographic regions: Mediterranean-Atlantic, Continental and Boreal, respectively, which could represent different ecotypes. Overall, our study indicates a complex phylogeographic history for *M. martes* indicating a mixed pattern of recolonization of northern Europe from both Mediterranean and non-Mediterranean refugia. The presence of CNE lineage, widespread across northern Europe, which did not correspond to the lineages present in any of the three peninsular refugia, suggests that the source of this lineages lie elsewhere, possibly in a northern cryptic refugia located in the Carpathians, as it has been previously proposed by paleontological data. These results provide new insights into the evidence that in addition to traditional southern refugia for temperate species, cryptic refugia existed in Northern Europe during glacials. However, this does not exclude the importance of Mediterranean peninsulas as a relevant source of diversity for pine martens. Moreover, a highly divergent phylogroup has been discovered in Fennoscandia-Rusia, wich comprises specimens of both *M. martes* and *M. zibellina* morphospecies, which appears characterized by very different demographic history as compared to the other European phylogroups. Our incipient results about this phylogroup suggest that Pleistocene conditions played an important initiating phylogeographic differentiation as well as sculpting this pre-existing phylogeographic variety into today's sister species' pine marten and sable. However, attention should be paid in the future to this region and a deep and needed study will probably give useful information concerning *Martes* complex evolution in this area.

Finally, the linked phylogeographic patterns found between the pine marten and the bank vole, provided clear evidence about the Quaternary effects on the evolution of forest dwelling species and suggest that coevolved prey-predator relationships lead to a stronger congruence of their phylogeographic history.

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Appendix S1 List of origins and collectors of pine marten samples used in this study. The sample code of museums and of samples from our collection is included with the correspondent haplotype found. See Table 1 for remaining correspondences.

Species	Country	ID	Sample code	Country_Code	Sample Location	UTMX	UTMY	Institution and/or Collector	Haplotype
Eueopean pine marten (*Martes martes)*									
	Spain	1	**8T**	SP_NE	Navarra; Etulain-Anue N-121 Km 18	42,93763387	-1,60867352	Fermin Urra.VRFN. Gobierno de Navarra	Mm1
		2	**9T**	SP_NE	Navarra; Enderitz; N-121 Km 12'5	42,89066785	-1,60616621	Fermin Urra.VRFN. Gobierno de Navarra	Mm1
		3	**11T**	SP_NE	Navarra;Cruce Inbuluzqueta N-135 Km 16'8	42,92489700	-1,54141837	Fermin Urra.VRFN. Gobierno de Navarra	Mm1
		4	**14T**	SP_NE	Navarra; Enderitz; N-121 Km 12'2	42,89318802	-1,60622056	Fermin Urra.VRFN. Gobierno de Navarra	Mm1
		5	**15T**	SP_NE	Navarra; Esparza NA-178 Km 40	42,85387300	-1,10017200	Fermin Urra.VRFN. Gobierno de Navarra	Mm1
		6	**8BU**	SP_NE	Burgos;Aostri de Losa-Tunel Peña Angulo	42,93879694	-3,10801844	Aitor Valdeón	Mm1
		7	**14AP**	SP_NE	Lleida;La Guingueta d Áneu;Cruce de estaron	42,53022900	1,19150193	Pere Aymerich	Mm1
		8	**SL**	SP_NE	Guipuzkoa;Salinas de Leniz	42,98705089	-2,57253164	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm1
		9	**199/05**	SP_NE	Álava; Ozaeta	42,91867282	-2,50151183	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm1
		10	**037/06**	SP_NE	Álava;Ozaeta	42,91867282	-2,50151183	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm1
		11	**MA1103**	SP_NE	Álava	42,90974855	-2,89371216	Museo de Ciencias Naturales de Álava	Mm1
		12	**431/06**	SP_NE	Álava; Legutiano	42,98052195	-2,64509459	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm1
		13	**10-TF**	SP_NE	Alava; Zuya; Altube	42,97636399	-2,86927798	Txema Fernández. IAN	Mm1
		14	**016/07**	SP_NE	Alava; Llodio	43,12529689	-2,98821596	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm1
		15	**67KA**	SP_NE	Bizkaia; Mañaria	43,12155152	-2,65138170	Julio Ruiz Guijarro	Mm1
		16	**14-EA**	SP_NE	Alava; Amurrio	43,00646502	-2,94023940	Enrique Arberas Mendibil	Mm1
		17	**160/07**	SP_NE	Alava; Aguiñiga	43,12529689	-2,98821596	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm1
		18	**253/09**	SP_NE	Alava; Amezaga; N-622 pk23;5	42,96125248	-2,84315285	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm1
		19	**1-ESK**	SP_NE	Alava; Escota	42,83752108	-2,94442004	Iker Ayala	Mm1
		20	**13T**	SP_NE	Navarra;Egués NA-150 Km 3'3	42,82427081	-1,55257416	Fermin Urra.VRFN. Gobierno de Navarra	Mm2
		21	**071/07**	SP_NE	Alava; Arzubiaga; N-1 PK357	42,87793161	-2,61897701	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm2
		22	**128-OR**	SP_NE	Huesca;Torla; Ordesa	42,64928800	-0,05779499	Fernando Carmena. Parque Nacional de Ordesa y	Mm3

						Monteperdido.	
23	**481/07**	SP_NE	Alava; Legutiano; A-2620 KM19	42,99377749	-2,63755586	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm4
24	**M154**	SP_NW	Asturias	43,29761456	-5,28447506	Montserrat Bosch. Institut de Recerca i Tecnologia Agroalimentàries	Mm5
25	**M 768/08**	SP_NW	Asturias; Piloña	43,32024600	-5,35208800	Lida Mamán/Christian Gortázar. Instituto de Investigación en Recursos Cinegéticos	Mm5
26	**1-JB**	SP_NW	Lugo; O corgo; N-546 km7	42,92882818	-7,48015053	Juan Fernando Berezo	Mm6
27	**6-JB**	SP_NW	Lugo; O corgo; N-546 km6;6	42,93428649	-7,48351762	Juan Fernando Berezo	Mm6
28	**7-JB**	SP_NW	Lugo; O corgo; N-546 km6;4	42,93691954	-7,48630786	Juan Fernando Berezo	MM6
29	**8-RP**	SP_NW	Lugo; Bóveda	42,60446666	-7,45921666	Rubén Portas Pérez	Mm7
30	**Mma19RO**	SP_NW	Galicia	42,27444466	-7,12388891	Rita Oliveira. CIBIO. (Vicente)	Mm8
31	**19 -SG**	SP_NW	Lugo;A fonsagrada; As rodas	43,12977133	-7,08367270	Manuel Arzúa Piñeiro. Sociedad Galega de Historia Natural	Mm9
32	**17-SG**	SP_NW	A Coruña;Cerdido; Rebordaos	43,62383500	-7,99496099	Manuel Arzúa Piñeiro. Sociedad Galega de Historia Natural	Mm9
33	**18-SG**	SP_NW	A coruña; Mera; Ortigueira	43,67571353	-7,84246369	Manuel Arzúa Piñeiro. Sociedad Galega de Historia Natural	Mm9
34	**23-SG**	SP_NW	Lugo; A fonsagrada	43,12299165	-7,07350302	Manuel Arzúa Piñeiro. Sociedad Galega de Historia Natural	Mm9
35	**26-SG**	SP_NW	A coruña; Cedeira; Meizoso	43,70355400	-7,95118899	Manuel Arzúa Piñeiro. Sociedad Galega de Historia Natural	Mm9
36	**33-SG**	SP_NW	A coruña; Cedeira; Esteiro	43,63527589	-8,04385823	Catuxa Varela Sociedad Galega de Historia Natural	Mm9
37	**32-SG**	SP_NW	Lugo; Fazai (Carballido)	43,04032479	-7,45345505	Manuel Arzúa Piñeiro. Sociedad Galega de Historia Natural	Mm9
38	**1JNA**	SP_NW	Asturias; Narcea; Calabazos	43,39460400	-6,07794899	Alberto Fdz. Gil. EBD-CSIC. Universidad de Oviedo	Mm9
39	**3JNA**	SP_NW	Asturias; San Antolín de Beón	43,03735976	-6,86972424	Alberto Fdz. Gil. EBD-CSIC. Universidad de Oviedo	Mm9
40	**6JNA**	SP_NW	Asturias; Cangas del Narcea; Portiella	43,39460400	-6,07794899	Alberto Fdz. Gil. EBD-CSIC. Universidad de Oviedo	Mm9
41	**10JNA**	SP_NW	Asturias	43,24782365	-5,11365973	Alberto Fdz. Gil. EBD-CSIC. Universidad de Oviedo	Mm9
42	**11JNA**	SP_NW	Asturias;Santo Adriano; Tuñon	43,29174898	-5,98186293	Alberto Fdz. Gil. EBD-CSIC. Universidad de Oviedo	Mm9
43	**13JNA**	SP_NW	Asturias	43,24782365	-5,11365973	Alberto Fdz. Gil. EBD-CSIC. Universidad de Oviedo	Mm9
44	**15JNA**	SP_NW	Asturias;Cangas del Narcea; La linde	43,06308705	-6,50702975	Alberto Fdz. Gil. EBD-CSIC. Universidad de Oviedo	Mm9
45	**16JNA**	SP_NW	Asturias	43,24782365	-5,11365973	Alberto Fdz. Gil. EBD-CSIC. Universidad de Oviedo	Mm9
46	**JNA01**	SP_NW	Asturias; Teverga;Taja	43,29174898	-5,98186293	Alberto Fdz. Gil. EBD-CSIC. Universidad de Oviedo	Mm9
47	**408/04**	SP_NE	Álava; Villlamaderne	43,26901068	-3,28426301	Patricia Lizarraga/ Laura Elorza. Centro de Recuperación de Fauna de Mártioda. DFA	Mm9
48	**34KA**	SP_NW	Bizkaia; Karrantza	43,26901068	-3,28426301	Igor Aguinaco Amigo	Mm9
49	**44-SG**	SP_NW	Lugo; Angueiro	43,01632300	-7,48073297	Manuel Arzúa Piñeiro. Sociedad Galega de Historia Natural	Mm9
50	**53-SG**	SP_NW	Lugo; Xermade; A Fraguela	43,35138328	-7,80821744	Manuel Arzúa Piñeiro. Sociedad Galega de Historia Natural	Mm9
51	**M405/08**	SP_NW	Asturias; El pando	43,37807400	-5,31081300	Lida Mamán/Christian Gortázar. Instituto de Investigación en Recursos Cinegéticos	Mm9
52	**CPV-01**	SP_NW	Leon; Posada de Valdeón; Collada Solano	43,12387191	-4,99164176	Parque Nacional de Picos de Europa	Mm9
53	**79EA(II)**	SP_NW	Cantabria; Praves	43,26901068	-3,28426301	Igor Aguinaco Amigo	Mm9
54	**Mma12RO**	SP_NW	Asturias; Cortrella	43,39460400	-6,07794899	Rita Oliveira. CIBIO. (Universidad de Oviedo)	Mm9

	55	**Mma13RO**	SP_NW	Asturias;Somiedo	43,11872900	-6,28008600	Rita Oliveira. CIBIO. (Universidad de Oviedo)	Mm9
	56	**Mma14RO**	SP_NW	Asturias;Somiedo	43,12180876	-6,26139072	Rita Oliveira. CIBIO. (Universidad de Oviedo)	Mm9
	57	**Mma15RO**	SP_NW	Asturias; Calabazos	43,39460400	-6,07794899	Rita Oliveira. CIBIO. (Universidad de Oviedo)	Mm9
	58	**Mma16RO**	SP_NW	Galicia; Muñiz	43,13032850	-6,89816883	Rita Oliveira. CIBIO. (Pablo Siena)	Mm9
	59	**Mma20RO**	SP_NW	Galicia	42,41527777	-7,22222222	Rita Oliveira. CIBIO. (Vicente)	Mm9
Portugal	60	**04-0045**	POR	National Parc Peneda Geres	41,88073377	-8,23275132	BTVS-ICN	Mm9
	61	**05-0014**	POR	National Parc Peneda Geres; Terras de Bouro	41,74559200	-8,16972899	BTVS-ICN	Mm9
	62	**07-0108**	POR	National Parc Peneda Geres; Terras de Bouro	41,71407188	-8,29960650	BTVS-ICN	Mm9
	63	**08-0084**	POR	National Parc Peneda Geres; Terras de Bouro	41,71407188	-8,29960650	BTVS-ICN	Mm9
Italy	64	**M1IT3**	IT_CS	Viterbo	42,39333954	12,09319886	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm10
	65	**M1IT9**	IT_CS	Viterbo; Lago di vico	42,34100751	12,15811383	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm10
	66	**M1ITL2**	IT_CS	Viterbo	42,36014230	12,07199528	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm10
	67	**Mma 27**	IT_CS	Viterbo	42,37349444	12,08214117	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm10
	68	**Mma 28**	IT_CS	Viterbo	42,39108129	12,11295292	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm10
	69	**Mma18**	IT_CS	Viterbo; Lagp di vico	42,34100751	12,15811383	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm10
	70	**M1IT6**	IT_CS	Roma	42,04875250	12,67613778	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm11
	71	**Mma19**	IT_CS	Roma; RN Macia dil Barco	42,04875250	12,67613778	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm11
	72	**Mma12**	IT_CS	Umbria	42,52842098	12,69643916	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm12
	73	**M1IT14**	IT_CS				Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm13
	74	**M1IT15**	IT_CS				Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm13
	75	**6-SAR**	IT_IS	Sardinia; North	40,98009438	9,23969070	Paolo Casula. Ente Foreste della Sardegna Direzione Generale	Mm14
	76	**4-SAR**	IT_IS	Sardinia; North	40,98009438	9,23969070	Paolo Casula. Ente Foreste della Sardegna Direzione Generale	Mm14
	77	**M5IT48**	IT_IS	Sardinia; Cagliari	39,43216161	9,22946860	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm14
	78	**M5IT57**	IT_IS	Sardinia; Nuoro;Urzulei	40,09453700	9,50861100	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm14
	79	**M5IT53**	IT_IS	Sardinia; Cagliari;Fluminimaggiore	39,44478297	8,49820097	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm15
	80	**M5IT54**	IT_IS	Sardinia; Nuoro;Lanusei	39,87920199	9,54136699	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm15
	81	**M5IT56**	IT_IS	Sardinia; Nuoro;Barisardo	39,85494709	9,64836872	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm15
	82	**M5IT58**	IT_IS	Sardinia; Nuoro;Loceri	39,86016701	9,58501799	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm15

	83	**M5IT60**	IT_IS	Sardinia; Cagliari;Guspini	39,54604904	8,61804930	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm15
	84	**1999-1002**	IT_CS	Varese; Zenna;Lago di vico	46,10173488	8,75477319	Adriano de Faveri. ISPRA (E. Lenzo)	Mm16
	85	**M1IT8**	IT_CS	Varese; Zenna;Lago di vico	46,10173488	8,75477319	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm16
	86	**M1ITL4**	IT_N	Cuneo	44,37075659	7,31307139	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm17
	87	**M1ITL6**	IT_N	Torino; Piobesi Torinesi	44,94046082	7,61058238	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm17
	88	**Mma 8**	IT_N	Weissmatteu	45,74671245	7,82974144	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm18
	89	**Mma 9**	IT_N	Weissmatteu	45,74671245	7,82974144	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm18
	90	**Mma 10**	IT_N	Weissmatteu	45,74671245	7,82974144	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm18
	91	**Mma 11**	IT_N	Weissmatteu	45,74671245	7,82974144	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm18
	92	**M1ITL7**	IT_N	Alessandria	44,97905929	8,43353631	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm47
	93	**MM1-AB**	IT_N	Riserva della Garzaia di Valenza	45,19260555	8,71495019	Alessandro Ballestrieri. Dipartimento di Biologia Animale, Università di Pavia.	Mm47
	94	**M1ITL5**	IT_N	Cuneo; Centallo	44,45001818	7,61965020	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm48
	95	**MM2-AB**	IT_N	Riserva della Garzaia di Valenza	45,19260555	8,71495019	Alessandro Ballestrieri. Dipartimento di Biologia Animale, Università di Pavia.	Mm48
	96	**13451**	IT_IS	Toscana; Livorno; Isola de Elba	42,83555283	10,40609787	Museo Zoologico "la Specola" Sezione del MSN-Universita degli studio di Firenze	Mm9
	97	**2009-0027**	IT_IS	Colle d'Orano-Marciana; Isola de Elba	42,78769899	10,11273900	Adriano de Faveri. ISPRA (F. Gannini)	Mm9
	98	**2-SAR**	IT_IS	Sardinia; North	40,98009438	9,23969070	Paolo Casula. Ente Foreste della Sardegna Direzione Generale	Mm9
	99	**M1ITL1**	IT_CS	Pisa	43,41975358	10,66944733	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm9
	100	**M5IT55**	IT_IS	Sardinia; Nuoro;Villagrande/Fonni	40,10529427	9,25232293	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm9
	101	**M5IT59**	IT_IS	Sardinia; Cagliari;gonnosfanadiga	39,48447939	8,66104444	Licia Colli. Istituto di Zootecnica - Facoltà di Agraria Università Cattolica del S. Cuore	Mm9
Croatia	102	**1-CR**	CRO	Near Zagreb	45,72817047	15,92560545	Ana Galov.Department of Animal Physiology. Faculty of Science. Zagreb (Konjevié)	Mm19
	103	**2-CR**	CRO	Near Zagreb	45,72817047	15,92560545	Ana Galov.Department of Animal Physiology. Faculty of Science. Zagreb (Konjevié)	Mm19
	104	**3-CR**	CRO	Near Zagreb	45,72817047	15,92560545	Ana Galov.Department of Animal Physiology. Faculty of Science. Zagreb (Huber. Konjevié)	Mm19
	105	**4-CR**	CRO	Near Zagreb	45,72817047	15,92560545	Ana Galov. Department of Animal Physiology. Faculty of Science. Zagreb (Konjevié)	Mm19
Ireland	106	**1-DA**	IR		53,41291000	-8,24388999	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham (J. Higgins)	Mm20
	107	**2-DA**	IR		53,41291000	-8,24388999	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham (J.	Mm20

							Higgins)	
	108	**3-DA**	IR		53,41291000	-8,24388999	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham (Congella McGuire)	Mm20
	109	**4-DA**	IR	Clare	52,85897400	-9,11004899	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham (Paddy Sleeman)	Mm20
	110	**5-DA**	IR		53,41291000	-8,24388999	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham (Pat Smiddy)	Mm20
	111	**6-DA**	IR		53,41291000	-8,24388999	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham (Pat Smiddy)	Mm20
Scotland	112	**7-DA**	SC	Benderloch Road; Oban	56,41041002	-5,46972300	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham	Mm28
	113	**8-DA**	SC	Benderloch Road; Oban 20	56,41041002	-5,46972300	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham	Mm28
	114	**9-DA**	SC		56,41041002	-5,46972300	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham	Mm28
	115	**10-DA**	SC	Field Barcaldine; Oban 22	56,41041002	-5,46972300	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham	Mm28
	116	**11-DA**	SC	Road near Oban	56,41041002	-5,46972300	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham	Mm28
	117	**21-DA**	SC	A9 road opposite Crubenmore Junction	57,00774112	-4,16618402	Angus Davison.Institute of Genetics, School of Biology, Queen's Medical Centre, University of Nottingham	Mm28
	118	**Mma 20**	SC	North Western Seabord	57,11662923	-5,12257116	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm28
	119	**Mma 21**	SC	North Western Seabord	57,11662923	-5,12257116	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm28
	120	**Mma 22**	SC	Northern Highlands	57,00774112	-4,16618402	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm28
	121	**Mma 23**	SC	Northern Highlands	57,00774112	-4,16618402	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm28
	122	**Mma 24**	SC	Speyside	57,00774112	-4,16618402	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm28
	123	**Mma 25**	SC	Inverness	57,42135742	-4,25429108	Ettore Randi. Conservation Genetics Laboratory. ISPRA	Mm28
France	124	**1999-316**	FR	Rambouillet (National Road 306)	48,64291551	1,82584801	Geraldine Veron. Muséum National d'Histoire Naturelle. Département Systématique et Evolution (R. Michel-Kerneur)	Mm29
	125	**TC-166**	FR	Landes; road Biscarrosse to lac of Navarosse	44,41987426	-1,17303537	Geraldine Veron. Muséum National d'Histoire Naturelle. Département Systématique et Evolution (Y. Grugier)	Mm21
	126	**TC-174**	FR	Cher; Neuvy sur Barangeon	47,30777631	2,45025979	Geraldine Veron. Muséum National d'Histoire Naturelle. Département Systématique et Evolution (R. Dohogne et Y. Grugier)	Mm22
	127	**TC-271**	FR	France (carrefour N 17 D 205; Nantiat; Haute Vienne)	46,00977400	1,17575300	Geraldine Veron. Muséum National d'Histoire Naturelle. Département Systématique et Evolution (R. Dohogne et Y. Grugier)	Mm31
	128	**1051**	FR	Boissise-la-Bertrand; Seine et Marne	48,61890900	2,97564000	Geraldine Veron. Muséum National d'Histoire Naturelle. Département Systématique et Evolution (R. Cornette)	Mm31
	129	**6759**	FR	Forêt domaniale de Chinon	47,16604588	0,23811010	Geraldine Veron. Muséum National d'Histoire Naturelle.	Mm31

	130	**M242**	FR	Near Dijon	47,51140500	5,28013900	Département Systématique et Evolution (Office National de la Chasse) Montserrat Bosch. Institut de Recerca i Tecnologia Agroalimentàries	Mm31
Luxembourg	131	**1-LUX**	LUX	Bridel	49,65564185	6,05601978	Jan Herr.Department of Biology and Environmental Science.University of Sussex.UK	Mm31
	132	**2-LUX**	LUX		49,68762102	6,12912355	Jan Herr.Department of Biology and Environmental Science.University of Sussex.UK	Mm26
	133	**3-LUX**	LUX	Steinsel	49,59883146	6,30241122	Jan Herr.Department of Biology and Environmental Science.University of Sussex.UK	Mm42
	134	**4-LUX**	LUX	Canach	49,59883146	6,30241122	Jan Herr.Department of Biology and Environmental Science.University of Sussex.UK	Mm29
	135	**5-LUX**	LUX	Bous	49,55431689	6,35190424	Jan Herr.Department of Biology and Environmental Science.University of Sussex.UK	Mm31
	136	**6-LUX**	LUX	Wiltz	49,94910426	5,91456533	Jan Herr.Department of Biology and Environmental Science.University of Sussex.UK	Mm31
Austria	137	**2003/261**	AU		48,50000000	14,26666666	Barbara Herzig. Mammal Collection. The Natural History Museum Vienna (Braunschmid Otto)	Mm32
	138	**2004/343**	AU		48,43333333	14,46666666	Barbara Herzig. Mammal Collection. The Natural History Museum Vienna (Plass Jurgen)	Mm32
	139	**2006/398**	AU		48,25000000	13,80000000	Barbara Herzig. Mammal Collection. The Natural History Museum Vienna	Mm25
Netherlands	140	**93/046**	NTH	Hulshorst	52,36370443	5,73225469	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm18
	141	**94/043**	NTH	Garderen	52,21845546	5,72483899	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm29
	142	**99/035**	NTH	Motor way N-304; near Apeldoorn; NL	52,18600411	5,91938830	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm29
	143	**99/044**	NTH	Motor way A28; near 't Harde	52,41337751	5,90082814	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm30
	144	**00/369**	NTH	Wekerom	52,10190800	5,72655039	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm29
	145	**04/006**	NTH	Veluwe	52,14657115	5,88543480	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm29
	146	**04/029**	NTH	Eerbeek	52,09795507	6,06849646	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm30
	147	**04/175**	NTH	Motor way N786; between Laag Soeren and Eerbeek	52,09244944	6,08905671	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm29
	148	**06/002**	NTH	Heelsum; NL	51,98049300	5,75599400	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm29
	149	**06/004**	NTH	Motor way A1; near Apeldoorn; NL	52,17307025	5,97146268	Hein van Grouw. National Museum of Natural History, Naturalis. Lieden, Netherlands	Mm30
Germany	150	**M4008**	DE	Niesky 2km O; Strabe Ritchung Horka	51,28768652	14,85677038	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	Mm18
	151	**ALE219**	DE	Hohenzell/Kreis Main-Kinzig;Spessart	50,32333611	9,53581388	Franz Müller	Mm27
	152	**3-FM**	DE	Niederrode ;Krifulda; Hessen	50,52138888	9,60416666	Franz Müller	Mm29

	153	**4-FM**	DE	Wickers;Krifulda; Hessen	50,54282222	9,98613333	Franz Müller	Mm29
	154	**ALE204**	DE	Rhön-Grabfeldkreis; Lange Rhön	50,54166666	10,06388888	Franz Müller	Mm29
	155	**ALE205**	DE	Rhön-Grabfeldkreis; Lange Rhön	50,50861111	10,10555555	Franz Müller	Mm29
	156	**ALE206**	DE	Rhön-Grabfeldkreis; Lange Rhön	50,47472222	10,02500000	Franz Müller	Mm29
	157	**M3997**	DE	Lieske südlich B156	51,32097643	14,53358656	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	Mm31
	158	**M5422**	DE	Ostritz Hirschfelde B99 Klosterwald	50,96617874	14,88957468	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	**Mm31**
	159	**M5623**	DE	Maukendorf; Abzweig B96 Spohla; Richtung Hoyerswerda	51,40770937	14,29062200	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	**Mm31**
	160	**ALE208**	DE	Zell-Barl/Mosel	50,03336388	7,14998333	Franz Müller	Mm31
	161	**ALE209**	DE	Hunsnick; Morbad	49,81071388	7,11984722	Franz Müller	Mm31
	162	**ALE212**	DE	Rhön-Grabfeldkreis; Hausen/Lange Rhön	50,50277777	10,06666666	Franz Müller	Mm32
	163	**M4222**	DE	Klosterwald B99 zwischen Schelegel und Marienthal	50,98366191	14,89154142	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	Mm33
	164	**ALE218**	DE	Limeshain-Hainchen; Wetteran-Kreis	50,27044166	8,99528611	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	Mm33
	165	**M4223**	DE	Niesky Aral Tankstelle B115	51,27904931	14,81818783	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	Mm34
	166	**M4007**	DE	KaupPa; Strabe am DelitsZcher Teich	51,25220174	14,48862585	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	Mm34
	167	**ALE207**	DE	Kreis Marburg-Biedenkopf; Wetter	50,44059444	8,76850000	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	Mm37
	168	**2-FM**	DE	Altenfeld/kuifulda; Hesse	50,45809722	9,87573888	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	Mm41
	169	**M3959**	DE	Cablau Zerna	51,28149297	14,23630794	Herman Ansorge. Staatliches Museum fur Naturkunde Gorlitz. Germany	Mm9
	170	**1-FM**	DE	Obrelsbach/ Rhön-Grabfeldkreis; Bayern	50,48333333	10,03333333	Franz Müller	Mm9
Czech Republic	171	**NM303**	CR	Steudenec	49,21666666	16,06666666	Natalia Martinkova.Institute of Vertebrate Biology, Academy of Science of the Czech Republic.	Mm9
Poland	172	**21-POL**	PL		53,83333333	23,25007777	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm24
	173	**84-POL**	PL		51,28333333	19,66666666	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm31
	174	**5-POL**	PL		52,33333333	20,75000000	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm33
	175	**23-POL**	PL		54,08333333	23,09969444	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm33
	176	**81-POL**	PL		53,75000000	21,91691666	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm33
	177	**2-POL**	PL		53,58333333	22,75000000	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm34
	178	**18-POL**	PL		51,57623102	23,55001938	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm34

	179	**26-POL**	PL		53,58346388	21,00000000	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm34
	180	**44-POL**	PL		51,78333333	20,08333333	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm34
	181	**48-POL**	PL		52,08333333	21,16666666	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm34
	182	**65-POL**	PL		51,16666666	23,20000000	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm34
	183	**74-POL**	PL		52,08333333	21,16666666	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm34
	184	**27-POL**	PL		53,83333333	21,50000000	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm35
	185	**30-POL**	PL		52,33333333	20,50000000	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm35
	186	**41-POL**	PL		49,75000000	22,25000000	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm36
	187	**6-POL**	PL		53,95000000	23,08335277	Malgorzata Pilot. Museum and Institute of Zoology, Polish Academy of Sciences.	Mm44
Latvia	188	**1-LT**	LT		55,75500000	26,23000000	Robert Myslajek	Mm23
Estonia	189	**6-EST**	EST	Hiuma Island	58,88090343	22,69212648	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm33
	190	**7-EST**	EST	Hiuma Island	58,88090343	22,69212648	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm33
	191	**8-EST**	EST	Hiuma Island	58,88090343	22,69212648	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm33
	192	**11-EST(1)**	EST	Hiuma Island; Körgessaare	58,98205801	22,47103186	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm33
	193	**11-EST(2)**	EST	Hiuma Island; Körgessaare	58,98205801	22,47103186	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm33
	194	**13-EST(1)**	EST	Hiuma Island; Suuremoisa	58,87107853	22,94396588	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm33
	195	**16-EST**	EST	Hiuma Island; Määvli	58,94729400	22,77162200	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm33
	196	**17-EST**	EST	Hiuma Island; Käina	58,82722800	22,77641199	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm33
	197	**2-EST**	EST	Hiuma Island	58,88090343	22,69212648	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm40
	198	**3-EST**	EST	Hiuma Island	58,88090343	22,69212648	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm40
	199	**4-EST**	EST	Hiuma Island	58,88090343	22,69212648	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm40
	200	**5-EST**	EST	Hiuma Island	58,88090343	22,69212648	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm40
	201	**12-EST(1)**	EST	Hiuma Island; Leluselja	58,81344300	22,56266999	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm40
	202	**1-EST**	EST	Selja	59,28944842	24,53996106	Asun Gómez. TRAGSEGA	Mm50

	203	**10-EST**	EST	Hiuma Island; Laasi	58,89824909	22,31818988	Maddis Podra.Institute of Mathematics and Natural Sciences, Tallinn University	Mm50
Hungary	204	**87.71.1**	HU	Baranya;Pécs	46,09237287	18,22513205	Gabor Csorba. Hungarian Natural History Museum	Mm44
	205	**20241**	HU	Borsod-Abaúj-Zemplén	48,28404276	20,64434073	Gabor Csorba. Hungarian Natural History Museum	Mm38
	206	**4090**	HU	Csongrád;Makó	46,16918629	20,28222455	Gabor Csorba. Hungarian Natural History Museum	Mm43
	207	**97.31.3**	HU	Györ-Sopron	47,61008268	16,80659278	Gabor Csorba. Hungarian Natural History Museum	Mm29
	208	**86.7.1.**	HU	Zala; Keszthely	46,76803410	17,21430563	Gabor Csorba. Hungarian Natural History Museum	Mm43
Romania	209	**1-RU**	RO	South Romania	43,99382476	24,45744393	Dumitru Murariu. "Grigore Antipa" National Museum of Natural History	Mm39
Sweden	210	**NRM20035102**	SW	Falsterbo; Falsterbonäset;Skåne	55,39108868	12,84022203	Peter Mortensen. Swedish Museum of Natural History	Mm18
	211	**NRM20055214**	SW	Vellinge; Foteviken; Bernstorp;Skåne	55,44861761	12,97323506	Peter Mortensen. Swedish Museum of Natural History	Mm18
	212	**NRM20075092**	SW	Vellinge; Bernstorp;Skåne	55,44633740	12,97735054	Peter Mortensen. Swedish Museum of Natural History	Mm18
	213	**NRM985128**	SW	Skoghall;Västmanland	59,33585674	13,47392284	Peter Mortensen. Swedish Museum of Natural History	Mm18
	214	**NRM20035099**	SW	Falsterbo; Falsterbonäset;Skåne	55,39719335	12,82884889	Peter Mortensen. Swedish Museum of Natural History	Mm31
	215	**NRM915124**	SW	Strängnäs; Södermanland	59,29713428	17,09430090	Peter Mortensen. Swedish Museum of Natural History	Mm31
	216	**NRM985172**	SW	Filipstad; Värmland	59,67238588	14,03150943	Peter Mortensen. Swedish Museum of Natural History	Mm31
	217	**NRM995178**	SW	Stockholm; Norra Djurgården; Uppland	59,35795744	18,06153989	Peter Mortensen. Swedish Museum of Natural History	Mm45
	218	**NRM20035159**	SW	Ösmo - Muskö; Södermanland	58,98933335	18,09881774	Peter Mortensen. Swedish Museum of Natural History	Mm45
	219	**NRM20055085**	SW	Vissefjärda; Hulan; Småland	56,55138699	15,67207999	Peter Mortensen. Swedish Museum of Natural History	Mm45
	220	**NRM20075094**	SW	Vellinge; Bernstorp;Skåne	55,45109543	12,97441338	Peter Mortensen. Swedish Museum of Natural History	Mm45
	221	**NRM985120**	SW	Road E18; Närke	59,24024556	14,61522526	Peter Mortensen. Swedish Museum of Natural History	Mm45
	222	**NRM985574**	SW	Bofors target rage; Värmland	59,35504330	14,54297605	Peter Mortensen. Swedish Museum of Natural History	Mm45
	223	**NRM985582**	SW	Närkes Kil; Närke	59,37543690	15,12697889	Peter Mortensen. Swedish Museum of Natural History	Mm45
	224	**NRM985586**	SW	Bofors target rage; Värmland	59,35504330	14,54297605	Peter Mortensen. Swedish Museum of Natural History	Mm45
	225	**NRM985639**	SW	Närkes Kil; Närke	59,36883208	15,07351632	Peter Mortensen. Swedish Museum of Natural History	Mm45
	226	**NRM985187**	SW	Fredriksberg; Dalarna	60,12893525	14,39594607	Peter Mortensen. Swedish Museum of Natural History	Mm45
	227	**NRM985198**	SW	Fredriksberg; Dalarna	60,12893525	14,39594607	Peter Mortensen. Swedish Museum of Natural History	Mm45
	228	**NRM985146**	SW	Blidsberg; Västergötland	57,92933532	13,49247522	Peter Mortensen. Swedish Museum of Natural History	Mm46
	229	**NRM985179**	SW	Blidsberg; Västergötland	57,92933532	13,49247522	Peter Mortensen. Swedish Museum of Natural History	Mm46
	230	**NRM985601**	SW	Björsjö; Dalarna	60,04679012	15,34237103	Peter Mortensen. Swedish Museum of Natural History	Mm67
	231	**NRM20035073**	SW	Luovaure; Snesudden; Lule lappmark	66,14525598	20,36294469	Peter Mortensen. Swedish Museum of Natural History	Mm69
	232	**NRM845006**	SW	Luleå; Norrbotten	65,58415700	22,15474900	Peter Mortensen. Swedish Museum of Natural History	Mm69
Finland	233	**M277**	FIN	Kihnio¨	62,20369302	23,17845301	Montserrat Bosch. Institut de Recerca i Tecnologia Agroalimentàries	Mm58
	234	**M273**	FIN	Kihnio¨	62,20369302	23,17845301	Montserrat Bosch. Institut de Recerca i Tecnologia Agroalimentàries	Mm58
	235	**M275**	FIN	Kihnio¨	62,20369302	23,17845301	Montserrat Bosch. Institut de Recerca i Tecnologia Agroalimentàries	Mm58

	236	**M278**	FIN	Padasjoki	61,35012100	25,27454015	Montserrat Bosch. Institut de Recerca i Tecnologia Agroalimentàries	Mm69
	237	**M279**	FIN	Padasjoki	61,35012100	25,27454015	Montserrat Bosch. Institut de Recerca i Tecnologia Agroalimentàries	Mm58
	238	**Mma17RO**	FIN	Central Finland	64,35136365	26,38804286	Rita Oliveira. CIBIO. (University of Jyväskylä)	Mm59
	239	**Mma18RO**	FIN	Central Finland	64,61541603	27,41689010	Rita Oliveira. CIBIO. (University of Jyväskylä)	Mm69
Norway	240	**NW-11365**	NW	Hedmark county; Fuggdal	61,63244887	8,12179595	Øystein Wiig. Mammal Collection. Natural History Museum. University of Oslo	Mm68
	241	**NW-11425**	NW	Hedmark county; Hummelneset	60,66666666	10,00000000	Øystein Wiig. Mammal Collection. Natural History Museum. University of Oslo	Mm68
	242	**NW-11849**	NW	Hedmark county;Fiskebekklia	60,66666666	10,00000000	Øystein Wiig. Mammal Collection. Natural History Museum. University of Oslo	Mm68
Russia	243	**T3-DS**	RUS	Kirov; Falyonskiy	58,28645716	51,72576388	Dimitry V. Skumatov	Mm49
	244	**T2-DS**	RUS	Kirov; Falyonskiy	58,28645716	51,72576388	Dimitry V. Skumatov	Mm50
	245	**3-DS**	RUS	Kirov;Kirov	58,64113102	49,73890856	Dimitry V. Skumatov	Mm50
	246	**1-DS**	RUS	Kirov;Kotelnich	57,90956709	48,42841075	Dimitry V. Skumatov	Mm50
	247	**25-DS**	RUS	Kirov;Omutninsk	58,68192200	52,45993112	Dimitry V. Skumatov	Mm50
	248	**26-DS**	RUS	Kirov;Omutninsk	58,50550889	52,20951833	Dimitry V. Skumatov	Mm50
	249	**10-DS**	RUS	Kirov;Rogovoe	58,55322641	50,72889005	Dimitry V. Skumatov	Mm50
	250	**11-RUS**	RUS	Leningrad ; Vyritsa	59,40242953	30,34069745	Dimitry V. Skumatov	Mm50
	251	**12-RUS**	RUS	Leningrad Province	59,83960937	32,60710947	Dimitry V. Skumatov	Mm50
	252	**T4-DS**	RUS	Kirov; Falyonskiy	58,28645716	51,72576388	Dimitry V. Skumatov	Mm51
	253	**T5-DS**	RUS	Kirov;Falyonskiy	58,28645716	51,72576388	Dimitry V. Skumatov	Mm51
	254	**T6-DS**	RUS	Kirov;Falyonskiy	58,28645716	51,72576388	Dimitry V. Skumatov	Mm51
	255	**T7-DS**	RUS	Kirov;Falyonskiy	58,28645716	51,72576388	Dimitry V. Skumatov	Mm51
	256	**7-DS**	RUS	Kirov; Kilmez	57,10802810	50,35695684	Dimitry V. Skumatov	Mm51
	257	**24-DS**	RUS	Kirov;Omutninsk	58,60975100	52,15365141	Dimitry V. Skumatov	Mm51
	258	**27-DS**	RUS	Kirov;Omutninsk	58,47725498	52,53821763	Dimitry V. Skumatov	Mm51
	259	**28-DS**	RUS	Kirov;Rogovoe	58,55322641	50,72889005	Dimitry V. Skumatov	Mm51
	260	**13-DS**	RUS	Komi; Vuktyl	63,86481284	57,49370460	Dimitry V. Skumatov	Mm51
	261	**16-DS**	RUS	Komi;Vuktyl	63,74323549	57,22442054	Dimitry V. Skumatov	Mm51
	262	**17-DS**	RUS	Komi;Vuktyl	63,79284541	57,85138037	Dimitry V. Skumatov	Mm51
	263	**31-DS**	RUS	Komi	63,45077326	58,13947871	Dimitry V. Skumatov	Mm51
	264	**32DS**	RUS	Kirov; Kilmez	56,93952999	51,07117099	Dimitry V. Skumatov	Mm51
	265	**14-DS**	RUS	Komi;Vuktyl	64,08524410	57,78018880	Dimitry V. Skumatov	Mm52
	266	**9-RUS**	RUS	Novgorod; Valdai Upland	58,17450399	33,25446861	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mm52
	267	**T1-DS**	RUS	Kirov; Falyonskiy	58,28645716	51,72576388	Dimitry V. Skumatov	Mm53

		268	**2-DS**	RUS	Kirov; Kirov	58,87975907	49,30391681	Dimitry V. Skumatov	Mm53
		269	**22-DS**	RUS	Kirov;Kotelnich	58,11629476	48,04265553	Dimitry V. Skumatov	Mm53
		270	**9-DS**	RUS	Kirov;Slobodskoy	58,55322641	50,72889005	Dimitry V. Skumatov	Mm53
		271	**15-DS**	RUS	Komi;Vuktyl	63,97211679	57,94858758	Dimitry V. Skumatov	Mm53
		272	**15-RUS**	RUS	Ural; Perm ; Kizel	59,00919378	57,67843556	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mm53
		273	**33DS**	RUS	Kirov; Kilmez	56,93952999	51,07117099	Dimitry V. Skumatov	Mm53
		274	**7-RUS**	RUS	Leningrad ; Podporozhye	60,91789328	34,18951020	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mm54
		275	**20-RUS**	RUS	Chelyabinsk; Krasnoarmeyskiy	55,31316016	61,99983177	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mm55
		276	**16-RUS**	RUS	Tver ; Bezhetsk	57,78443061	36,72009810	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mm56
		277	**17-RUS**	RUS	Tver ; Bezhetsk	57,78443061	36,72009810	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mm56
		278	**4-DS**	RUS	Kirov; Rogovoe	58,55322641	50,72889005	Dimitry V. Skumatov	Mm57
		279	**14-RUS**	RUS	Penza; Sosnovka Village	53,27628074	45,27853761	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mm60
		280	**Rsep9**	RUS	Near Kirov city	58,60344169	49,53794759	Dimitry V. Skumatov	Mm61
		281	**Rsep7**	RUS	Near Kirov city	58,60344169	49,53794759	Dimitry V. Skumatov	Mm62
		282	**T8-DS**	RUS	Kirov;Falyonskiy	58,28645716	51,72576388	Dimitry V. Skumatov	Mm63
		283	**Rsep8**	RUS	Tyumen region	55,45643150	69,43747698	Dimitry V. Skumatov	Mm64
		284	**Rsep11**	RUS	Tyumen region	55,45643150	69,43747698	Dimitry V. Skumatov	Mm65
		285	**19-RUS**	RUS	Chelyabinsk; Krasnoarmeisk	55,61168551	62,14651528	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mm66
		286	**Rsep6**	RUS	Tyumen region	55,45643150	69,43747698	Dimitry V. Skumatov	Mm66
		287	**10-RUS**	RUS	Leningrad; Boksitogorsk	59,46937200	33,86022200	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mm69
Sable (*Martes zibellina)*									
	Russia	288	**18-DS**	RUS	Tomsk;Alexandrovskoe	59,17328866	79,08158564	Dimitry V. Skumatov	Mz1
		289	**2-MZ**	RUS	Kamtchatka	54,61531892	158,39028767	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mz2
		290	**4-MZ**	RUS	Transbaikalia; Buryatia South; Dzhida Area	54,22023171	111,47659877	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mz3
		291	**19-DS**	RUS	Tomsk;Alexandrovskoe	59,17328866	79,08158564	Dimitry V. Skumatov	Mz4
		292	**1-MZ**	RUS	Transbaikalia; Buryatia North; Barguzin Area	54,22023171	111,47659877	Alexei Abramov. Laboratory of Mammalogy, Zoological Institute, Russian Academy of Sciences	Mz5
		293	**20-DS**	RUS	Tomsk; Alexandrovskoe	59,17328866	79,08158564	Dimitry V. Skumatov	Mz6
		294	**21-DS**	RUS	Tomsk; Alexandrovskoe	59,17328866	79,08158564	Dimitry V. Skumatov	Mz7

	295	**Rsep1**	RUS	Tomsk; Alexandrovskoe	59,10532493	79,15567035	Dimitry V. Skumatov	Mz8
	296	**Rsep2**	RUS	Tomsk; Alexandrovskoe	59,10532493	79,15567035	Dimitry V. Skumatov	Mz9
	297	**Rsep3**	RUS	Tomsk; Alexandrovskoe	59,10532493	79,15567035	Dimitry V. Skumatov	Mz10
Unknow	298	**FJ429093**						Mz11
	299	**NC011579**						Mz11

CHAPTER

3

Species Identification

PAPER III*

A non-invasive genetic method to identify the sympatric mustelids pine marten (*Martes martes*) and stone marten (*Martes foina*): preliminary distribution survey on the northern Iberian Peninsula

**The applicability and the usefulness of this method for improving our knowledge of different bio-ecological traits of sympatric marten species has been demonstrated in other three different researches which are included as Appendix A (Paper IIIa), B (Paper IIIb) and C (Paper IIIc).*

PAPER III

A NON-INVASIVE GENETIC METHOD TO IDENTIFY THE SYMPATRIC MUSTELIDS PINE MARTEN (*Martes martes*) AND STONE MARTEN (*Martes foina*): PRELIMINARY DISTRIBUTION SURVEY ON THE NORTHERN IBERIAN PENINSULA

ABSTRACT

The closely related mustelids European pine marten (*Martes martes*) and stone marten (*Martes foina*) sympatrically inhabit a large area of Europe. However, given our limited knowledge of their bioecological relationships, their extremely elusive behaviour and the fact that their faeces cannot be distinguished on the basis of morphology alone, it is very difficult to monitor their populations. In this study, we describe a reliable non-invasive polymerase chain reaction (PCR)–restriction fragment length polymorphism (PCR-RFLP) method for distinguishing between *M. martes* and *M. foina* based on the analysis of deoxyribonucleic acid extracted from faeces samples. The method was specifically designed to avoid possible interference from potential prey mammals and other sympatric carnivores. The procedure consists of PCR amplifying a mitochondrial D-loop region followed by digesting the resulting 276-bp-long amplicons with the restriction enzymes *Hae*III and *Rsa*I. To assess the efficiency of this technique, we conducted a preliminary field study across the potential sympatric distribution areas of both marten species in the northern Iberian Peninsula. Out of 359 faeces samples collected, we identified 80 as specimens from the stone marten and 235 from the pine marten. Unequivocal species identification was thus possible in 88% of the faeces samples collected. These findings reveal the combined use of non-invasive genetic sampling and GIS technology to be a reliable and cost-effective procedure for improving our knowledge of the spatial distributions of sympatric marten species. This protocol could also be used to identify and improve information gaps, to develop effective research and management programmes and in population and landscape genetics studies of marten species.

Keywords Non-invasive genetic sampling, Genetic species identification, Faecal DNA

INTRODUCTION

Accurate species identification is a key step in conservation biology and the basis for any study concerned with wildlife management and conservation (Frankham et al. 2002). However, obtaining this kind of information for elusive or cryptic carnivore species can be logistically difficult, particularly if relying entirely on field signs such as hair, faeces or tracks (e.g. see Piggott and Taylor 2003 for a recent discussion). These types of signs have been traditionally subjected to a variety of morphological analyses to establish their species of origin (Kohn and Wayne 1997). Nevertheless, there are situations in which samples deposited by carnivores of similar body size living in sympatry with the target species preclude any reliable identification at the species level on the basis of morphology alone (Davison et al. 2002; Birks et al. 2004). Thus, the misidentification of species from scats is a common event, as has been indicated for several sympatric carnivore species (Davison et al. 2002; Dalén et al. 2004; Riddle et al. 2003; Pilot et al. 2006).

The European pine marten (*Martes martes*) and stone marten (*Martes foina*), similar in terms of their morphology, feeding and behaviour, live sympatrically across a large area of Europe. *M. martes* is a threatened or scarce species in many countries where forest habitat loss and fragmentation are major threats for conservation. In contrast, the number of stone martens has increased in many countries of their current distribution range (Proulx et al. 2004).

Traditionally, the presence of martens has been determined through a variety of techniques such as roadkill or hunting information, live trap and sighting surveys (Messenger and Birks 2000), the use of track plates (Zielinski and Kucera 1995), camera traps (Zielinski and Kucera 1995), hair-snagging devices (Messenger and Birks 2000; Lynch et al. 2006) or scat-based surveys (Birks et al. 2004). Each of these methods has its relative merits or biases depending on the size of the area surveyed, the sampling effort and the cost and efficiency of the method (Birks et al. 2004). The monitoring of martens inferred from records of their scats seems to be the most effective for widespread surveys, as the animals are minimally disturbed. However, a necessary step in this type of survey is the reliable identification of the species from which the faeces was derived (Riddle et al. 2003; Dalén et al. 2004; Gómez-Moliner et al. 2004). Unfortunately, these two marten species cannot be reliably identified solely on the basis of faeces morphology (Marchesi et al. 1989; Pilot et al. 2006) and may be easily mistaken for species such as the red fox (*Vulpes vulpes*; Davison et al. 2002), European polecat (*Mustela putorius*), commom genet (*Genneta genneta*) and their related mustelid species stoats (*Mustela erminea*), American minks (*Mustela vison*; Birks et al. 2004) and European minks (*Mustela lutreola*).

Several genetic species identification methods have been recently developed for studies on carnivores based on noninvasive sampling. These methods include the amplification and direct sequencing of mitochondrial deoxyribonucleic acid (mtDNA; Höss et al. 1992; Murakami 2002; Davison et al. 2002), the use of species-specific mtDNA primers (Dalén et al. 2004), microsatellite allele frequencies (Randi and Lucchini 2002), species-specific allele lengths of a given microsatellite (Domingo-Roura 2002; Kalz et al. 2006; Pilot et al. 2006) or polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) methods (Hansen and Jacobsen 1999; Riddle et al. 2003; Gómez-Moliner et al. 2004; Vercillo et al. 2004; Colli et al. 2005). However, little attention has been paid to the reliability and utility of these laboratory techniques for non-invasive field studies or conservation programmes (Schwartz et al. 1998; Taberlet et al. 1999; Piggott and Taylor 2003; Kalz et al. 2006). Moreover, most of these reports only describe and present the particular molecular technique. There is therefore a need for studies assessing the field use of DNA techniques in species surveys to confirm their validity for widespread use in monitoring or conservation programmes (e.g. Mowat and Paetkau 2002; Pilot et al. 2006).

Recent molecular methods described for the genetic identification of *M. martes* and *M. foina* have been based on microsatellite analysis (Domingo-Roura 2002; Pilot et al. 2006) or PCR-RFLP of the Cyt-b gene (Vercillo et al. 2004; Colli et al. 2005). Pilot et al. (2006) proposed a method based on the simultaneous use of the microsatellites Mel 10 (Domingo-Roura 2002) and Ma 18 that is suitable for non-invasive samples and takes into account the two main sources of error in non-invasively collected material (allelic dropout and false alleles; Taberlet et al. 1996; Broquet and Petit 2004). However, the lower amplification rate and need for a higher number of replicates of nuclear DNA (nDNA) compared to mtDNA is a significant drawback of the broad-scale application of this method to large numbers of faecal samples.

In contrast, the PCR-RFLP methods developed by Vercillo et al. (2004) and Colli et al. (2005) have been mainly used on tissue samples, their feasibility for use on faeces has not yet been analyzed and their utility has not been tested in a real field study. We considered these techniques, but both require the use of primers known to work on a wide variety of vertebrates (Kocher et al. 1989). Given that the DNA of non-target species such as prey or other carnivores is often amplified during the identification procedure (personal observation), the use of these methods on faeces samples can compromise the quality of the data provided (Piggott and Taylor 2003).

Thus, the aim of this study was to develop a reliable and unambiguous non-invasive PCR-RFLP method for distinguishing between the scats of *M. martes* and *M. foina* through the analysis of mtDNA. We intended to avoid the interference from potential mammalian prey species or other carnivore species showing similar scat morphology because this is a common source of error when using field samples. Our

analysis focused on the mtDNA control region because of its rapid evolution rate, generating different haplotypes even among closely related species and populations (Tamura 2000), and because it relies upon the high copy number of mtDNA. Finally, to assess the field suitability of the technique, we conducted a preliminary study across the potential distribution area of both marten species in the northern Iberian Peninsula. Our findings revealed the presence and range of the two marten species and suggest the efficiency of the technique for use on large sample numbers.

MATERIALS AND METHODS

Design of PCR-RFLP assay

Sampling and DNA extraction

Fresh tissue and hair specimens from roadkill animals were used to isolate DNA from the target marten species, *M. martes* and *M. foina*. Marten specimens were collected mainly from several regions of the northern Iberian Peninsula but were also obtained from other European countries (France, Luxembourg, Estonia, Finland, Belarus and Russia). DNA was also isolated from several prey species and other sympatric carnivore species showing a similar scat morphology that could easily be misidentified as martens. The species, sample numbers and sample types are provided in Supplement 1. DNA was isolated from tissues and hairs using the Qiagen DNeasy ® Tissue DNA extraction kit according to the manufacturer's instructions.

Primer design

To design primers for the *Martes* species, we used the CLUSTAL X programme (Thompson et al. 1997) to check and align the mitochondrial D-loop sequences available from GenBank for the target marten species, other carnivore species, prey species and humans (*Homo sapiens*). Supplement 2 provides the GenBank accession numbers for the control region nucleotide sequences for all the species included in the alignment. Inter-specific D-loop sequence differences were targeted as potential marten-specific primer sites. We designed a forward primer Mm_L1 (5 - CCCAAAGCTGACATTCTAAC-3 adapted for the Martes genus from the L1607 primer of Davison et al. 1999) and a reverse primer designed specifically for this study, Mm_H1 (5 -ATGGGCCCGGAGCGAGAAGAGGTACAC-3). The primers were designed to amplify a short fragment of 276 bp to maximize the probability of amplifying degraded DNA as expected in faecal samples.

To verify the specificity of the primers designed for the two target species, we searched for primer binding sites using Amplify 3.1 software on all the sequences listed in Supplement 2. Their specificity was also evaluated using the primers on DNA extracted from fresh tissue and hair samples for all the species listed in Supplement 1.

PCR amplification of the D-loop region

A 2-µL volume of the DNA extraction mixture was added to 23 µL of the PCR mixture containing 0.5 µL of forward primer Mm_L1 and 0.5 µL of reverse primer Mm_H1 (20 pmol/µL), 2.5 µL 10× reaction buffer, 0.75 µL MgCl2 (50 mM), 1.4 µL deoxynucleotide triphosphates (2.5 mM), 1 µL of bovine serum albumin (10 mg/mL), 16.15 µL of sterile water and 0.2 µL *Taq* polymerase (5 U/µL). After incubation for 5 min at 94°C, the samples were subjected to 35 amplification cycles in a BIO-RAD iCycler (Version 3.021, BIO-RAD Laboratories) consisting of denaturation for 1 min at 94°C, annealing for 1 min at 63.5°C and a final extension stage of 1 min at 72°C. Annealing temperature was selected after temperature gradient PCR on tissuederived DNA. Four microlitres of the final amplified product was analysed by electrophoresis on 2% agarose gel. Negative controls were used to check for contamination.

DNA sequencing

PCR products were purified using the QIAquick® Tissue PCR Purification kit according to the manufacture's instructions and sequenced using the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in an ABI PRISM Model 310 Genetic Analyzer (Applied Biosystems). The new nucleotide sequences reported in this paper were deposited in GenBank under accession numbers EF200700 (*M. martes*), EF200701 and EF200702 (*M. foina*).

Restriction enzyme analysis

The CLUSTAL X programme (Thompson et al. 1997) was used to check and to align the mitochondrial D-loop sequences and their different haplotypes for the two target marten species and for the carnivore species whose DNA sequences were also amplified by the primer pairs developed (*M. putorius*, *M. lutreola*, *M. erminea* and *M. vison*; Supplement 2). The sable (*Martes zibellina*) was also included, as its distribution range overlaps that of *M. martes* around the Ural Mountains in central Russia, where hybridisation between the two species is common (Grakov 1994; Helldin 1998; Davison et al. 2001).

The red fox and common genet were not included in the RFLP test because their DNA was not amplified by the primers developed.

Polymorphic sites were determined using In silico software (Bikandi et al. 2004). The restriction enzymes *Rsa*I and *Hae*III were identified as those generating different RFLP patterns for each species (Fig. 1). The robustness of the assay was tested using the selected enzymes to digest PCR products obtained from all the species listed in Supplement 1.

DNA digestions by endonucleases were run in 14-µL volumes, consisting of 1.4 µL of the appropriate 10× reaction buffer (supplied by manufacturer with the respective enzyme), 0.2–0.4 µL of restriction enzyme solution (10 U/µL), 5 µL of the PCR product and the remaining volume of pure water. Incubations were performed for 6 h at 37°C, followed by a 3% agarose gel electrophoresis of 10 µL of the digestion products. Ethidium bromide-stained bands were visualized and photographed using an UVIdoc LCD system (UVITEC).

Fig. 1 Aligned sequences of the amplified mitochondrial D-loop fragment in *Martes martes* and *Martes foina*. The primer sequences are shown in bold. The restriction sites for *Hae*III (recognition sequence GGCC) and *Rsa*I (recognition sequence GTAC) are underlined and in bold. Restriction patterns (Table 1) are indicated *in brackets* for each species.

```
Martes martes (AA)    CCCAAAGCTGACATTCTAACTAAACTATTCCCTGATTTCCTCTCCCTATGTCTTAATTCA 60
Martes martes (AB)    ............................................................ 60
Martes foina  (BC)    .................................C.........C.C..T........ 60

Martes martes (AA)    TATATTTAATAACATTTACTGTGCCTCCCCAGTATGTACTTTTTCCCCACCCCTATGTAT 120
Martes martes (AB)    .................................GTAC.............GTAC 120
Martes foina  (BC)    .............CC....GTAC.........GTAC.C......AC.......... 120

Martes martes (AA)    ATCGTGCATTAGTGGTTTGCCCCATGCATATAAGCATGTACATGTTATGCTTGATCTTGC 180
Martes martes (AB)    .................................GTAC..A.C...T..A....... 180
Martes foina  (BC)    .................................GTAC.............T..A. 180

Martes martes (AA)    ATTCGTGCACCTCACTTAGATCACGAGCTTAATCACCAGGCCTCGAGAAACCATCAACCC 240
Martes martes (AB)    ..................................GGCC.................. 240
Martes foina  (BC)    .........TT....C....C.........G.......A..................... 240

Martes martes (AA)    TTGCCCGATGTGTACCTCTTCTCGCTCCGGGCCCAT 276
Martes martes (AB)    ..................................... 276
Martes foina  (BC)    ....TA..C.............................. 276
```

Use of the PCR-RFLP assay on faecal samples: preliminary field study of the genus Martes *in the northern Iberian Peninsula*

The faeces identification technique was tested in a study performed across the current sympatric distribution range of the two *Martes* species in the northern Iberian Peninsula (Proulx et al. 2004). From September 2002 to September 2006, we collected 359 faeces samples initially identified as Martes sp. based on scat morphology. Sampling was conducted along linear features, such as forest trails and paths, for which pine martens show a preference. Universal Transversal Mercator (UTM) coordinates were recorded for all the samples collected using a global positioning system (Garmin eTtrex). The faecal samples were stored in autoclaved tubes containing ethanol 96% and frozen at –20°C until processed.

The faecal DNA extraction procedure was based on the protocol described by Gómez-Moliner et al. (2004). Extraction blanks were included to check for crosscontamination and were processed as samples in the subsequent amplifications. DNA extractions were carried out in a specialized laboratory to avoid contaminating DNA.

The PCR-RFLP assay described above for tissue/hair samples was used with the necessary modifications on faecal samples. These modifications comprised of increasing DNA extraction solution from 2 to 4 µL and inclusion of positive controls obtained from tissue/hair samples of known M. martes and M. foina specimens in all processes. The PCR protocol was also slightly modified to a touchdown PCR procedure. This involved eight extra cycles after the first denaturation step at 94°C for 2 min of 1 min at 95°C, 40 s at 72°C (reducing the temperature 0.5°C per cycle) and 45 s at 72°C to enhance the enrichment of the specific product over any non-specific one. Finally, the UTM coordinates corresponding to each sample were projected onto a GIS (Arcview 9.0. ESRI) along with the species identification data provided by the PCR-RFLP assay.

RESULTS

PCR-RFLP assay

Primer design and PCR amplification

The accuracy of the newly designed D-loop primers Mm_L1 and Mm_H1 was tested on sequences for the two target species (Supplement 2.1) using the Amplify 3.1 programme and on sequences obtained from the tissue/hair specimens from the 50 *M. martes* and 32 *M. foina* roadkill individuals collected from different regions of the martens' current European distribution area (Supplement 1.1). In all virtual analyses (Amplify v.3.1), the primer pair Mm_L1/ Mm_H1 consistently generated the expected amplicons of 276 bp length, which it subsequently also did in reality in all tissue/hair DNA samples tested (Fig. 1).

In contrast, when Amplify 3.1 was used on the other carnivore species sequences (Supplement 2.2), no virtual amplification products were obtained except for *M. putorius*, *M. lutreola*, *M. erminea* and *M. vison* (Table 1). Moreover, of the potential prey species tested (Supplement 2.3), only *Sorex araneus* rendered an amplification product of 900 bp using Amplify 3.1. These theoretical results were checked by running assays on DNA extracted from tissue and hair specimens of all the carnivore and prey species (Supplement 1). No amplification products were obtained for any of the species, except the four *Mustela* species and *Sorex coronatus*. This crosscheck confirmed the high specificity of the markers developed for *Martes* and

Mustela species, whose scats are similar in size and shape. The primers failed to amplify DNA corresponding to the potential marten prey species examined with the exception of the *Sorex* spp. that rendered a 900-bp amplicon. Notwithstanding, this 900-bp amplicon was not produced when working with degraded DNA, such that we could rule out any interference effects of prey species when the PCR assay was used on faeces samples. No human DNA amplicons were generated by the PCR assay (Supplements 1.4, 2.4)

Table 1 Amplicon lengths in base pairs and diagnostic restriction enzyme patterns generated using *Hae*III and *Rsa*I

	Species	Amplicon length (bp)	*Hae*III fragment size (bp)	*Hae*III Restriction Pattern	*Rsa*I Fragment size (bp)	*Rsa*I Restriction pattern	Combined restriction pattern
Marten species	*Martes martes*	276	*220, 51, 5*	A	*97,94, 62,* 23	A	AA
					97,94, 41, 21, 23	B	AB
	Martes foina	276	*271,* 5	B	*94, 82, 62,* 23, 15	C	BC
	Martes zibellina	276	*271,* 5	B	*97,94, 41,* 21, 23	B	BB
			220, 51, 5	A	*158, 94,* 24	D	AD
Carnivore species showing similar scat morphology[a]	*Mustela putorius*	280–287	*275–282,* 5	B	*121–8, 93, 41,* 25	E	BE
					93, 82, 40,41, 25	F	BF
	Mustela lutreola	280–281	*275–6,* 5	B	*134, 121–122,* 25	G	BG
					82, 39–40, 134, 25	H	BH
	Mustela vison	286–287	*281–2,* 5	B	*128–9, 117, 41*	G	BG
					117, 99, 41, 30	E	BE
	Mustela erminea	276–277	*135–6,136,* 5	C	*118–19, 94, 41,* 23	E	CE

Fragment sizes that could be visually identified on agarose gel for each enzyme are shown in italics. Fragments italicized and set in bold could not always be identified on agarose gel. [a] The red fox and common genet were not included because their DNA was not amplified by the PCR method.

Restriction fragment length polymorphism

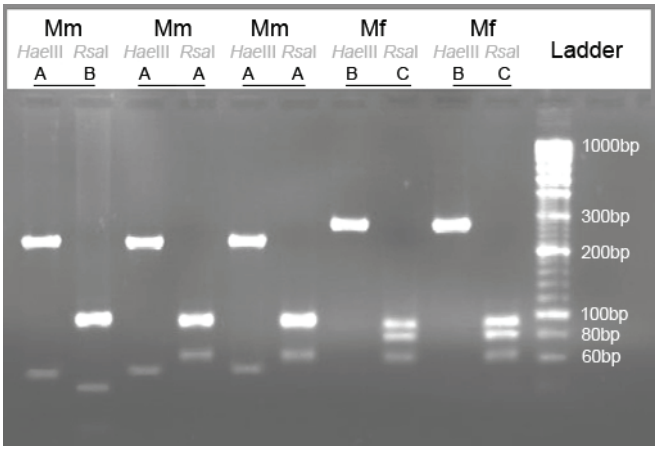
Through a detailed comparison of the restriction maps of the sequences obtained here and those from GenBank (Supplement 2), the position of cleavage sites for the restriction endonucleases *Hae*III and *Rsa*I were found to be suitable for differentiating *M. martes* from *M. foina* (Fig. 1), from the other carnivore species whose DNA was amplified by the selected primers (*M. putorius*, *M. lutreola*, *M. erminea* and *M. vison*) and from *M. zibellina* (Table 1). *M. martes* displayed a 220- and a 51-bp fragment (restriction pattern A) after digestion with HaeIII (the remaining 5-bp-long band was not visible on agarose gel). Further, M.martes rendered two restriction patterns after mitochondrial D-loop digestion with RsaI because of a base substitution (T–C) in the cleavage site of the enzyme (Fig. 1). Digestion pattern A

included a thick electrophoretic band corresponding to 97- and 94-bp-long fragments and a second 62-bp-long band. Digestion pattern B was a thick electrophoretic band corresponding to the 97- and 94- bp-long fragments and a second band of 42 bp length. *M. foina* showed a 271-bp-long fragment (restriction pattern B; the remaining 5-bp-long band was not visible on agarose gel) after digestion with *Hae*III. *Rsa*I digestion produced five fragments of 94, 82, 62, 23 and 15 bp length appearing as three electrophoresis bands (restriction pattern C). All the RFLP patterns are shown in Fig. 2.

Thus, *Hae*III enzyme digestion served to differentiate the two species, but to avoid false identifications and obtain species-specific restriction patterns, the simultaneous use of *Rsa*I was required.

Unambiguous interpretation of the results could be achieved visually without the need of computer analysis. Using this technique, two haplotypes can be discriminated for *M. martes* (AA, AB) and one for *M. foina* (BC; Fig. 2). Restriction digestion patterns that could not be ascribed to any of the species investigated were not observed. Moreover, other European mustelids producing morphologically similar faeces showed different RFLP haplotypes (Table 1). In 115 out of 119 samples analysed (Supplement 1; *M. martes*, n=52; M. foina, n=32; *M. putorius*, n=10; *M. lutreola*, n=10; M. erminea, n=5; M. vison, n=10), our RFLP analysis yielded results that matched the theoretical RFLP patterns obtained using In silico software (Bikandi et al. 2004). One of the exceptions corresponded to a specimen erroneously identified as stone marten (*M. foina*), which showed pine marten (*M. martes*) restriction patterns. On the other hand, one specimen labelled as pine marten from Finland was identified as *M. zibellina* by our PCRRFLP method.

Fig. 2 Photograph of a 3% agarose gel showing ethidiumbromide stained bands and diagnostic restriction enzyme patterns (AB; AA; BC) generated using *Hae*III and *Rsa*I for *Martes martes* (Mm) and *Martes foina* (Mf)



Use of PCR-RFLP on faecal samples: a preliminary field study of the Martes *genus in the northern Iberian Peninsula*

Out of 359 faecal samples collected from the entire sympatric distribution range of both marten species in the northern Iberian Peninsula (Proulx et al. 2004), 316 were classified as one of the target species by our PCR-RFLP method. Thus, unequivocal species identification was possible in 88% of the samples. This represents a high species identification rate for the technique used on faecal samples. In the remaining 12%, the DNA extracted was not amplified by the primers used.

The geographical locations for the 316 correctly identified faecal samples in the Iberian Peninsula are shown in Fig. 3a. We effectively identified 80 faecal samples as stone marten and 235 as pine marten. One sample was identified as the European polecat, indicating visual confusion of the scat in the field survey. While in some forested sampling areas, the prevalence of the pine marten was clear (sampling areas 1, 2, 3 and 6), in other forested areas, the ranges of both marten species fully overlapped (sampling areas 4 and 5). The overlapping distribution ranges of the martens in sampling area 4 are depicted in Fig. 3b. This area has been selected because of the high number of samples of both Marten species (N=190) analysed.

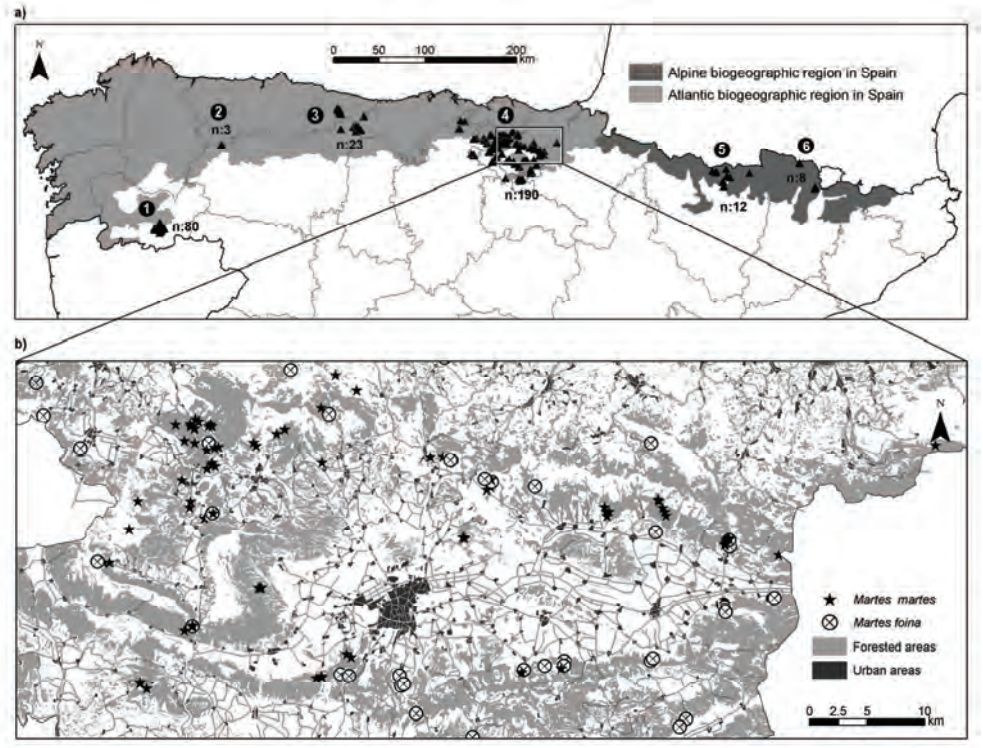


Fig. 3 a GIS determined geographical locations and number of samples ascribed to the target species after applying the PCR-RFLP method to faecal samples collected in the northern Iberian Peninsula. Sampling areas are designated by successive numbers (1–6). b Detail of the overlapping distribution of *Martes martes* and *Martes foina* within sampling area 4. Forest and urban areas appear shaded in grey and black, respectively. Black stars and circles represent the geographical locations of faecal samples identified as *Martes martes* and *Martes foina* by the proposed PCR-RFLP method, respectively

DISCUSSION

PCR-RFLP method

The use of the technique developed here on all published partial D-loop sequence haplotypes and tissue samples of the target martens and other carnivores from several regions of Europe included in our study revealed the method to be highly robust. Our assay was also highly reliable, as positive results were obtained only for *Martes* and *Mustela* species. Other sympatric carnivore species, in particular the red fox and common genet, whose scats can be easily mistaken for those of martens, rendered no amplicons. A wide screen for potential mammal preys of the martens of interest gave no amplicons except for *Sorex* spp. However, *Sorex* spp. amplicons were very different in size (900 bp) compared to those yielded by the mustelids, thus precluding misidentification. Moreover, a large 900-bp fragment is unlikely to provide an intact template for amplification (Kohn and Wayne 1997; Broquet et al. 2006). Thus, interference because of the presence in faeces samples of genetic material from species consumed by the martens can be in principle ruled out.

When we tested the method in faecal samples collected in the field survey, a high species identification rate was obtained (88%). The remaining 12% of samples did not amplify. The possibility that some samples had been incorrectly identified in the field as marten scats would mean an even higher correct identification rate. Particularly, confusing fox as marten scats is a common error, as indicated by Davison et al. (2002).

One of the advantages of our protocol is that it relies on the high copy number of mtDNA for improved species resolution in low-quantity, low-quality samples. Further, the use of multiple restriction sites reduces the risk of misidentification. In addition, a short DNA fragment (276 bp) is more likely to provide an intact template for amplification (Kohn and Wayne 1997; Broquet et al. 2006) when working with degraded DNA and can be effective even in old and/or rain-washed samples.

Some of the restriction patterns yielded by the method were found to be shared by *M. vison*, *M. lutreola* and *M. putorius*. Thus, previously described specific methods (Gómez-Moliner et al. 2004) should be used to distinguish between these species. Moreover, the discrimination of all the mustelid species treated in the present paper might already become possible using *Rsa*I digestion alone with polyacrylamide gels, which would allow visualizing DNA fragments differing in a few bases length. However, the application of agarose gels facilitates the use of this method even in basic field laboratories.

Comparison with other methods

The PCR-RFLP method described in this paper was especially designed for non-invasively collected samples and specifically for faecal material. With this purpose in mind, we were able to reliably distinguish other carnivore species whose scats could be confused as marten scats. Besides, interference because of prey DNA was avoided through the use of mustelid-specific primers. Similar PCR-RFLP methods based on Cyt-b (Vercillo et al. 2004; Colli et al. 2005) have been mainly developed for tissue samples, and their feasibility or efficiency for use on faecal DNA has not been tested. Notwithstanding, given (1) that the primers used by both sets of authors are generic for vertebrates (Kocher et al. 1989; personal observation), (2) that both protocols give rise to similar diagnostic fragments using faecal DNA from other carnivores (Pilot et al. 2006; personal observation) and (3) that potential prey DNA (e.g. from *Cletryonomys glareolus*, *Microtus agrestis*, *Mus musculus*) will be amplified and interfere with the restriction patterns for the martens (personal observation), these methods are unlikely to be useful for identifying martens in scat surveys. The approach recently developed by Pilot et al. (2006) for the genetic identification of both marten species by microsatellite analyses has the main advantage that the costs of species identification would be entirely absorbed in the pursuit of intraspecific genetic analysis by genotyping of faeces. Nevertheless, the success rate of the PCR-RFLP method proposed based on the mitochondrial D-loop region is higher than that based on microsatellites: 88 vs 53.4%. This can be explained by the higher copy numbers of mtDNA compared to nDNA, conferring a greater success rate to the method for screening large numbers of samples across broad geographical areas. The amplification of nDNA requires two or even three replicates to obtain a valid result when working with degraded DNA (Taberlet et al. 1996; Miller et al. 2002; Broquet and Petit 2004). This would mean an increased time and cost expenditure for the nDNA analysis compared to our PCR-RFLP technique.

Preliminary field study of the genus Martes *in the northern Iberian Peninsula*

The development of non-invasive DNA techniques for species identification has recently offered researchers the possibility to design reliable programmes for monitoring elusive species as an alternative to the use of traditional sources of distribution data.

In the case of the sympatric marten species examined here, there is a clear need to monitor their presence and distribution across their distribution ranges (Proulx et al. 2004). These two species show inverse demographic trends, a different population status and probably compete for the same resources. Thus, their management needs are likely to differ. Moreover, given our limited knowledge of their bioecological

relationships, their extremely elusive behaviour and the fact that their faeces are morphologically indistinguishable, it is not easy to monitor populations of these mustelids.

Most scat surveys on the pine marten conducted in its sympatric distribution area with the stone marten have assumed that stone martens rarely penetrate forests (e.g. Delibes 1983; Russell and Storch 2004). This assumption, however, is clearly an oversimplification of the complex sympatric relationship between the two species and can lead to false interpretations of their distribution patterns (Pilot et al. 2006).

The results obtained in the present preliminary field study indicate that sympatric relationships differ depending on the habitat type, forest structure, as well as on landscape effects of forest fragmentation and human disturbance of the surrounding habitat. Despite the fact that, as a general rule, the pine marten has a clear dominance over the stone marten in late-successional forests (Fig 3a sampling areas 1, 2, 3 and 6) and displaces the stone marten to surrounding areas, both species are known to co-exist in many forested areas (Fig 3a sampling areas 4 and 5). Our results suggest that the increasing effects of forest fragmentation and current inverse demographic trend of the two species have led to a high complexity of their bioecological relationships. These factors have determined that the co-existence of both species has become common on forest patches within mosaic habitats (Fig. 3b).

The results provided by the combined use of non invasive genetic analysis of faecal samples and GIS technology tested in this preliminary field study suggest that our protocol would serve to assess the effects of human activities on distribution patterns, identify and resolve information gaps and design effective research and management programmes.

The information obtained in our field study requires confirmation through an extended sampling effort. However, it is a first step towards improving our knowledge of distribution patterns of sympatric marten species, at both broad and small scales in the northern Iberian Peninsula.

In conclusion, our results indicate that the PCR-RFLP method proposed for use on non-invasive samples (faeces or remotely plucked hair) is a reliable, efficient, time-saving and cost-effective procedure for improving our knowledge of the spatial distributions of sympatric marten species. This method has applications in studies focusing on the population genetics, phylogeographic variation and landscape genetics of marten species.

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SUPPLEMENTARY MATERIAL

S1 Hair/Tissue samples analyzed [a]

1. Marten target species

Martes martes (European pine marten; n:50, h:22-t:28); *Martes foina* (stone marten; n:32, h:11-t:21).

2. Carnivore species showing a similar scat morphology that could easily be misidentified as martens

Vulpes vulpes (red fox; n:10, h:2-t:8) *Mustela putorius* (European polecat; n:10, h:1-t:9); *Genneta genneta* (commom genneta*;* n:10 h:8-t:2)*; Mustela erminea* (stoat; *n:5, h:5); Mustela vison* (American mink; n:10, h:1- t:9) *Mustela lutreola (*European mink; n:10, h:8-t:2).

3. Potential mammalian prey species

*Apodemus flavicolis (*yellow-necked field mouse; n:3, t:3); *Apodemus sylvaticus (*European woodmouse; n:4, t:4*)*; *Crocidura russula (*white-toothed shrew; n:4; t:4); *Mus musculus (*house mouse; n:4, t:4); *Microtus agrestis* (short-tailed field vole; n:4, t:4); *Microtus arvalis (*common vole; n:4, t:4); *Sorex coronatus* (crowned shrew; n:5, t:5); *Suncus etruscus* (white-toothed pygmy shrew; n:3, t:3); *Neomys fodiens* (Eurasian water shrew; n:5, t:5*)*; *Clethryonomis glareolus* (bank vole, n:4, t:2-h:2); *Glis glis* (fat dormouse; n:3, h:3); *Sciurus vulgaris* (Eurasian red squirrel; n:4, h:1-t:3); *Elyomis quercinus* (garden dormouse; n:2, h:2); *Talpa europaea (*European mole; n:4, t:4); *Erinaceus europaeus* (western European hedgehog; n:5, t:5); *Cervus elaphus* (red deer; n:5, t:5), *Capreolus capreolus* (western roe deer; n:5, t:5); *Sus scrofa* (wild boar; n:5, t:5).

4. Human

Homo sapiens (Human; n:4, h:4)

[a] n: number of samples analysed; t: tissue sample; h: hair sample

S2 GenBank accession numbers of D-loop sequences analyzed

Newly obtained sequences of *Martes martes* and *Martes foina* were analysed together with sequences from the following mammalian species for the PCR-RFLP design. The partial sequences that did not fully incorporate the fragment analysed were included with complete sequences to check for possible changes in the D-loop sequences.

1. Marten Species

Martes martes (European pine marten; AF336949-AF336964, AF336968, AF336969 AJ585357); *Martes foina (stone marten;* AF336973, EF200701, EF200702); *Martes zibellina (*Sable; AF336965-AF336967, AF336970, EF200700).

2. Carnivore species showing similar scat morphology [a]

Vulpes vulpes (red fox*;* AJ585358, AF487753, AF487752, AF487736-AF487746, AF338789, AF338802-AF338790 D83639, AF098155, AM181037); *Mustela putorius* (European polecat; AY962022-AY962045, AF207718, AF207726, AF068560, AF068566-AF068568, AF068570); *Mustela erminea (*stoat; AB061215, AB049782-AB049788, AB049777-AB049780; AJ585353-AJ585356, AB006729-AB006733); *Mustela vison (*American mink; AJ585352-AJ585350, AB052720*); Mustela lutreola (*European mink; AF207720-AF207725, AJ548803-AJ548814).

3. Potential mammalian prey species

Sciurus vulgaris (Eurasian red squirrel; AJ238588, AB249881, NC_002369, AF111001-AF111026); *Apodemus sylvaticus* (European woodmouse; AY588255, AY588252, AY588264, AY588253, AJ410624, AJ410626-AJ410628,); *Apodemus flavicolis* (yellow-necked field mouse; AY588253, AY588264, AJ410624-AJ410631); *Mus musculus* (house mouse; NC_005089, AB042809, AB049357, AB042523, AB042524); *Mus spretus* (western wild

mouse; AF287305, U47539); *Rattus norvegicus* (Norway rat; X57080, X57081, DQ897633-DQ897638, DQ673907-DQ673917, AJ428514, NC_001665, AC_000022, X14848, AY172581, X52757, X04733, X04734); *Rattus rattus* (black rat; AB211039, DQ009781-DQ009794, X04735); *Microtus agrestis* (short-tailed field vole; F267270, AY948542) *Microtus rosameridionalis* (southern vole; DQ323955, NC_008064, DQ015676); *Microtus liechtensteini* (AF267260-AF267266, AF267278-AF267283); *Microtus subterraneus* (European pine vole; AF267246, AF267247, AF267250, AF267271- AF267275); *Microtus oeconomus* (root vole; AF267268, AF267269); *Microtus arvalis* (common vole; AJ009883, DQ121418, AY708394-AY708459, DQ181947-DQ182012, AF267285) *Microtus nivalis* (snow vole; AF267284); *Clethrionomys glareolus* (bank vole; AJ236833, Y07543, AY185801-AY185810, AY133323-AY133326, AY133303-AY133315, AF165533) *Erinaceus europaeus* (western European hedgehog; NC002080, X88898, AF379703-AF379749); *Talpa europaea* (European mole; Y19192); *Galemys pyrenaicus* (Iberian desman; DQ305040); *Crocidura russula* (white-toothed shrew; NC006893, AY918341-AY918368, AY769264 AY769263, AF343009-AF343017, X90952); *Sorex araneus* (European shrew; X90951, AY781981-AY781060, AH014450-AH0144849); *Oryctolagus cuniculus* (European rabbit; AJ001588, AJ535817-AJ535822, AJ535791-AJ535792, AJ535785, AJ535784, AJ563720-AJ563721, AF534080-AJ563710, AJ293843-AJ293832, AJ293837, AJ293839, Z83356-Z83363, Z83351, Z83347, Z83345, Z83367, Z83353, Z83355, Z83349, Z83340-Z83344, AF157459, U62924-U62927, AJ535793-AJ535816, AJ535786-AJ535790, AJ535786-AJ535788, AJ563722, AJ563711-AJ563719, AJ293838-AJ293840, AJ293841-AJ293842, AJ293836-AJ293831, Z83364-Z83366, Z83352, Z83354, Z83358, Z83350, Z83346, AF003189-AF003195); *Lepus castroviejoi* (Broom Hare; DQ883207); *Lepus europaeus* (European hare; DQ645450, DQ883196-DQ8832016, AY876080 -AY876118, AY466782-AY466853, AY300032-AY300036, AY1546661-AY154666, AY103494-AY103531, AY163356-AY163376, AF1574363-AF157454, DQ645432 -DQ645449, AJ421471, Y15315); *Cervus elaphus* (red deer; AM419026, DQ386106-DQ386110, AM279271, DQ452075-DQ452087, NC007704, AB012385, AF016972-AF016973); *Capreolus capreolus* (roe deer; AM419028, DQ114784, DQ384640-DQ384708, DQ114745-DQ114783, AM279273, AY625732-AY625892, Z70318, AJ287365-AJ287383); *Sus scrofa* (wild boar; D17739, D16483, AJ854456).

4. Human

Homo sapiens (Human; AF254896 and D38112).

[a] As there were no available D-loop sequences for *Gennetta gennetta* in Genbank, the PCR procedure was only tested on tissue/hair samples.

CHAPTER

4

RELIABLE FAECAL DNA GENOTYPING OF SYMPATRIC MARTENS

PAPER IV

Reliable faecal DNA genotyping of sympatric marten species (*Martes martes* and *Martes foina*): The impact of sample collector field experience on species and individual identification success rates

PAPER IV

RELIABLE FAECAL DNA GENOTYPING OF SYMPATRIC MARTEN SPECIES (*Martes martes* AND *Martes foina*): THE IMPACT OF SAMPLE COLLECTOR FIELD EXPERIENCE ON SPECIES AND INDIVIDUAL IDENTIFICATION SUCCESS RATES

ABSTRACT

Non-invasive wildlife research using DNA from faeces has become increasingly popular. In this study, we assessed the reliability and success of using genetic techniques to determine species and individual identification of sympatric martens (*Martes martes* and *M. foina*) by genotyping non-invasively collected faecal samples. First, we developed a novel and accurate multiplex panel of 15 microsatellites *loci*, selected by cross species amplification of 41 *loci*. The application of this panel facilitated species distinction, discarding the presence of putative hybrids. Further, in order to evaluate the impact of sample collector experience on DNA quality, we conducted a genetic survey all over the sympatric range of both marten species in the Iberian Peninsula by three different kinds of sample collectors (wildlife biologist; trained volunteers and technical staff from natural parks). In order to achieve this goal, we evaluate the a) success of PCR-RFLP identification of *Martes* sp. faecal samples and b) genotyping success and error rates of faecal *M. martes* samples. Our results show that the difference in the level of expertise between sample collectors significantly influence the success rate of microsatellite genotyping of pine marten faecal samples (mean 45% n=317) but not the species identification success rate (mean 84%, n=634). Based on our results, we recommend conducting sampling by an experienced biologist to maximize non-invasive sampling and DNA quality so as to ensure accurate genotyping success. Application of our methods to field collected scats can be used in a cost-effective way so as to investigate distribution, patterns of genetic diversity and structure as well as to estimate population abundance for sympatric martens.

Keywords Non-invasive genetic sampling, Microsatellites, Genotyping success, Faecal DNA, Genetic species identification, *Martes*

INTRODUCTION

The European pine marten (*Martes martes*) and stone marten (*Martes foina*), species, which are similar in morphology and feeding habits, are closely related mustelids that live to a large extent sympatrically over an extensive area of Europe (Proulx et al. 2004). The North Iberian Peninsula constitutes the present south-western distribution limit of the pine marten and consequently the southernmost sympatric area with the stone marten (López-Martín, 2007). *M. martes* is either threatened or scarce in many countries where forest habitat loss and fragmentation are major threats (Bright 1999). By contrast, the number of stone martens has increased in many countries where they are currently distributed (Proulx et al. 2004) and they have even invaded urban areas (Herr et al. 2009). However, given our limited knowledge of their ecological niches in sympatric areas, their extremely elusive behaviour, and the fact that their faeces are morphologically indistinguishable, there is considerable difficulty in studying and monitoring their populations (Ruiz-González et al. 2008). As these inverse demographic trends and probable competition between the two species may lead to the reduction of the pine marten's range (Goszczyński et al. 2007) and the geographic expansion of the stone marten, it is important to monitor the presence and numbers of these species in different parts of Europe (Proulx et al. 2004).

Moreover, molecular phylogenetic studies confirmed that these two species are closely related, although they are not the most related within the subgenus *Martes* (Stone & Cook 2002; Sato et al. 2003; Marmi et al. 2004; Koepfli et al. 2008). Hybridization between *Martes* species has been previously documented (Davison et al. 2001; Kyle et al. 2003; Grakov et al. 1994). Nevertheless, sympatric, *M. martes* and *M. foina* do not interbreed, or at least this has not been previously reported (Davison et al. 2001).Thus, additional analyses, including population genetic-level sampling, will be very useful in confidently resolving relationships among these recently evolved species (Koepfli et al. 2008) and verifying possible hybridization events.

Molecular methods incorporating non-invasive sampling via the collection of scats or hairs have become common for population monitoring of carnivores, providing valuable information about species and individual identification (Taberlet & Luikart 1999; Piggott & Taylor 2003; Waits & Paetkau 2005; Schwartz & Monfort 2008; Beja-Pereira et al. 2009).

The standard method for monitoring marten populations are scat transect surveys (Birks et al. 2004). Consequently, faeces should provide a plentiful non-invasive source material that is easy to collect for genetic surveys (Beja-Pereira et al. 2009; Ruiz-González et al. 2008). However, the applicability of non-invasive techniques to a new species must be explored before embarking on large-scale studies (Taberlet &

Luikart 1999; Waits & Paetkau, 2005; Valiere et al., 2007). The drawbacks of non-invasively collected faecal samples of mustelids are (a) that species determination of faecal samples may be misidentified even by experienced collectors (Davison et al. 2002; Birks et al. 2004) and (b) extracts from field-collected faeces usually yield little target DNA and may contain polymerase chain reaction (PCR) inhibitors, potentially leading to false allele amplification (FA) and/or allelic drop-out (ADO) on microsatellite genotyping procedures (Waits & Paetkau, 2005). Consequently, working with non-invasively obtained faecal samples of sympatric martens requires a previous determination of species identity so that we can thereafter apply a valuable and informative microsatellite panel useful on faecal samples. In this context, previous studies have recommended that any study requiring a non-invasive genetic method should be preceded by a pilot study in which the probability of identity, as well as genotyping errors, should be assessed (Taberlet & Luikart 1999; Valière et al.2007).

Recent progress in molecular techniques has supplied several non-invasive genetic methods for the species identification of martens and related mustelids (e.g. Gómez-Moliner et al. 2004; Livia et al. 2007; O´Reilly et al. 2008; Ruiz-González et al. 2008) which can be applied to field monitoring. However, there are currently no microsatellites developed specifically for *M. martes* and *M.foina* for individual identification, which could provide valuable information for population censuses and to address questions regarding the population genetics of free ranging marten populations (Beja-Pereira 2009). Nevertheless, cross-amplification of loci identified in related species should be used. Previous studies focused on pine marten have given information about the genetic variability and structure of several European populations (Kyle et al. 2003; Mergey 2007; Pertoldi et al. 2008; Mullins et al. 2010). However, information regarding a valuable microsatellite panel for Iberian Peninsula populations of pine martens or stone martens is unavailable. Moreover, theses studies were mainly focused on tissue (Kyle et al. 2003, Mergey 2008; Pertoldi et al. 2008) or remotely plucked hair (Mullins et al. 2010) so no information about the effectiveness of microsatellite markers on faecal marten samples is currently available.

In addition, to effectively and efficiently apply faecal DNA analysis in large-scale studies, it is important to identify the variables that impact PCR amplification success (Waits & Paetkau 2005; Murphy et al. 2007). Although laboratory procedures can be optimised to reduce the impact of genotyping errors and increase genotyping success [e.g. faecal preservation method (Wasser et al. 1997; Murphy et al. 2000; Murphy et al. 2002; Piggott & Taylor 2003) DNA extraction method (Flagstad et al. 1999; Goossens et al. 2000; Frantz et al. 2003; Wehausen et al. 2004), and amplification method (Goossens et al. 2000; Bellemain & Taberlet 2004; Piggott et al. 2004)], other extrinsic field factors [e.g. age of the faecal sample (Lucchini et al. 2002; Piggot 2004, Santini et al. 2007; Brinkman et al. 2010), weather conditions (Farrell et al. 2000;

Lucchini et al. 2002; Piggot 2004; Brinkman et al. 2010), diet (Murphy 2003; Murphy et al. 2007) and/or season (Maudet et al. 2004; Hajkova et al. 2006)], are usually difficult to control in non-invasive field surveys (Piggot & Taylor, 2003). Also, the considerable remaining variability among studies implies that other unidentified parameters are acting (Broquet et al. 2007). In many different non-invasive genetic studies the sample collection procedure was conducted by different staff with very different field experience [e.g. game wardens and hunters (Bellemain et al. 2005); experienced field volunteers (Jacob et al. 2010), specialised wildlife biologists (Rossellini et al. 2008) rangers and technical staff from protected areas and/or a combination of different personnel (Fabri et al. 2007)]. The difference in the level of expertise between sample collectors potentially impacts accuracy in the species identification of faecal samples (Davison et al. 2002; Zuercher et al. 2003; Prugh & Ritland 2005) and results in population size estimation surveys of rare or elusive species (e.g. Jacob et al. 2010). This bias may be limited if volunteers have the opportunity to train and acquire more experience in species identification and fieldwork (Prugh & Ritland 2005; Jacob et al. 2010). In spite of the clear relevance of this field parameter for reliable and cost effective non-invasive genetic studies, to our knowledge the effect of sample collector experience on species identification via mitochondrial DNA and on genotyping success and errors rates has not previously been evaluated.

Thus, the aims of this study were: a) First, to develop a reliable and accurate mutiplex panel of microsatellites for individual identification of *M. martes* and test its applicability to *M. foina* in both tissue and scat samples. b) Second, to apply this panel for the detection of possible hybridization events between both species in sympatric areas and c) finally, to assess the impact of the field experience of sample collectors on the success of PCR-RFLP identification of *Martes* sp. faecal samples and d) on genotyping success and the error rates of faecal pine martens samples by conducting a genetic survey across the sympatric area of both marten species in the northern Iberian Peninsula.

MATERIAL AND METHODS

Sample collection and DNA extraction

Tissue samples

Fresh tissue specimens from roadkilled animals were used to isolate DNA from the target marten species, *M. martes* and *M. foina*. Marten specimens were collected across a wide geographic area in the northern Iberian Peninsula which covers the sympatric distribution range of both species (López-Martín 2007).

DNA was isolated from tissues and hairs using the Qiagen DNeasy Tissue DNA extraction kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions.

Faecal samples

The faecal sampling was conducted between 2006 and 2009 all over the sympatric range of both marten species in the northern Iberian Peninsula (López-Martín 2007).

To assess the impact of sample collector experience on the results of microsatellite genotyping and species identification rates, samples were collected opportunistically by three different kinds of staff: a) Wildlife biologists with recognized experience in non-invasive surveys of carnivores (WB), b) Volunteers who received a training course on how to identify *Martes* sp. scat samples (TV) and c) rangers and technical staff from different National Parks (TS). The wide study area and long-term programme did not permit the standardization of sampling efforts across space and time. However, the proportion of the samples obtained for each kind of sample collector was similar in each season.

Before sampling, we mailed a brief description of the project, the sampling protocol and material for sample collection (individually labelled 2 ml autoclaved tubes containing ethanol 96%) to each of the collaborators. European pine martens, stone martens and other carnivores use forest roads and frequently defecate on them as a way of visual-scent marking (Barja 2005). Thus, sampling was conducted systematically along linear features, such as forest trails and paths. Morphological characteristics, such as size and shape, were used to presumably distinguish faeces of the genus *Martes* from those of medium-sized carnivores such as the red fox and the wildcat (Ruiz-González et al. 2008).

For each faecal sample, sampling date, sample collector and Universal Transversal Mercator (UTM) coordinates using a global positioning system (Garmin eTtrex) were recorded. Collaborators were warned to collect only fresh samples (<5 days). Fresh faeces were characterised by the presence of a mucus layer and lack of any sign of dehydration (Rosselinni et al. 2009). Moreover, to assess the validity of the fresh designation in each faecal sample a pre-selection procedure was applied by evaluating a photograph of each faecal sample, discarding samples typified as fresh that presumably correspond to this category.

A portion of each "marten like" faecal sample was picked up by with stick and preserved in 2 ml autoclaved tubes containing 96% ethanol and preserved at room temperature until processed (Gómez-Moliner et al. 2004; Ruiz González et al. 2008). A different stick and tube were used for each sample.

In each extraction step, special care was taken to avoid cross-contamination, which is an important problem especially when handling DNA from non-invasive samples (Taberlet et al. 1999; Pompanon et al.

2005). Thus, extractions were performed in a dedicated laboratory on a sterile bench, using filter tips throughout. To monitor potential contamination, we included one negative extraction control per extraction process. DNA was then extracted using the DNA Stool MiniKit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer's protocol for DNA extraction from stool samples. The DNA was eluted from the silica membrane twice using 100 µL buffer AE (Qiagen).

Species identification

Martes species identification of each faecal sample was accomplished by a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method, providing for an effective genetic identification of sympatric marten species (Ruiz-González et al. 2008). A small fragment (276 bp) of mitochondrial DNA (mtDNA), D-loop region, was amplified with specific primers developed for *Martes* and *Mustela* by PCR. The primers were designed to amplify small fragments to maximise the probability of amplification of degraded DNA. Following DNA amplification, PCR products were digested with the restriction enzymes *Hae*III and *Rsa*I. The combined use of both enzymes produced a species-specific banding pattern allowing the scats of *M. martes* and *M. foina* to be discriminated (see Ruiz-González et al. 2008 for further details).

Microsatellite analysis

Development of a microsatellite multiplex protocol for M.martes *and* M.foina *useful for faecal DNA genotyping*

Forty one microsatellite loci identified in the genomic DNA of other mustelid species were tested for cross-species amplification and polymorphism in *M. martes* and *M. foina*. The 41 microsatellite primer sets were originally developed for *M. americana* (Ma1, Ma2, Ma3, Ma4, Ma5, Ma8, Ma9, Ma10, Ma11, Ma15, Ma18, Ma19; Davis & Strobeck 1998), *Gulo gulo* (Gg7, Gg14, Davis & Strobeck 1998; Gg454, Walker et al. 2001) *Meles meles* (Mel1, Mel6, Bijlsma et al. 2000; Mel10, Domingo-Roura et al. 2003), *Mustela lutreola* (MLUT27, MLUT04, Cabria et al. 2007), *Neovison vison* (Mvis020, Mvis022, Mvis072, Mvis075, Fleming et al. 1999; Mvi39, Mvi57, O'Connell et al. 1996), *M. erminea* (Mer 22, Mer 41 and Mer 95; Fleming et al. 1999); *Lutra lutra* (Lut604 and Lut615, Dallas & Piertney 1998) and for *Martes pennanti* (MP0059, MP0084, MP0100, MP0144, MP0175, MP0188, MP0200, MP0234, MP0247, MP0288, Jordan et al. 2007). These loci were selected for their high polymorphism and heterozygosity as show in previous studies of pine martens (Kyle et al. 2003; Pertoldi et al. 2008) or related species (Jordan et al. 2007).

PCR conditions were firstly optimised for each primer in singleplex and the annealing temperature was selected after temperature gradient PCR on tissue-derived DNA. Microsatellite amplifications were performed in a total volume of 15 µL with 2 µL DNA, 0.2 µL of each primers (20 pmol/µL), 1.7 µL 10× reaction buffer, 0.64 µL $MgCl_2$ (50 mM), 0.5 µL deoxynucleotide triphosphates (2.5 mM), 0.1 µL of bovine serum albumin (10 mg/mL), 11.56 µL of sterile water and 0.1 µL Taq polymerase (5 U/µL). The following PCR conditions were used for all amplifications: After incubation for 5 min at 94 °C, the samples were subjected to 40 amplification cycles in a BIO-RAD iCycler (Version 3.021, BIO-RAD Laboratories) consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 54-59 °C and a final extension stage of 1 min at 72 °C.

Loci with a low rate of amplification, monomorphic in both species, and with a high quantity of unspecific amplification were discarded. This first screening was evaluated with 15 tissues, and 10 faecal samples of each species from the Iberian Peninsula (data not shown).

From the 41 screened loci we selected a suite of 15 markers to design a Multiplex protocol with special emphasis on demonstrated variability in pine martens, amplification strength with small quantities of DNA (faecal DNA) and powerful enough to allow individual identification. Forward primers were fluorescently labelled with different dyes (6-FAM, NED, PET, VIC) to enable multiplex electrophoresis of microsatellite products. In order to avoid noise from variable adenylation during the PCR, the 'pigtail' sequence GTTTCTT was added to the 5 -end of each reverse primer (Brownstein et al. 1996). PCR multiplex amplifications were carried out with QIAGEN Multiplex PCR kits using the manufacturer's protocol in a total volume of 10 µL with 2 µL of DNA and 2 pmol of each fluorescence labelled forward and unlabelled reverse primers. We applied a hot-start thermocycling protocol. The initial polymerase activation (HotStart PCR) was done at 95 °C for 15 min, followed by 42 cycles (35 for tissue samples) of denaturation at 94 °C for 30s, primer annealing at 57 °C for 90s, and sequence extension at 72 °C for 60s, and a final extension step at 60 °C for 30 min.

In addition to the negative controls for extraction, negative PCR controls were included as proposed by Pompanon et al. (2005). Additionally, we amplified a reference sample as a positive control and to test that the electrophoretic mobility of the fragments was consistent across runs (Davison & Chiba 2003).

In non-invasive genetic sampling, genotyping errors occur due to increased rates of null alleles, allelic dropout (ADO) and false alleles (FA) (Taberlet et al. 1999; Pompanon et al. 2005). Therefore, we followed a modified multiple-tube approach from Taberlet et al. (1996), amplifying each DNA extract in four replicates and in separate rooms dedicated to low DNA-content samples.

First, to test the applicability of the multiplex protocol designed on non-invasive genetic surveys, 21 PCR-RFLP identified faecal samples of each species were genotyped four times per locus to estimate error rates and genotyping success. These samples were randomly chosen from each kind of sample collector (7 from WB, 7 from TV and 7 from TS). Moreover, 10 of the 35 tissue samples of each species were amplified four times per locus to estimate error rates for tissue samples. As errors were almost absent from tissue samples (see Results), the remaining 25 tissue samples were amplified only once per locus.

Later, in order to assess genotyping success and error rates on field studies for each of the different kinds of samples collector, all the faecal samples identified by the PCR-RFLP method as pine marten were genotyped at 15 *loci*. DNA quality was initially screened by PCR-amplifying each DNA sample four times at four loci (Multiplex 1: MP0188; MP0059; Gg-7; Ma1). Only samples showing >50% positive PCRs were further amplified four times for the remaining 10 loci. Samples with ambiguous results after four amplifications per *locus* or with <50% successful amplifications across *loci* were removed from further analyses as they were not considered reliable genotypes.

Multiplex PCR products were run on an ABI (Foster City, CA) 3130XL automated sequencer (Applied Biosystems), with the internal size standard GS500 LIZ™ (Applied Biosystems). Fragment analyses were conducted using ABI software GENEMAPPER 4.0.

Data analysis

Probability of identity, genotype checking, and individual identification

To test the discrimination power of the set of 15 microsatellites, we computed the probability of pairs of individuals bearing an identical multilocus genotype (P_{ID}) with GIMLET v 1.3.4 (Valière 2002) using a data set of genotypes obtained from tissue DNA of 35 pine and stone martens, respectively. PID calculations were performed with both the unbiased equation for small sample size and the equation for siblings. The more conservative P_{ID} for full-sibs ($P_{ID\text{-}Sib}$) was estimated as an upper limit to the probability that pairs of individuals would share the same genotype. As advocated in Waits et al. (2001), the observed probability in the population ranged from unbiased P_{ID} to $P_{ID\text{-}Sib}$ (Waits et al. 2001).

Consensus genotypes from four replicates were reconstructed using Gimlet v 1.3.4 (Valière 2002), accepting heterozygotes if the two alleles were seen at least in two replicates and homozygotes if a single allele was seen at least in three replicates. Gimlet was also used to estimate PCR success and errors: ADO (Taberlet et al. 1996), and FA (Gagneux et al. 1997).

We used GENETIX (Belkhir et al. 2004) to estimate observed (H_O) and expected heterozygosities (H_E), the number of total alleles (A), shared alleles (SA) and private alleles (PA) for each locus and for each of the tested species in this study (Mm= *Martes martes*; Mf= *Martes foina*).

Deviation from Hardy-Weinberg equilibrium (HWE) was tested using the exact test implemented in GenePop version 4.0 (Raymond & Rousset 1995; Rousset 2008). Statistical significance was evaluated by running a Markov Chain Monte Carlo (MCMC) consisting of 10,000 batches of 10,000 iterations each, with the first 10,000 iterations discarded before sampling (Guo & Thompson 1992). Significance levels were adjusted with sequential Bonferroni correction in order to correct for the effect of multiple tests (Rice 1989).

MICRO-CHECKER software (Van Oosterhout et al. 2004) was used to check for potential scoring errors and the presence of null alleles.

Bayesian admixture analysis of sympatric martens

STRUCTURE version 2.2 (Pritchard et al. 2000; Falush et al. 2003; Falush et al. 2007) was used to determine the level of admixture, calculated as the proportion of the genome of an individual that is originated from each of the two parental species. Simulations were run using a burn-in period of 10^5 sweeps followed by 10^6 MCMC iterations. Independent runs of K (i.e. number of clusters or gene pools assumed) were performed from one to four clusters and repeated ten times to check for consistency in the results. Admixture ancestry and correlated allele frequency models were used without prior knowledge of genetic information. Individual assignment probabilities refer to the proportion of the individual's genome which originated from European pine marten (q_i) or stone marten (q_j). Moreover, we calculated pair-wise FST values (Weir & Cockerham 1984) and tested for pairwise genetic differences among clusters identified by STRUCTURE using a randomization procedure implemented in FSTAT v 2.9.3.2 (Goudet 1995; Goudet 2001). Statistical significance at the 0.05 level was evaluated after the Bonferonni correction for multiple comparisons (Rice 1989)

Effect of sample collector experience on species identification and genotyping success

In order to evaluate the impact of the field experience of sample collectors on faecal DNA sample quality for genetic analysis, we evaluate five different parameters for each of the three different kind of sample collector: i) *Martes* sp. identification rate (proportion of samples identified by the PCR-RFLP method as *M. martes* or *M. foina*: Msp.), ii) microsatellite amplification rate (PCR replicates that yielded at least one scoreable allele: PCR+), iii) genotyping error rates (FA and ADO), iv) the proportion of samples that did not pass the microsatellite quality screening (SCR-), and v) the proportion of samples yielding a reliable

genotype (samples correctly genotyped at 15 loci: G+). The first parameter was calculated from all the analyzed samples and the other four from all the faecal samples identified as pine marten.

To compare the proportions obtained for each parameter and to determine whether they can be considered as equal for the different sample collectors or if at least two proportions are significantly different we used a Chi-square test based on 10,000 Monte Carlo simulations. The Marascuilo procedure (Marascuilo & Slaughter, 1981) was also used if the Monte Carlo simulation rejected H0, as the Marascuilo procedure compares all pairs of proportions, which enabled the pairwise differences to be identified. Statistical analysis was performed using XLSTAT (Addinsoft™, New York, USA). Results were considered statistically significant at p =0.05 level.

RESULTS

Reliability of a microsatellite multiplex panel for individual identification of M. martes *and* M. foina*: Genetic variability, probability of identity and genotyping errors*

Details of the multiplex panel of 15 microsatellites *loci*, selected by cross species amplification of 41 *loci*, are shown in Table 1. Allele frequencies at all loci were estimated based on tissue samples from 35 different individuals of each species. All but three of the analyzed microsatellite loci (MP0188, Mel-10 and Ma-19 were monomorphic in *M. foina)* were polymorphic in both mustelid species, with an average number of alleles per polymorphic locus of 4.13 and 3.31 (ranging from 2 to 6) in *M. martes* and *M. foina*, respectively (Table 1). The presence of private alleles is considered one of the most significant indicators of population or species distinction (Beaumont et al. 2001; Oliveira et al. 2008). Only 13 out of the 96 unique alleles found in both species (13.54%) were shared alleles, whereas 33 (34.38%) and 50 (52.08%) were private alleles for *M. martes* and *M. foina*, respectively. Interestingly, alleles found at 7 microsatellite loci were species-specific with slight differences in allele size (Table 1).

Comparison of H_E and H_O revealed slight differences between pine martens and stone martens (Table 1). Observed heterozygosity was lower than expected heterozygosity in both species. Mean expected heterozygosity over all polymorphic loci was 0.586 and 0.469 and mean observed heterozygosity was 0.552 and 0.462 for *M. martes* and *Martes foina*, respectively (Table 1).

Significant departure from HW equilibrium after Bonferroni correction was observed at two loci in pine martens (MP0059 and Lut-435), whereas only one locus deviated from HW equilibrium proportions in stone martens (Ma-1). The results of MICROCHECKER rejected the hypothesis that putative null alleles were causing bias in genetic diversity and population differentiation estimates.

High genetic differentiation was detected between the two mustelid species based on 15 microsatellite data (F_{ST}=0.485; P<0.001).

Table 1. Properties of the 15 microsatellite multiplexed loci used in this study and summary of the genetic variability assessed per locus and species. The table includes: number of alleles (N_A), shared alleles (S_A), private alleles (P_A), observed (H_O) and expected (H_E) heterozygosities, rates of positive PCR (PCR+), dropout (ADO) and false allele (FA) for each locus and for each of the species tested in this study (Mm= *Martes martes*; Mf= *Martes foina*).

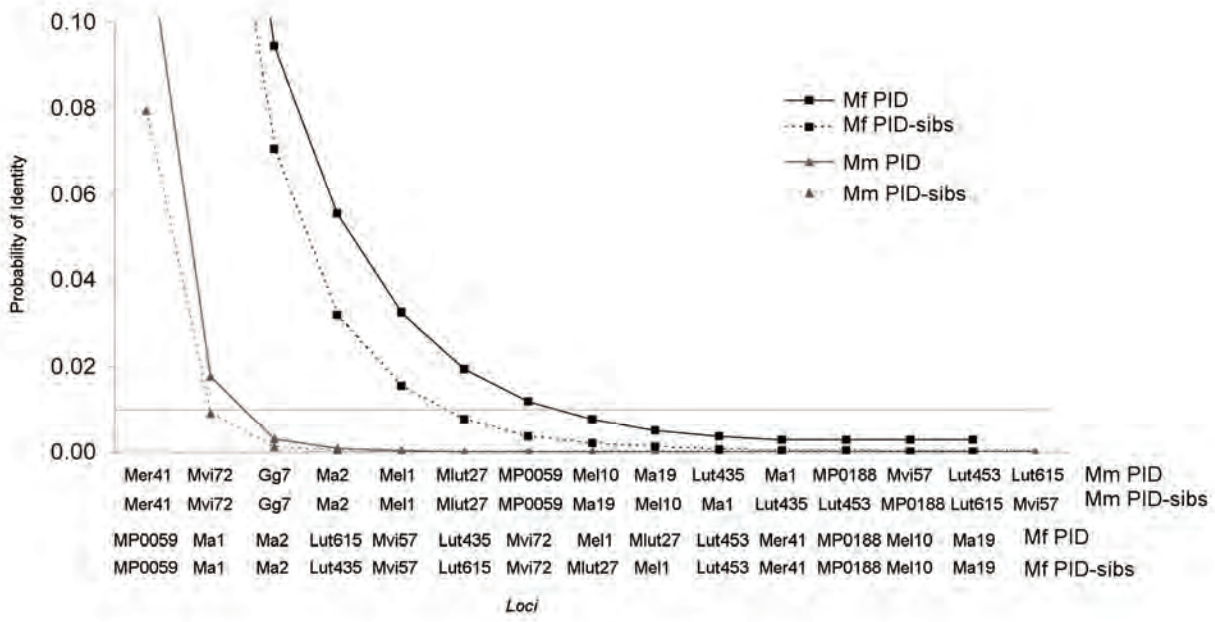
MULTIPLEX	Locus	DYE	Size range		N_A		S_A	P_A		H_E		H_O		PCR+		ADO		FA	
			Mm	Mf	Mf	Mm		Mf	Mm	Mf	Mm	Mf	Mm	Mf	Mm	Mf	Mm	Mf	Mm
MULT_1	Gg-7	PET	164-172	166-174	3	5	2	1	3	0.342	0.713	0.371	0.638	0.13	0.96	0.333	0.143	0	0
	Ma-1*	NED	204-210	203-213	6	4	0	6	4	0.675	0.520	0.629	0.444	0.94	0.91	0.345	0.167	0	0
	MP0059*	VIC	140-148	139-147	4	4	0	4	4	0.707	0.615	0.628	0.472	0.96	0.98	0.150	0.092	0	0.072
	MP0188	6-FAM	113-121	105	1	3	0	1	3	-	0.483	-	0.418	0.81	0.97	0	0.139	0	0
MULT_2	Lut-453	6-FAM	108-112	102-108	3	3	1	2	2	0.292	0.513	0.228	0.5000	1	0.96	0.400	0.093	0	0.033
	Mel-1	PET	265-275	264-272	3	5	0	3	5	0.378	0.643	0.371	0.667	0.98	0.87	0.281	0.174	0	0.009
	Mel-10	NED	158-166	127	1	5	0	1	5	-	0.536	-	0.472	1	0.88	0	0.375	0	0.014
MULT_3	Lut-435*	VIC	129-143	135-139	3	3	1	2	2	0.493	0.508	0.543	0.361	0.94	0.86	0.333	0.114	0.167	0.027
	Ma-19	PET	206-210	211	1	3	0	1	3	-	0.578	-	0.556	0.96	0.85	0	0.192	0	0
	Mvi-57	6-FAM	100-114	100-108	4	5	2	2	3	0.484	0.416	0.543	0.444	1	0.94	0.300	0.167	0	0.014
	Mvi072	NED	260-276	264-270	4	6	2	2	4	0.477	0.726	0.457	0.806	0.94	0.77	0.190	0.183	0	0.023
MULT_4	Lut-615	6-FAM	111-115	113-119	4	2	1	3	1	0.469	0.490	0.400	0.528	0.88	0.89	0.389	0.269	0	0.014
	Ma-2	NED	167-179	169-177	4	5	2	2	3	0.629	0.672	0.628	0.583	0.85	0.93	0.167	0.274	0	0.008
	Mer41	VIC	150-160	156-164	3	5	2	1	4	0.277	0.757	0.286	0.805	0.94	0.87	0.361	0.316	0	0
	Mlut-27	PET	198-204	186-194	2	4	0	2	4	0.408	0.616	0.457	0.583	0.94	0.91	0.325	0.333	0	0
Total					**46**	**62**	**13**	**33**	**50**										
Mean					**3.31**	**4.133**				**0.469**	**0.586**	**0.462**	**0.552**	**0.88**	**0.90**	**0.238**	**0.202**	**0.011**	**0.014**

Means for the number of alleles and Heterozigosities are computed over polymorph loci. Loci marked with an asterisk deviated from Hardy–Weinberg proportions at the p =0.05 level (Ma-1 for M. foina*; MP0059 and Lut-435 for* M. martes*).*

Probability of identity was calculated on the basis of allele frequencies estimated from the 35 tissue samples of each species. The overall P_{ID} using all loci for pine martens and stone martens was $5.42x10^{-11}$ and $5.42x10^{-7}$ and the overall PID-sibs was $4.47x10^{-5}$ and $1.95x10^{-3}$, respectively. When locus Gg-7 was excluded, which was the locus that was difficult to type for stone marten faeces samples, the overall PID became $1.21x10^{-6}$ and the overall PID-sibs was $2.79x10^{-3}$. Thus PID-sibs was again lower than the 0.01 threshold necessary to prevent the shadow effect, i.e. the presence of two or more individuals with the same multilocus genotype (Mills et al. 2000) (Fig. 1).

Thus, the 15 loci used in this study were necessary, yet sufficient, to distinguish with 99% certainty between sibling pine or stone martens (Mills et al. 2000) (Fig. 1). Moreover, the PID-sib showed that the proportion of individuals with identical profiles dropped to zero if the most informative loci were used (3 in *M. martes* and 8 in *M. foina*) (Fig. 1).

Figure 1. Probability of identity (P_{ID}) and probability of identity for full-sibling ($P_{id\text{-}sibs}$) values with the addition of loci in decreasing order of heterozygosity for *M. martes* (Mm) and *M. foina* (Mf). The 1% cut-off line represents the point where enough loci are typed to distinguish between individuals with 99% certainty.



Genotyping errors from tissue were estimated from 10 samples of each marten species. No ambiguous genotypes were detected. The proportion of PCR+s varied between 90 and 100% among loci and from 86

to 100% among samples for both species. Only 1 ADO was detected, at locus Gg-7 in pine martens and 3 in the same locus for the stone marten.

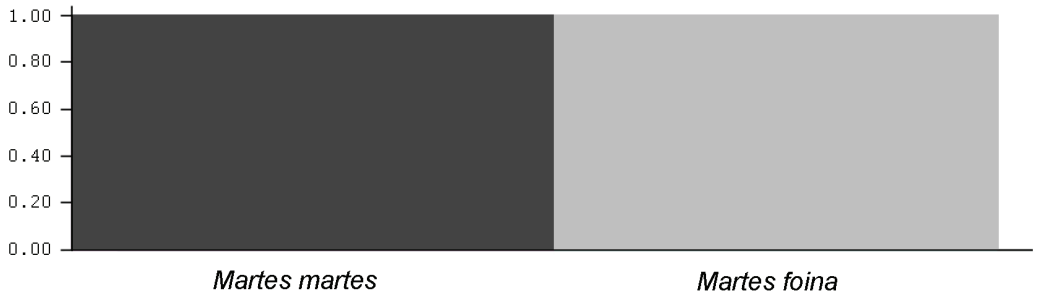
Genotyping success and error rates for faeces were preliminarily estimated from 42 samples (21 of each species). 13 faecal samples of *M. martes* (61.9%) and 12 (57.14%) of *M. foina* were completely and successfully genotyped. The other samples showed less than 50% amplification success or high genotyping error rates after 4 replications at 15 loci, so they were discarded as we were not able to resolve consensus genotypes from repeated attempts. Gg-7 was not valuable for faecal sample genotyping of stone martens because of the low amplification success rate (0.13).

The average proportion of positive PCRs for full multilocus microsatellite genotypes of faecal samples was 88% for the stone marten and 90% for the pine marten (Table 1) and varied among loci from 77% and 100% in both species. The mean ADO rate for *M. foina* and *M. martes* was 0.238 and 0.202, respectively. The ADO rates were not homogenous in each of the multiplexed reactions and each of the species (see Table 1). False allele rates averaged 0.011 for stone martens and 0.014 for pine martens (Table 1). Error types other than ADO or false allele FA, were also detected but they appeared at very low rates (< 0.001) and we considered them negligible.

Bayesian admixture analysis for species assignment

Analyses using STRUCTURE with both the admixture ancestry and correlated allele frequency models supported the existence of two distinct genetic clusters (k=2) based on the log probability of the data (ln [Pr(X/*K*)]) given the model. Moreover, the modal value of ΔK (Evanno et al. 2005) was also shown at K=2 (data not shown). Cluster I grouped all the samples identified as *M.martes* by both phenotype and PCR-RFLP method, with an estimated average proportion of membership (Q_I) higher than 0.99 (Figure 3). Cluster II included all the individuals identified as stone martens by phenotype and PCR-RFLP with Q_{II} > 0.99 (Figure 2).

Figure 2. Cluster identification by STRUCTURE without prior genetic information. Each individual is represented as a vertical line partitioned into two segments whose length is proportional to the individual's estimated membership coefficients. The analyses were performed assuming two (K=2) distinct genetic clusters.



Effect of sample collector experience on Martes *sp. identification and pine marten genotyping success of faecal DNA*

Sample collection and Martes *species identification*

Out of 849 faecal samples collected from the entire sympatric distribution range of both marten species in the northern Iberian Peninsula (López-Martín 2007), 215 (25.32%) were finally discarded as they were not assigned as fresh samples according to the pre-selection procedure (sample collector indications and photograph verification). The percentage of discarded samples was greater on samples collected by TS (29.38%) than by TV (28.97%) and WB (20.00%), respectively (Chi-square test, P=0.009) (Table 2). The Marascuilo procedure indicated that the difference between the proportions of discarded samples was statistically significant between WB and TS and between WB and TV. After sample pre-selection procedure, we obtained a total number of 634 fresh faecal DNA samples suitable for genetic analysis (Table 2). 532 were identified as one of the target species by our PCR-RFLP method. Thus, unequivocal species identification was possible in 83.91% of the samples. In the remaining 12%, the DNA extracted was not amplified by the primers used. The species identification success rate was similar for all three sample collectors with slightly higher values for WB (85.56%) and TV (84.58%) in comparison with TS (79.87%) (Figure 3, Table 2). However, differences were not significant (P=0.284).

Genotyping success and error rates of pine marten faecal samples

From the total scat samples identified as pine marten (n=317) we evaluated the effect of sample collector experience on microsatellite amplification rate (PCR+), genotyping error rates (FA and ADO), the

proportion of samples that failed to pass quality screening (SCR-) and samples yielding a reliable genotype (G+). The first quality-screening test was not passed by 121 samples (38.17%), which were immediately discarded. The proportion of the samples that did not pass quality-screening was higher, and statistically significant (P=0.003), in both TV (49.23%) and TS (46.83%) in comparison to WB (30.06%) (Table 2). The Marascuilo procedure identifies WB collected samples as most differentiated from both TV and TS samples (Table 2). The remaining 196 samples (61.83%) were amplified for the other 11 loci. After multiple-tube genotyping, 52 samples (16.4% of the total pine marten samples analysed) were then discarded because they showed <50% PCR success, or because of high failure rates. Full multilocus microsatellite genotypes were obtained for the remaining 144 samples. (73.47% from the samples that passed the screening and 45.43% from the total *M. martes* samples analyzed). After a regrouping procedure, we identified 114 individual genotypes (Table 2). The highest genotyping success rate (52.60%) was obtained for samples collected by WB, followed by TV (40.00%) and TS (34.18%). Chi-square test showed significant difference between the proportion of samples correctly genotyped across sample collectors (p=0.018), and the Marascuilo procedure showed that the only significant difference was that obtained between WB and TS collected samples. The overall PCR success rate (calculated only for correctly genotyped samples) was 95% with not significant differences (P=0.118) and similar values across sample collectors. The observed average error rates across loci were: ADO =0.189 and FA =0.021. The results obtained for the three sample collectors were very similar and not statistically significant (ADO, P=0.071; FA, P=0.527) (Figure 3 Table 2).

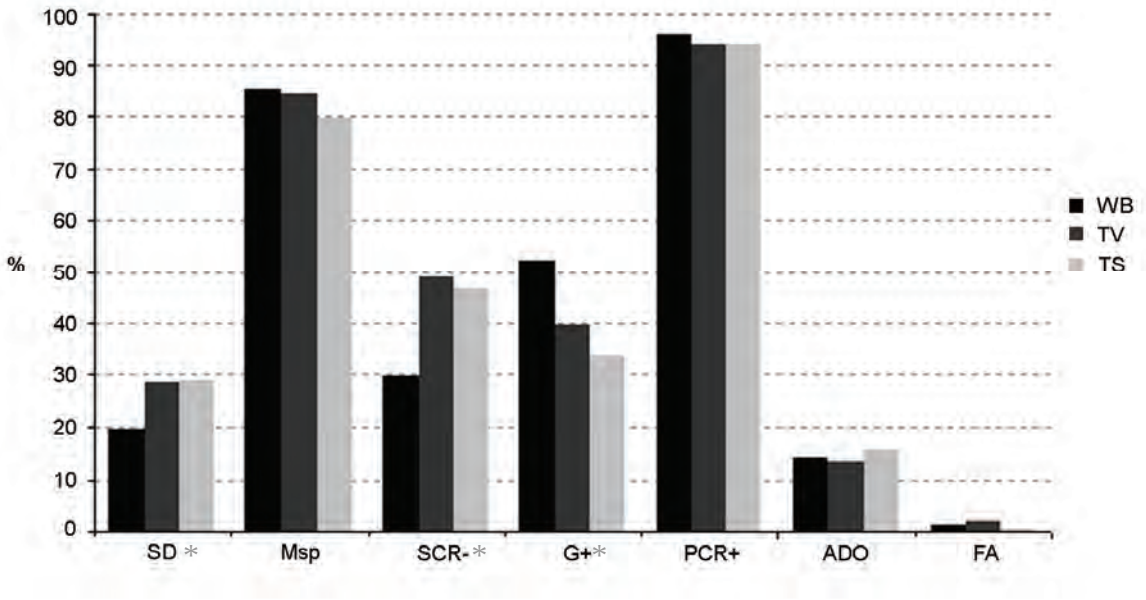
Table 2. Number of samples collected (SC), discarded (SD) and analyzed (SA); number (in brackets) and percentage of samples non-amplified by mtDNA (AMP-), identified as *M. martes* or *M. foina* (Msp), identified as *M. foina* (Mf), identified as *M. martes* (Mm); identified as *M. martes* that failed to pass screening (SCR-), not correctly genotyped (G-) and correctly genotyped (G+) and rates of positive PCR (PCR+), dropout (ADO) and false allele (FA) for each of the three different kinds of sample collectors: wildlife biologist (WB); trained volunteers (TRV) and technical staff (TS).

				Sample Collector			
				WB	TRV	TS	Total
SC				355	283	211	**849**
	SD*			71	82	62	**215**
	SA			284	201	149	**634**
		AMP-		14.44 (41)	15.42 (31)	20.13 (30)	**16.09 (102)**
		Msp		85.56 (243)	84.58 (170)	79.87 (119)	**83.91 (532)**
			Mf	24.65 (70)	52.24 (105)	26.85 (40)	**33.91 (215)**
			Mm	60.92 (173)	32.34 (65)	53.02 (79)	**50.00 (317)**
			SCR-*	30.06 (52)	49.23 (32)	46.83 (37)	**38.17 (121)**
			G-	17.34 (30)	10.76 (7)	18.99 (15)	**16.4 (52)**
			G+*	52.60 (91)	40.00 (26)	34.18 (27)	**45.43 (144)**
			PCR+	0.96	0.94	0.95	**0.95**
			ADO	0.142	0.137	0.159	**0.189**
			FA	0.014	0.02	0.015	**0.021**
			IDIND	69	18	27	**114**

**P-value < 0.05 for Chi-square test statistic.*
For positive Chi-square test, the Marascuilo procedure was used to determine which pairs of proportions, corresponding to each of the sample collectors, were statistically significant. For the three parameters in which the Chi-square test was positive the statistically significant pairs were:
SD (WB-TV; WB-TS); SCR- (WB-TS; WB-TS); G+ (WB-TS).

	Contrast	Value	Critical value	Significant
SD	|p(WB) - p(TV)|	0.090	0.084	Yes
	|p(WB) - p(TS)|	0.094	0.093	Yes
	|p(TV) - p(TS)|	0.004	0.101	No
SCR-	|p(WB) - p(TV)|	0.192	0.174	Yes
	|p(WB) - p(TS)|	0.168	0.162	Yes
	|p(TV) - p(TS)|	0.024	0.205	No
G+	|p(WB) - p(TV)|	0.126	0.175	No
	|p(WB) - p(TS)|	0.184	0.160	Yes
	|p(TV) - p(TS)|	0.058	0.198	No

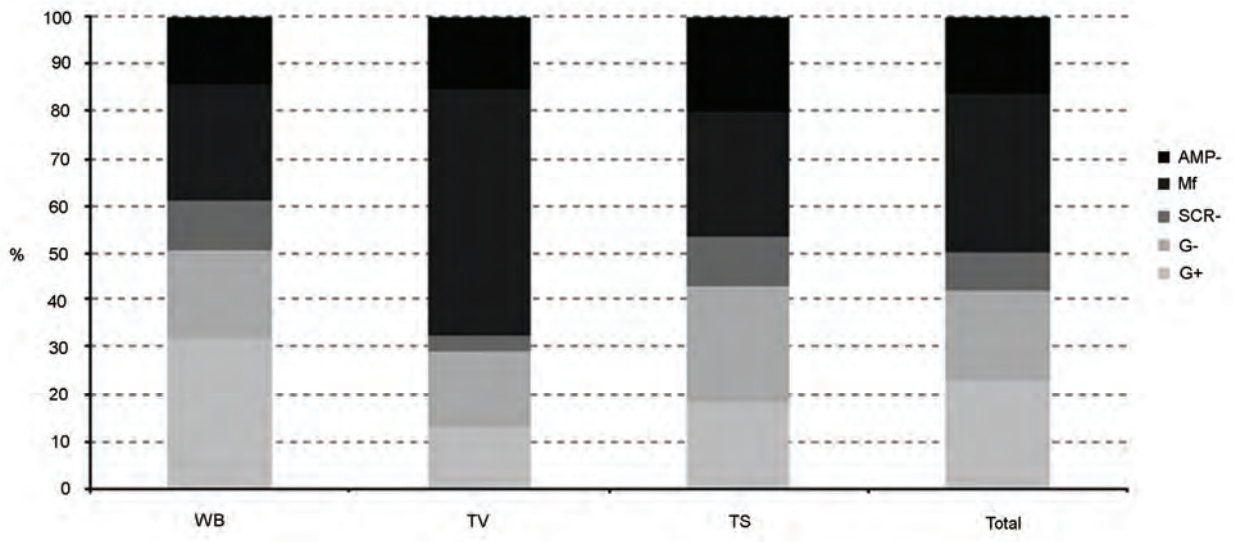
Figure 3. Comparison of percentage (%) of discarded samples by pre-selection procedure (SD), *Martes* species identification success (Msp), genotyping success (G+), positive PCR (PCR+), dropout (ADO) and false allele (FA) for each of the three different kinds of sample collectors: wildlife biologist (WB); trained volunteers (TRV) and technical staff (TS).



**P-value < 0.05 for Chi-square test statistic.*
For positive Chi-square test, the Marascuilo procedure was used to determine which pairs of proportions, corresponding to each of the sample collectors, were statistically significant. For the three parameters in which the Chi-square test was positive the statistically significant pairs were:
SD (WB-TV; WB-TS); SCR- (WB-TS; WB-TS); G+ (WB-TS).

From the total analyzed samples (n=634) 16.08% could not be assigned to species level, 33% were identified as *M. foina*, 8% failed to pass quality-screening and 19.08 % were not finally reliably genotyped. Thus, approximately 23% of all the analysed samples were correctly genotyped (45.43% if we only consider samples identified as *M. martes* by PCR-RFLP). The global and partial results for each sample collector are summarized in Figure 3. The partial results obtained for each sample collector are slightly different and mainly biased by the differences in the percentage of samples identified as *M. foina*.

Figure 4. Percentage (%) of samples from the total analyzed samples that were: non-amplified by mtDNA (AMP-), identified as *M. foina* (Mf), identified as *M. martes* that failed to pass quality-screening (SCR-), identified as *M. martes* not reliably genotyped (G-) and identified as *M. martes* correctly genotyped (G+) for each of the three different kinds of sample collectors (wildlife biologist=WB; trained volunteers=TRV and technical staff=TS) and for the whole dataset.



DISCUSSION

Research on non-invasive genetics of mustelids are limited and biased towards species identification methods (e.g. Gómez-Moliner et al. 2004; Livia et al. 2007; Ruiz-González et al. 2008). Microsatellite genotyping of non-invasive samples has also been carried out on a limited number of species and used mainly for individual identification and population size estimation (e.g. European otter, Hajkova et al. 2009; wolverine, Hedmark & Ellegren 2007; badger, Wilson et al. 2003; Frantz et al. 2006).

To our knowledge, this study is the first to investigate and evaluate a microsatellite panel for individual identification of both sympatric marten species (*M. martes* and *M. foina*) while also being valuable for faecal DNA genotyping. Moreover, this paper is the first attempt to elucidate genetic relationships between pine and stone martens by microsatellites. Additionally, this research is the first to compare the effect of sample collectors experience on *Martes* species identification and microsatellite genotyping success and error rates in non-invasively collected faecal samples.

Reliability of the microsatellite panel for individual identification and variability assessment of sympatric martens

Reliable amplification of microsatellite markers is a prerequisite for estimating the size and structure of populations within the landscape (Taberlet et al. 1999). In this paper, we standardized individual genetic identification of sympatric martens from faecal DNA with protocols offering some advantages over past efforts (Mullins et al. 2010). Firstly, species identification of the field-collected samples ensured that only target species samples were used for further analysis. This initial step towards species identification is crucial in landscapes where both species overlap extensively (Ruiz-González et al. 2008). In addition, the screening of a sufficiently large number of 41 microsatellite loci for the initial assessment of variability improved our ability to ascertain the combination of loci, resulting in higher precision in probability of identity. This enabled us to get an optimal combination of a few very variable loci. This study identified, by cross species amplification of 41 loci, a valuable panel of 15 microsatellite loci to consistently genotype faecal samples of sympatric pine and stone martens.

The most conservative estimate of PID, i.e. PID_{Sib} statistics, suggested that DNA profiles consisting of the 15 loci used in our study would be sufficient to distinguish between individuals of each of the study species, including siblings, with 99% certainty (Figure 1). The PID_{Sib} for the 15 loci (14loci in stone marten, after excluding the most difficult to type Gg-7) used is 2.79×10^{-3} for stone martens and 5.42×10^{-7} for pine martens and can be used to distinguish even closely related individuals. Therefore they can also be used in studies requiring individual identification for population size estimation based on non-invasive scat sampling.

The genotyping success (G+) and error rates obtained from the 21 faecal samples from each species for the microsatellite panel optimization procedure (G+: Mm=61.9%, Mf=57.14%; ADO: Mm=20.2%, Mf=23.8%; FA: Mm=1.4% and Mf=1.1%) were similar to what was obtained for the whole pine marten survey (GS: 45.43%; ADO: 1.89% FA: Mm=2.1%). Thus, the partial results of the pilot study reflected the magnitude of the success and error rates that could be obtained on more extensive research.

The success rate of pine marten microsatellite genotyping (presented here as percentage of faecal samples that provided consensus genotypes for all loci needed for reliable individual identification) in our study was 45.43%. This result is around average in comparison with many other studies undertaken on other carnivore species, which reported successful genotyping of faecal samples in the range of 14–79% [e.g. The badger (*Meles meles*): 62%, Frantz et al. 2003. The wolverine (*Gulo gulo*): 54% Hedmark & Ellegren 2007; 70%, Flagstad et al. 2004. Otter (*Lutra lutra*): 14% Lanszki et al. 2008; 20% Dallas et al. 2003;

21% Ferrando et al. 2008; 24% Kalz et al. 2006; 41% Prigioni et al. 2006; 44% Arrendal et al. 2007; 55-63% Hajkova et al. 2009; 65% Hung et al. 2004; 73%, Janssens et al. 2008. Coyote (*Canis latrans*): 28% Adams et al. 2007. Wolf (*Canis lupus*): 59%, Lucchini et al. 2002; 79 %, Creel et al. 2003; 52.8%, Marucco et al. 2009, 50-61%, Stenglein et al. 2010]. However, many of these studies used less than 10 microsatellites so the ease with which a complete genotype can be obtained is greater if we compare that complete genotyping with the 15 microsatellites used in this study.

Another important issue in non-invasive genetic studies is the detection and elimination of genotyping errors, as they can dramatically affect survey results, especially when estimating abundance via genetic sampling (Waits & Leberg 2000; Creel et al. 2003; Pompanon et al. 2005). The rate of genotyping errors, therefore, should always be carefully computed and clearly reported (e.g. Broquet & Petit 2004). This information can be considered as proof of data quality and thus a means of measuring the credibility of the results (Bonin et al. 2004). The frequency of allelic dropout and false alleles in our study (ADO =17.8%, FA =2.9%) was similar to that obtained in other non-invasive genetic studies of mustelids (e.g. ADO=15.8%, FA=2%, Ferrando et al. 2008; ADO=27%, FA=8% Frantz et al. 2003).

There are a few studies focused on the genetic variability of pine martens (Kyle et al. 2003; Mergey 2008; Pertoldi et al. 2008; Mullins et al. 2010), while no previous studies are available for *M. foina*. Kyle et al. (2003) documented the genetic structure of several European pine marten populations; Mergey (2008) studied the genetic structure of French populations inhabiting a fragmented landscape; while Pertoldi et al. (2008) assessed the genetic variation of Danish pine martens across both space and time. More recently, Mullins et al. (2010) developed a reliable and non-invasive method for censusing Irish pine marten populations from remotely plucked hair. Eleven of the 27 different microsatellite loci used in these previous studies have also been included in the present work.

Studying pine marten populations across Europe, Kyle et al. (2003) found microsatellite H_E values in the range of 0.56–0.64 for continental and 0.34-0.66 for insular populations (8 microsatellite loci). Mergey obtained similar values for French populations (11 loci, H_E=0.2). Pertoldi et al. (2008) found He values in the range of 0.67-0.79 in Danish populations(11 loci), while Mullins et al. (2010) found slightly lower values of H_E for the reduced and isolated Irish populations (0.35, 17 loci). In this study using 15 variable loci we detected H_E values of 0.586. So, taking into account the high number of variable loci used in this study we can estimate that in comparison to continental European populations, the Iberian population of pine martens has lower levels of genetic variability. However, caution is called for when trying to interpret differences in the level of genetic variability between different studies as these differences can also be attributed to the different number of microsatellites used.

The lower heterozigosity levels found in *M. foina*, in comparison to *M. martes* (Mf=0.469; Mm=0.586), could be explained by the lower polymorphism of microsatellite markers in stone martens (Mm: A=4.13; Mf: A=3.31). The microsatellite multiplex panel was primarily developed to be variable for pine martens as landscape genetic research on this species is ongoing (Ruiz-Gonzalez et al. in prep.). Thus, further optimisation of other polymorphic markers for *M. foina* may also be required for population/individual level genetic analysis in this species.

Reliable species identification: no evidence of genetic hybridization

The genetic analyses performed in this study were based on different molecular markers (mtDNA and nDNA), and gave consistent results that provided detailed information regarding reliable species identification of sympatric marten species. The microsatellite data confirmed the validity of the species identification results obtained by the PCR-RFLP method in mtDNA. The presence of species-specific microsatellite alleles and loci, together with Bayesian admixture analysis, facilitated pure-bred species distinction, discarding the presence of putative hybrids.

All the samples identified at species level by the PCR-RFLP method for the development of the microsatellite panel (Mm=48; Mf=47), were identified as pure European pine marten or stone marten based on microsatellite data analyses. The microsatellite panel generated in this study, together with Bayesian analysis, provided an efficient method for corroborating the pure ancestry of sympatric martens. These preliminary results, with the absence of phenotypically intermediate individuals described in the literature, indicated that there is no hybridization between these two sympatric marten species in the Iberian Peninsula.

Scats vs. hairs for microsatellite genotyping

Faecal or hair sampling methods have relative merits or biases depending on the size of the area surveyed, the sampling effort and the cost and efficiency of the method (Beja-Pereira 2009; Schwartz & Monfort 2008). In terms of estimating population size, intensive surveys in reduced areas may be more cost effective by genotyping remotely plucked hair as a result of requiring fewer amplifications to obtain reliable genotypes (Mullins et al. 2010). However, more sophisticated field work is needed to obtain hair samples in comparison to the easy collection of scats (Beja-Pereira 2009). Moreover, in areas with sympatric martens (i.e. most of Europe) scat-based surveys could be more cost effective as a distribution assessment and target DNA of each species can be obtained in one step. Thus, scats seem to be the most effective for widespread surveys of sympatric martens where a large number of samples can be obtained by

different collectors with a straightforward scat sampling protocol. Unfortunately, despite relatively easy sampling in the field, subsequent genetic analysis of pine marten faeces, using nuclear microsatellite *loci*, is challenging.

Effect of sample collector experience on Martes *sp. identification and pine marten genotyping success of faecal DNA*

Faecal samples could potentially result in highly variable DNA quality and quantity, resulting in different genotyping success and error rates (Beja-Pereira et al. 2009). The difference in the level of expertise among sample collectors can potentially impact the results of microsatellite genotyping as has been documented in this study.

The proportion of samples discarded from the total samples collected showed significant differences among samples collected by different personnel, demonstrating that the sampling conducted by wildlife biologists had the highest proportion of valuable fresh samples for genetic analysis. For example, a sampling conducted by WB increased the overall available fresh sample for consecutive genetic analyses by 9% and 10% in comparison to TV and TS, respectively.

An overall high species identification rate was obtained (mean 84%), confirming previous results of the application of the PCR-RFLP method on scat samples for monitoring the presence and distribution of sympatric marten species in the northern Iberian Peninsula (Ruiz-González et al. 2008; Rosellini et al. 2008) and Italy (Balestrieri et al. 2009). These results are above the mean in comparison to that found in Broquet et al. (2007), with an average amplification success rate of 73% of mtDNA reported in the non-invasive papers reviewed. *Martes* sp. identification rate was not statistically significant among different sample collectors (WB=85.56%; TV=84.58%; TS=79.87%). Thus, after a sample pre-selection procedure, sample collector experience had no significant effect on developing a reliable distribution survey of sympatric martens, as all three groups of samples collectors achieved similar identification rates. Thus, the work of non-specialized personnel may still be valuable in genetic studies focused on distribution assessment as mtDNA methods can effectively work with highly variable DNA quality and quantity.

On the other hand, we demonstrate that sample collector experience can have a significant effect on nuclear DNA quality and on the ability to obtain full genotypes from pine marten scats. Surprisingly, this effect has been largely ignored in the faecal DNA literature.

Five different measures were used to quantify nDNA quality of PCR-RFLP identified pine marten scat samples, but only two of these measures were significantly different among sample collectors.The

proportion of the samples that did not pass the quality-screening was lower, and statistically significant for wildlife biologist collected samples (30.06%) in comparison to trained volunteers (49.23%) and technical staff (46.83). Thus, samples collected by WB increased the overall available samples for further genotyping by 19.17% and 16.77% in comparison to TV and TS, respectively.

Genotyping success rates showed significant differences among samples collected by different personnel demonstrating that the degree of experience in collecting fresh quality samples increases the probability of obtaining a correct genotype. For example, a sampling conducted by WB increased the overall per sample finalized genotyping success by 12.6% and 18.3% in comparison to TV and TS, respectively. By contrast, PCR amplification success and error rates, because FA and ADO were not significantly different among sample collectors. However, these two measures are less important indicators of differences in DNA quality as PCR amplification products alone do not necessarily reflect true genotypes (Broquet & Petit 2004). Thus, in large-scale non-invasive genetic sampling projects, the field experience of sample collectors can significantly increase the number of completed genotypes and maximize output per unit effort.

Recommendations for faecal genetic surveys of martens in sympatric areas

As already suggested by previous non-invasive genetic studies conducted on carnivore species (Piggot 2004; Santini et al. 2007; Murphy et al. 2007), success rates are greatly improved by the use of very fresh samples. Thus, the effectiveness of sample collectors in meeting this objective is a crucial parameter. While sample quality is a limiting factor for microsatellite genotyping, it is not so for species identification rate by PCR-RFLP of mtDNA, after conducting a freshness sample pre-selection procedure.

The *Martes* sp. identification rate, based on a PCR-RFLP on mtDNA, was not influenced by sample collection staff. Thus, the development of a reliable distribution survey of sympatric martens could be done by different volunteers, independently of their previous experience, as the mtDNA methods seem not to be affected by the quality of fresh samples. This can be explained by the higher copy numbers of mtDNA compared to nDNA, conferring a greater success rate even with non-fresh samples that are not suitable for genotyping analysis. However, the success variability of faecal DNA genotyping reported in the present study for different sample collectors indicates that it is highly recommended that researchers carry out a pilot study prior to implementing a full study, where the experience of collaborators in field non-invasive surveys should be taken into account.

Factors that may influence success include intrinsic ones such as the individual, season and diet, over which the researcher has minimal control (Piggot & Taylor 2004). However, extrinsic sources of variation

in success such as sample collectors experience may be minimised by the collection of samples by experienced wildlife biologists.

Finally, the results of the present work give some guidelines which should be taken into account before starting faecal genotyping studies focused on pine martens living in sympatric areas with stone marten. In our study, 45.43% of the samples identified as pine marten were fully genotyped by the 15 microsatellites. However, when considering all the samples analysed, only 22.7 % were correctly genotyped (16.08% could not be assigned to species level, 33% were identified as *M. foina*, 8% did not pass the microsatellite screening, and the remaining 19.08 % were not fully genotyped). These results reveal the extent of the effort needed to develop non-invasive individual identification studies in species of the genus *Martes* with extensive overlapping areas. These observations could be extrapolated to non-invasive studies of other sympatric carnivores.

The difference in the level of expertise among sample collectors potentially impacts the results of microsatellite genotyping, while there are no significant differences in species identification rate. Non-invasive genetic studies may benefit from the help of different sample collectors with differences in the level of expertise, as exemplified in Bellemain et al. (2005), Jacob et al. 2010 or in our present study. However, in order to maximize non-invasive sampling and DNA quality which will insure accurate genotyping success, we recommend, whenever possible, that sampling be conducted by experienced biologists.

Application of our methods to field collected scats can be used in a cost-effective way to investigate species biology (including distribution, patterns of genetic diversity, relatedness and population connectivity) as well as to estimate population abundance for sympatric martens in the wild.

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CHAPTER

5

Landscape genetics

PAPER V

Non-invasive landscape genetics of the European pine marten (*Martes martes*): assessing spatial genetic structure and distribution in a heterogeneous landscape

PAPER VI

Landscape genetics as a tool for the empirical assessment of a regional ecological network: The European pine marten (*Martes martes*) as a target-species

PAPER V

NON-INVASIVE LANDSCAPE GENETICS OF THE EUROPEAN PINE MARTEN (*Martes martes*): ASSESSING SPATIAL GENETIC STRUCTURE AND DISTRIBUTION IN A HETEROGENEOUS LANDSCAPE

ABSTRACT

Non-invasive genetic sampling in combination with landscape genetics provides a valuable framework for the study of rare and elusive species in order to understand how landscape features influence population genetic structure. In this study, we used non-invasive methods to assess the distribution of sympatric martens (*Martes martes* and *M. foina*) and the spatial genetic structure of the forest dwelling European pine marten (*M. martes*) inhabiting a fragmented landscape, in the northern Iberian Peninsula. Out of 798 faeces samples analysed by a PCR-RFLP method, we identified 323 stone martens and 347 pine martens, which allowed us to determine species distribution. We used a number of different analytical approaches for identifying spatial genetic structure and to analyse patterns of gene flow for the 140 individual pine marten genotypes identified by multilocus microsatellite analyses of 15 *loci*. Results from spatial and non-spatial Bayesian clustering methods were mainly concordant and detected the presence of different genetic clusters distributed across the study area with non-overlapping distribution. The data reported in this study suggested that habitat loss and fragmentation could be one of the main causes of the spatial genetic structure found in a typical forest dwelling species. Other factors, such as, direct persecution, probable interspecific competition with the stone marten and the fact that the study area is situated at the limit of the species distribution, could be acting synergically to shape the current spatial genetic structure.

Keywords: Landscape genetics, Non-invasive genetic sampling, *Martes martes*, Bayesian analysis, Faecal DNA

INTRODUCTION

Landscape genetics has emerged as a synthetic discipline which integrates population genetics, landscape ecology and spatial statistics (Manel et al. 2003; Storfer et al. 2007; Holderegger & Wagner 2008). Landscape genetics explicitly quantifies the effects of landscape composition, configuration, and matrix quality on spatial patterns in neutral and adaptive genetic variation and underlying microevolutionary processes (Manel et al. 2003; Storfer et al. 2007; Holderegger and Wagner 2008; Balkenhol et al. 2009). All species are to some extent influenced by spatially heterogeneous landscapes (Segelbacher et al. 2010). Spatial heterogeneity affects, among other things, dispersal and, consequently, gene flow (Holderegger et al. 2006). An important issue is whether changes in landscape features create barriers to gene flow and as such lead to population structure. Multiple factors that act at various spatial scales affect spatial connectivity and rates of gene flow across a landscape (Anderson et al. 2010). These factors can include biotic (e.g. forest structure, landscape uses, and presence of prey, predators or sympatric competitors), abiotic (e.g. rivers, topography, environmental conditions) and/or anthropogenic features (e.g. roads, urban areas, crops). Thus, emergent patterns of gene flow are the result of the interactions between structural landscape connectivity and how organisms respond to landscape structure (Manel et al. 2003). In this context, assessing genetic population structure is crucial for defining appropriate conservation and management units (Frankham et al. 2002; Palsbøll et al. 2007), and for maintaining gene flow and genetic diversity (Moritz 2002).

Until recently, genetic structuring was studied by defining populations on an *a priori* basis, and gene flow was measured mainly by FST or similar parameters (Pearse & Crandall, 2004). However, landscape genetics has shifted towards individual-based sampling and analysis, especially when organisms are continuously distributed (Manel et al. 2007; Clark et al. 2008; Frantz et al. 2009; Cushman & Landguth 2010a,b; Segelbacher et al. 2010; Storfer et al. 2010). Recent landscape genetic approaches largely focus on describing and mapping populations (e.g. Guillot et al. 2005; Dionne et al. 2008) and on identifying factors that influence rates and patterns of gene flow within and between populations (e.g. McRae et al. 2005; Coulon et al. 2006; Cushman et al. 2006; McRae and Beier 2007; McCairns & Bernatchez 2008; Schwartz et al. 2009), and deducing from these which features restrict or promote movements of individuals. Bayesian clustering algorithms (Pritchard et al. 2000; Guillot et al. 2005; Francois & Durand 20010) have become popular tools for characterizing population genetic structure based on individual genotypes. These methods probabilistically assign individuals to groups based on their multi-locus genotypes by minimizing Hardy–Weinberg and linkage disequilibria, without presuming pre-defined populations (Pearse & Crandall 2004). A recent extension of these methods addresses the spatial nature of

the problem of locating genetic discontinuities by including the geographical coordinates of individuals in their prior distributions (Francois & Durand 2010). These models offer a powerful tool to answer questions in ecology, conservation and wildlife management, as genetic discontinuities within populations can be correlated with landscape features (Manel et al. 2003). These methods lead to more meaningful population delineations, and they are particularly useful for describing population structure in highly mobile or continuously distributed species, such as large and midsized carnivores (e.g. Cegelski et al. 2003; McRae et al. 2005; Pilot et al. 2006; Riley et al. 2006; Millions & Swanson 2007; Gula et al. 2009; Zalewski et al. 2009; Mucci et al. 2010; Williams & Scribner 2010). However, sufficient sample collection for these purposes is a difficult task, especially in rare and elusive species, in which sampling is a limiting factor (Schwartz & Monfort 2008). In this context, molecular methods incorporating non-invasive sampling via the collection of scats or hairs have become common for population monitoring of carnivores, providing valuable DNA sources for genetic surveys (Taberlet & Luikart 1999; Piggott & Taylor 2003; Waits & Paetkau 2005; Schwartz & Monfort 2008; Beja-Pereira et al. 2009).

The pine marten (*Martes martes* L. 1758) is a typical woodland-dwelling mustelid that occurs throughout most of Europe and northern and central Asia, extending from northern Portugal to western Siberia (Proulx et al. 2004). The pine marten is generally associated with forest habitats, mainly mature coniferous and mixed forests (Delibes 1983, Buskirk 1992, Proulx et al. 2004). Nonetheless, they have also been recently reported in fragmented landscapes where woods consist of isolated, small fragments within an agricultural landscape matrix (Pereboom et al. 2008; Balestrieri et al. 2010). Deforestation and forest fragmentation have been reported to affect the distribution and density of pine martens (Brainerd et al. 1994, Kurki et al. 1998), which are believed to need a minimum woodland area to survive (Zalewski and Jędrzejewski 2006) and tend to avoid treeless areas (Storch et al. 1990, Brainerd and Rolstad 2002, Pereboom et al. 2008, Ruiz-González et al. 2008). Given their strong associations with structural complexity in forests, the species is particularly sensitive to human influences on their habitats, including habitat loss, and landscape-scale effects of habitat fragmentation (Brainerd 1990; Bright 1993; Pereeboom et al. 2008). Consequently, the pine marten is a well suited species for carrying out studies focused on the effects of forest fragmentation on genetic structure and gene flow. However, little is known about the genetic population structure (Kyle et al. 2003) and the influence of habitat fragmentation on pine marten gene-flow (Mergey 2007). Previous studies focused on the pine marten have given information about the genetic variability and structure of several European populations (Kyle et al. 2003; Mergey 2007; Pertoldi et al. 2008; Mullins et al. 2010). However, information about the genetic variability and population structure of the Iberian populations found at the southern edge of the species distribution is negligible.

Previous studies focused on forest dwelling species (e.g. Roe deer, Coulon et al. 2004, Coulon et al. 2006; American marten, Broquet et al. 2006; Wasserman et al. 2010) supported the hypothesis that the landscape has a significant influence on the structuring of the populations under study. Indeed, recent works based on a least cost distance approach, indicated that several landscape features act as moderators of gene flow because of a high resistance to pine marten movements (Mergey 2008; Ruiz-Gonzalez et al. in prep.). Thus, this study could provide a new insight and help to validate previous results obtained in forest associated species.

Terrestrial organisms respond to complex landscape structure at their own unique set of spatial and temporal scales (Anderson et al. 20010), based on their inherent dispersal abilities and sensitivity to environmental change (D'Eon et al. 2002). Landscape spatial heterogeneity not only influences, directly or indirectly, landscape connectivity (Spear et al. 2010), but also the probability of site occupancy (Goszczyński et al. 2007; Ruiz-González et al. 2008). Moreover, the spatial organization of a species in a landscape is influenced, at least in part, by the presence of sympatric competitors (Linnell & Strand 2000). In this context, the interference interactions between sympatric carnivores can potentially influence population genetic structure as they may compete for the same resources and consequently synergically increase the effects of other biotic or abiotic factors. Thus, the probable interspecific competition between the pine marten and the more widespread sympatric stone marten (*Martes foina*) may influence pine marten distribution range (Delibes 1983; Goszczyński et al. 2007) and hence affect population structure.

Consequently, *prior* to conducting any landscape genetic study focused on the pine marten via non-invasive sampling of scats in sympatric areas with the stone marten, a distribution assessment through the application of a species identification genetic method is an essential previous step (Ruiz-González et al. 2008). Although some studies on the distribution of sympatric martens have been conducted in the Iberian Peninsula (Rosellini et al. 2008; Ruiz-González et al. 2008), there is a remarkable absence of information on the distribution of the European pine marten and on the bio-ecological relationships between these species (Proulx et al. 2004). The north Iberian Peninsula, houses the south-western distribution limits of a number of temperate forest species (e.g. *Glis glis*, *Myodes glareoulus*) including the focal pine marten (Palomo et al. 2007). Thus, this area has remarkable conservation priorities for the pine marten and for biodiversity conservation in general, as distributional limits are thought to be particularly important as long-term stores of genetic diversity and hot spots for speciation (Hampe & Petit 2005). Moreover, the study area plays an important connector role constituting a natural link between the Cantabrian Mountains and the Pyrenees and is considered of strategic importance for the conservation of ecological connectivity in south-western Europe (Worboys et al. 2010).

Thus, the main aims of our study were to i) first, determine the spatial distribution of pine marten and stone marten and infer sympatric relationships between both species through non-invasive genetic sampling of scats. As pine marten faeces cannot be distinguished from those of the sympatric stone marten (*Martes foina*), which is widespread in the study area (López-Martín, 2008), and can also be easily confused with those of other carnivores (Davison et al. 2002; Harrington et al. 2010), molecular techniques were applied for the identification of faecal samples (Ruiz-González et al. 2008). Our second objective was to ii) characterize the spatial genetic structure of the European pine marten using 15 highly variable genetic markers on previously PCR-RFLP indentified faecal DNA. Therefore, we inferred population structure in the pine marten by comparing two genetic assignment approaches that use a Bayesian statistical framework to delineate genotype clusters without the need to infer gene flow with *a priori* subjective groupings. Complementarily, spatial autocorrelation analyses were used to infer spatial genetic structure. Barriers to gene flow among populations were tested combining landscape derived data with GIS. This study will allow us to detect which spatial and landscape features influence pine marten gen-flow and should be considered as potential barriers for the migration movements of individuals, with particular respect to the differentiation of management units.

As habitat specialist species are usually considered highly vulnerable to habitat fragmentation (Bright 1993; Devictor et al. 2008) we predicted that, as a forest dwelling species, pine martens would be better represented in forested areas and would present spatial genetic structure due to the fragmented nature of the landscape under study.

MATERIAL AND METHODS

Study area

The Basque Country and Navarre are located in the northern Iberian Peninsula, bordering the Cantabrian Sea and sit between the mountain ranges of the Pyrenees and the Cantabrian Mountains (Figure 1). They belong to the Atlantic and Mediterranean biogeographic regions, with the exception of the Northeastern area of Navarre, which belongs to the Alpine region.

The Basque Country comprises an area of 7,235 km^2 and has a high population density of 298 inhabitants per square kilometer (INE, 2008) and a dense road network. The area is still covered by remnant zones of natural and semi-natural vegetation composed of deciduous oak and beech forests. However, extensive industrial development and increasing urbanization together with an increased density in communication infrastructures have given rise to a heterogeneous landscape characterized by a clear pattern of forest loss

and fragmentation. Natural forest covers approximately 28% of its area, forestry plantations 29%, non-wooded mountains 24%, cultivated land 14%, and urban land and infrastructures 5.7%. Contrastingly, Navarre comprises an area of 10,390 km^2 and has a low population density of 60 inhabitants per square kilometer (INE, 2008). The area is covered by a non-fragmented natural forest system, concentrated in the North, while cultivated land and urban areas are located in the South. Natural forest covers nearly 36% of its area, forestry plantations 6%, non-wooded mountains 10%, cultivated land 46%, and urban land and infrastructures 1.4%.

Non-invasive sample collection and DNA extraction

As the pine marten is an elusive, scarce and stress sensitive species (Barja et al. 2007), we obtained DNA from non-invasive sampling of faeces. This DNA source is regularly used for non-invasive genetic studies of carnivores (Schwartz & Monfort 2008; Beja-Pereira, 2009) and was used in previous pine marten studies (Ruiz-González et al. 2008; Rosellini et al. 2009; Balestrieri et al. 2010). We conducted a multi-stage sampling scheme, in which samples from a pilot study were used to assess the appropriateness of the sampling with respect to the research questions (Balkenhol 2009). Thus, two scat-based surveys were conducted between 2004 and 2009 all over the sympatric range of both marten species in the study area (López-Martín 2007). The first one, conducted in the 2004-2005 period was used to initially estimate the distribution range of the two species of the genus *Martes* in the study area. The second one, conducted between 2006 and 2009 was used to refine species distribution assessment and to obtain a higher number of fresh samples of *M. martes* for microsatellite genotyping after a PCR-RFLP species identification process. Fresh faeces were characterised by their strong smell, the presence of a mucus layer and lack of any sign of dehydration. European pine martens, stone martens and other carnivores use forest roads and frequently defecate on them as a means of visual-scent marking (Barja 2005). Thus, sampling was conducted systematically along linear features, such as forest trails and paths. Morphological characteristics, such as size and shape, were used with a view to distinguishing faeces of the genus *Martes* from those of other medium-sized carnivores such as the red fox and the wildcat (Rosellini et al. 2008; Ruiz-González et al. 2008). The territory of the European pine marten has been calculated to be nearly 1.3 km^2 for males and 1.0 km^2 for females in some forested areas of the study area (O. Berdión, pers. comm.). The mean territory size of the stone marten is similar to that of the European pine marten, being about 0.96 km^2 (López-Martín 2003). Thus, in order to minimize multiple sampling of the same individual and to maximize the number of sampled individuals, samples were collected if separated by a spatial distance of ≥ 1km from the next nearest sample. Transects were uniformly distributed throughout the study area in an

effort to survey all the territory, with special emphasis on forested areas. Universal Transversal Mercator (UTM) coordinates were recorded for all the samples collected using a global positioning system (Garmin eTtrex). The faecal samples were stored in autoclaved tubes containing ethanol 96% and frozen at –20°C until processed (Gómez-Moliner et al. 2004; Ruiz González et al. 2008).

Sampling was carried out thanks to the collaboration of more than 30 volunteers and students, personnel of the regional Natural parks and a specialized field biologist. The widespread study area and long-term programme did not permit the standardization of sampling in space and time. Collaborators were trained to collect only fresh samples (<5days) (Ruiz-González et al. 2008).

At each extraction step, special care was taken to avoid cross-contamination, which is an important problem especially when handling DNA from non-invasive samples (Taberlet et al. 1999; Pompanon et al. 2005). Thus, extractions were performed in a dedicated laboratory on a sterile bench, using filter tips throughout. To monitor potential contamination, we included one negative extraction control per extraction process. DNA was then extracted using the DNA Stool MiniKit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer's protocol for DNA extraction from stool samples. The DNA was eluted from the silica membrane using twice 100 µL buffer AE (Qiagen).

Additionally, fresh tissue specimens from road-kill pine martens were included in the study, when possible. DNA was isolated from tissues using the Qiagen DNeasy Tissue DNA (Qiagen, Hombrechtikon, Switzerland) extraction kit according to the manufacturer's instructions.

Species identification and spatial distribution

The specific identification of faecal samples was accomplished by a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method, providing for an effective genetic identification of sympatric marten species (Ruiz-González et al. 2008). A small fragment (276 bp) of mitochondrial DNA (mtDNA), D-loop region, was amplified with specific primers developed for *Martes* and *Mustela* by PCR. The primers were designed to amplify small fragments to maximise the probability of amplification of degraded DNA. Following DNA amplification, PCR products were digested with the restriction enzymes *Hae*III and *Rsa*I. The combined use of both enzymes produced a species-specific banding pattern allowing the scats of *M. martes* and *M. foina* to be discriminated (see Ruiz-González et al. 2008 for further details). To assess the spatial distribution of both species in the study area the UTM coordinates corresponding to each sample were projected onto a GIS (Arcview 9.0. ESRI) along with the species identification data provided by the PCR-RFLP method. Finally, we characterized the location of each species with regards to

landscape uses. Landscape use data was obtained in vector format from the most recent Spanish forestry map (Spanish Ministry of the environment, 2006) while the road network map was obtained from the Spanish National Geographic Institute (2008).

DNA amplification and microsatellite genotyping

The extracted DNA was PCR-amplified and genotyped using 15 microsatellite primers (Ma1, Ma2, Ma19 Gg7, Davis and Strobeck 1998; Mel1, Bijlsma et al. 2000; *Mel10*, Domingo-Roura et al. 2003; MLUT27, Cabria et al. 2007; Mvis072, Mer 41 Fleming et al. 1999; Mvi57 O'Connell et al. 1996; Lut 615 Dallas & Piertney 1998; MP0059, MP0188, Jordan et al. 2007). These loci were selected by cross species amplification of 41 loci and based on demonstrated variability in Iberian pine marten populations, amplification strength with small quantities of DNA (faecal DNA), and powerful enough to allow individual identification according to Ruiz-González et al. (in prep.).

Forward primers were fluorescently labelled with different dyes (6-FAM, NED, PET, VIC) to enable multiplex electrophoresis of microsatellite products (Ruiz-González et al. in prep.). In order to avoid noise from variable adenylation during the PCR, the 'pigtail' sequence GTTTCTT was added to the 5 -end of each reverse primer (Brownstein et al. 1996). PCR multiplex amplifications were carried out with QIAGEN Multiplex PCR kits using the manufacturer's protocol in a total volume of 10 µL with 2 µL of DNA and 2 pmol of each fluorescence labeled forward and unlabelled reverse primers. We applied a hot-start thermocycling protocol. The initial polymerase activation (HotStart PCR) was done at 95°C for 15 min, followed by 42 cycles (35 for tissue samples) of denaturation at 94°C for 30s, primer annealing at 57°C for 90s, and sequence extension at 72°C for 60s, and a final extension step at 60°C for 30 min.

In addition to the negative controls for extraction, negative PCR controls were included as proposed by Pompanon et al. (2005). Additionally, we amplified a reference sample as a positive control and to test that the electrophoretic mobility of the fragments was consistent across runs (Davison & Chiba 2003).

In non-invasive genetic sampling, genotyping errors occur due to increased rates of null alleles, allelic dropout (ADO) and false alleles (FA) (Taberlet et al. 1996; Pompanon et al. 2005). Therefore, we followed a modified multiple-tube approach from Taberlet et al. (1996), amplifying each DNA extract in four replicates and in separate rooms dedicated to low DNA-content samples.

All the faecal samples identified by the PCR-RFLP method as pine marten were genotyped at 15 *loci*. DNA quality was initially screened by PCR-amplifying each DNA sample four times at four loci (Multiplex 1: MP0188; MP0059; Gg-7; Ma-1). Only samples showing >50% positive PCRs were further

amplified four times at the remaining 11 *loci*. Samples with ambiguous results after four amplifications per *locus* or with <50% successful amplifications across *loci* were removed from further analysis as they were not considered reliable genotypes.

Multiplex PCR products were run on an ABI (Foster City, CA) 3130XL automated sequencer (Applied Biosystems), with the internal size standard GS500 LIZ™ (Applied Biosystems). Fragment analyses were conducted using the software ABI software GENEMAPPER 4.0.

Probablity of identity, genotyping checking and individual identification

To test the discrimination power of the set of 15 microsatellites, we computed the probability of pairs of individuals bearing an identical multilocus genotype [i.e. probability of identity (P_{ID})] with the software GIMLET v 1.3.4 (Valière 2002). P_{ID} calculations were performed with both the unbiased equation for small sample size and the equation for siblings. The more conservative P_{ID} for full-sibs ($P_{ID\text{-}Sib}$) was estimated as an upper limit to the probability that pairs of individuals would share the same genotype.

Consensus genotypes from four replicates were reconstructed using GIMLET v 1.3.4 (Valière 2002), accepting heterozygotes if the two alleles were seen at least in two replicates and homozygotes if a single allele was seen at least in three replicates. Gimlet was also used to estimate PCR success and errors (ADO and FA).

Data analysis

We used different analytical approaches for identifying spatial genetic structure and for analysing patterns of genetic diversity and gene flow across the entire study area. As suggested in previous studies (e.g. Pearse & Crandall, 2004, Frantz et al. 2009; Francois & Durand, 2010) we applied two Bayesian model-based clustering algorithms, one spatial and one non-spatial to infer population structure (i.e. number of clusters, K) and to assign individuals (probabilistically) to populations (or clusters) without *a priori* knowledge of population units and limits: GENELAND v 3.2.2 (Guillot et al.2005a,b; Guillot 2008; Guillot et al. 2008) and STRUCTURE v 2.1 (Pritchard et al. 2000; Falush et al. 2003; Falush et al. 2007). Both of these approaches assume that populations are panmictic units with distinct allele frequencies. We checked ex-post that the inferred groups were significantly differentiated by FST values and at Hardy-Weinberg equilibrium (HWE). Moreover, to test whether individual dispersal was restricted in space, we conducted spatial autocorrelation analysis. Finally, past bottleneck and recent migration among populations (within the last few generations) was assessed using BOTTLENECK (Cornuet & Luikart 1996; Piry et al. 1999) and a Bayesian model implemented in MIGRATE software version 3.0.3

(Beerli & Felsenstein 2001; Beerli 2006), respectively. Barriers to gene flow among populations were tested combining landscape derived data with GIS. Details for all analyses are given below.

Genetic clustering analyses

GENELAND procedure

First we analysed population genetic structure using GENELAND version 3.2.2 (Guillot et al. 2005a, b Guillot 2008; Guillot et al. 2008) an extension of program R 2.11.1 (Ihaka & Gentleman 1996). GENELAND implements a Bayesian clustering algorithm similar to STRUCTURE and uses an MCMC re-sampling method to estimate unknown parameters including the number of genotype clusters. GENELAND integrates the spatial coordinates of individuals together with the genetic information and so provides an improved definition of the spatial genetic units when compared with non-spatial clustering methods. This model assumes that populations are spatially organized as a set of non-overlapping polygons with no gaps (Guillot et al. 2005a, b). Thus, areas of genetic discontinuity were also detected as geographical areas of global low posterior probability of population membership. Unlike STRUCTURE, GENELAND treats the number of genotype clusters as an additional parameter (Guillot et al. 2005a). Due to substantial algorithm improvement implemented in the recent versions of GENELAND software (from version 3.0.0 onwards), we used the correlated frequency model that allowed us to detect subtle structures in the presence of low genetic differentiation that would probably remain undetected using an uncorrelated frequencies model (Guillot 2008).

The number of clusters was determined by running the MCMC iterations 50 times, allowing K to vary, with the following parameters: 500,000 MCMC iterations, maximum rate of the Poisson process fixed to 150, uncertainty attached to the spatial coordinates fixed at 1 km, minimum K = 1, maximum K = 10, maximum number of nuclei in the Poisson–Voronoi tessellation fixed to 450, bur-in of 100,000 in the post-processing, as well as the option to filter null alleles.

We included uncertainty (1 km) in the spatial coordinates for each individual to account for any measurement error, movement of individuals, and the potential for observed locations to reflect the true locations inaccurately (Guillot et al. 2005a). The uncertainty associated with the spatial coordinates was set to 1 km, based on a 1 km^2 estimate of home range size in the study area.

Each run provided (in a single step) an estimate of K and a map of the estimated populations. We calculated the mean logarithm of posterior probability for each of the 50 runs. The runs were then sorted according to their mean posterior density and only the best 3 runs were considered in the analysis. These 3

runs were then post-processed (with a burn-in of 100,000 iterations) in order to obtain posterior probabilities of population membership for each individual and each pixel of the spatial domain. We finally checked visually for the consistency of results across these 3 runs.

STRUCTURE procedure

STRUCTURE version 2.2 (Pritchard et al. 2000; Falush et al. 2003; Falush et al. 2007) was also used to investigate the genetic structure of the pine marten. STRUCTURE is currently the most widely adopted method of determining population structure for groups of individuals with unknown population affinities, and thus provides a standard with which to compare the results produced by GENELAND. We used an admixture model, which allows for multiple genetic sources of individuals, with correlated gene frequencies (Falush et al. 2003). Simulations were run using a burn-in period of 10^5 sweeps followed by 10^6 MCMC iterations. Independent runs of K (i.e. number of clusters or gene pools assumed) were performed from one to eight clusters and repeated twenty times to check for consistency in the results.

To determine the optimal number of clusters, a plot of the estimated log probabilities for each K value (Pr(X|K)) was examined. If the plot showed a clear peak at one K value, this value with the highest probability was chosen. If the plot increased gradually, and no clear peak exists, the smallest K value where the plot reaches a plateau was chosen as the most parsimonious number of populations (Pritchard et al. 2000). Additionally, we computed the standardized second order rate K of change of Ln P(X|K) (Evanno et al. 2005), to identify the K value that produces the greatest gain in probability of the model. Once the true K was selected, the fractional membership of each individual in each cluster (q), averaging q over the 20 runs, was plotted on a map of the study region to assess geographical congruence of the clusters and contrast the results obtained from the spatially explicit GENELAND results.

Moreover, to discuss the relationship between genetic discontinuities and landscape features, we plotted the results of the modal population for each pixel of the study area according to GENELAND and the mean membership coefficients of each individual for each cluster according to STRUCTURE.

Population genetic analysis of the inferred clusters

We summarized genetic variation through the number of alleles per locus (A) and expected (HE) and observed (HO) heterozygosities using GENETIX v 4.05.2 (Belkhir et al. 2004) for each of the inferred clusters (based on spatially explicit GENELAND results) and for the full dataset. Estimates of pairwise linkage disequilibria for each pair of loci in each population and deviation from HWE was tested using the exact test implemented in GenePop version 4.0 (Raymond & Rousset 1995 Rousset, 2008). Statistical

significance was evaluated by running a Markov Chain Monte Carlo (MCMC) consisting of 10,000 batches of 10,000 iterations each, with the first 10,000 iterations discarded before sampling (Guo & Thompson 1992). Significance levels were adjusted with sequential Bonferroni correction in order to correct for the effect of multiple tests (Rice 1989).

We calculated pair-wise FST values (Weir & Cockerham 1984) and tested for pairwise genetic differences among clusters identified by Bayesian models using a randomization procedure implemented in FSTAT v 2.9.3.2 (Goudet 1995; Goudet 2001). Statistical significance at the 0.05 level was evaluated after the Bonferonni correction for multiple comparisons (Rice 1989). Moreover, analysis of molecular variance (AMOVA) was also conducted to test the significance of the inferred population structure with the software ARLEQUIN v 3.1 (Excoffier et al. 2005) at three levels: among populations (considering Navarre and the Basque country) among individuals within populations and within individuals. MICRO-CHECKER software (Van Oosterhout et al. 2004) was used to check for potential scoring errors, the presence of null alleles and linkage disequilibrium.

Spatial autocorrelation analysis

We assessed fine-scale spatial genetic structure (SGS) using spatial autocorrelation analyses. Spatial autocorrelation analyses, i.e. the analyses of genetic relatedness between pairs of individuals as a function of geographical distance, were conducted using SPAGeDI 1.2 (Hardy & Vekemans 2002). These were performed on the 15 polymorphic *loci* with kinship coefficients (Fij) (Loiselle et al. 1995). As suggested by Vekemans and Hardy (2004), Fij was chosen as a pair-wise estimator of genetic relatedness, as it is a relatively unbiased estimator with low sampling variance. The slope (b) of this linear regression does not depend on an arbitrary choice of distance classes and, therefore, provides a good estimator of the degree of SGS at this scale (Hardy & Vekemans 2002).

Because there is no consensus regarding how to generate distance classes, we used the recommendations of Hardy and Vekermans (2002). In all cases, more than 50% of all individuals were represented at least once in each interval, and the coefficients of variation of the number of times each individual was represented was less than one (Hardy & Vekemans 2002). Thus, to illustrate the pattern of spatial autocorrelation, the number of spatial distance classes was set to 22, leading to a minimum of 148 pair-wise comparisons per distance class. A jackknife procedure over loci was used to estimate standard errors for each distance class and 10,000 randomizations of individual spatial locations were performed to test for the overall spatial structure (Hardy & Vekemans 2002).

Bottleneck detection

Statistical methods have been developed to infer the demographic history of a population from a single genetic sample. The program BOTTLENECK version 1.2.02 (Cornuet & Luikart 1996; Piry et al. 1999) was applied to test for recent reductions in effective population size. During population bottlenecks, rare alleles are lost due to drift at a rate faster than loss of heterozygosity. This disparity is used to detect past bottlenecks. The analyses were performed under the three microsatellite mutational models available: infinite allele model (IAM), stepwise (SMM) and two-phase model of mutation (TPM) (with 95% stepwise mutation).

Migration rates among clusters

Evidence of recent migration events across clusters was assessed using the MIGRATE software version 3.0.3 (Beerli & Felsenstein 2001; Beerli 2006;). This software estimates the number of migrants (4Nm) exchanged between populations per generation using an expansion of the coalescent theory in a Bayesian approach. Following the recommendations of Beerli (2004), we did an initial run on our data set using FST to find the start parameters, and we used the output of the initial run as the start parameters of our second run. Because there were only minor differences between the outputs from the first and the second runs, we presented only the output from the second run.

RESULTS

Non-invasive sample collection and species identification

Out of 977 faecal samples collected from the entire study area, 179 were discarded because they were not fresh or because they presumably belong to the same individual (samples separated by < 1km). 670 out of 798 analyzed samples were classified as one of the target species by our PCR-RFLP method. Thus, unequivocal species identification was possible in 84% of the samples. In the remaining 16%, the DNA extracted was not amplified by the primers used. We effectively identified 323 faecal samples as stone marten and 347 as pine marten.

Additionally, we obtained 74 tissue samples of pine marten and 109 of stone marten from road-kill animals. Thus we obtained a total number of 421 samples of pine marten and 432 of stone marten. The geographical locations for the 670 correctly identified faecal samples and 183 tissue samples of both species in the study area are shown in Figure 1. Geographic information system (GIS) technology revealed that areas of high quality pine marten habitat (natural forest) were interspersed in varying configurations

with non-forested areas and human- made non-suitable habitat (roads; urban areas, reservoirs) (Fig. 1). In this heterogeneous landscape the stone marten is widely distributed across the whole study area while the pine marten is restricted to the main forested areas.

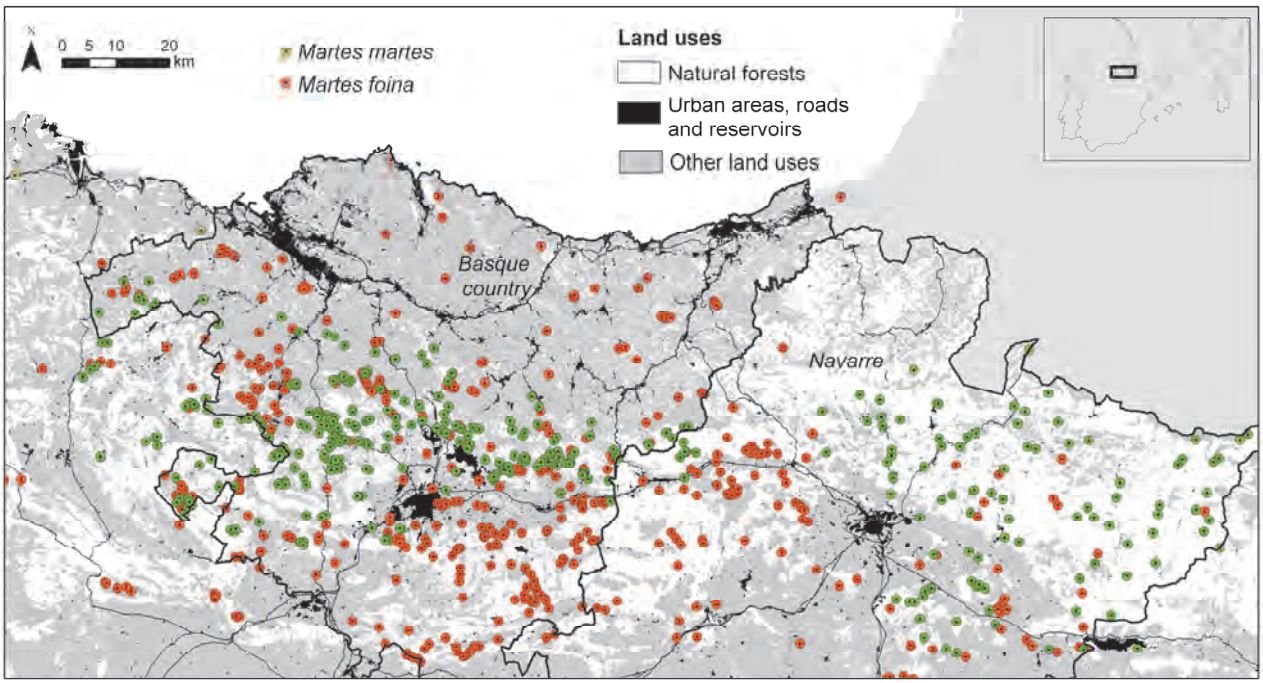

Fig. 1. Study area, located in the regions of the Basque country and Navarre, Northern Spain. Grey and white circles represent the geographical locations of samples identified as *Martes martes* and *Martes foina* by the PCR-RFLP method, respectively (Faecal samples: n=670; Tissue samples: n=183). Natural forest appears shaded in white, urban areas, roads and man-made reservoirs in black and other land-uses in grey.

Individual identification and genotype checking

Out of 347 faecal samples identified as pine marten 120 were not included in the microsatellite genotyping procedure. These samples corresponded to the sampling period between 2004-2005 and were used for a first distribution assessment of sympatric martens in the study area. Thus, 301 pine marten samples (227 faecal samples and 74 tissue samples) were used for microsatellite genotyping. The first quality-screening test was not passed by 103 non-invasive samples (45.4%), which were immediately discarded. The remaining 124 samples (54.6%) were amplified at the other 11 *loci*. After multiple-tube genotyping 41 samples from this sub-set (33.06%) were also discarded because they showed < 50% PCR success, or because of high failure rates. Full multilocus microsatellite genotypes were obtained for the remaining 83 samples (66.93% from the samples that passed the screening and 36.56 from the total samples analyzed). The observed average error rates across loci were: ADO = 0.178 and FA = 0.029. The average proportion of positive PCRs (calculated only for positive samples) was 91% and varied among loci

from 78 to 98% and among samples from 68 to 100%. PID analysis showed that the set of 15 *loci* would produce an identical genotype with a probability of 9.83×10^{-12}, and with a probability of 2.09×10^{-5} for a full-sib. Thus, only 2.09 pine martens in 100,000 siblings are expected to share by chance an identical genotype, suggesting no "shadow effect" (i.e. all the genotypes identify distinct individuals; Mills et al. 2000), and that matching genotypes are recaptures of the same individual.

After a regrouping procedure we identified 66 individual genotypes from faecal samples. The 74 tissue samples were correctly genotyped at 15 *loci* and provided new individuals. In total we identified 157 genotypes that correspond with 140 different individuals. The number of times each individual was detected in the survey varied from 1 to 3 with a total number of 17 re-samplings.

Clustering analyses

Bayesian clustering with spatial information (GENELAND)

The three GENELAND runs giving the highest average posterior probability suggested the presence of four geographically coherent genetic clusters in the study area. The modal population for each pixel of the study area inferred by GENELAND indicated that there is a clear spatial pattern with a West-East subdivision between the four inferred pine marten populations (Fig 2). The second and third best runs gave rise to similar clusters (results not shown). Individuals from Navarre (almost continuously forested area) formed a unique genetic cluster (N) located in the Eastern part of the study area. Individuals from the Basque country (highly fragmented forested area) formed three different clusters: Eastern Basque (EB) Central Basque (CB) and Western Basque (WB) clusters (Fig 2).

Bayesian clustering without spatial information (STRUCTURE)

Structure provided consistent results over 20 replicated runs tested for each K. The STRUCTURE analysis showed that the likelihood of the data increased quickly up to $K = 3$ and then reached an asymptote (Figure 3a). Calculation of ΔK value (Evanno et al. 2005) from the STRUCTURE output produced a distinct apex value (300.75) when $K = 3$ (Fig. 3a), implying the likely presence of three genetically distinct groups. The assignment of individuals to populations for $K = 3$ is presented in Fig. 3b and their spatial location is provided in Fig 2.

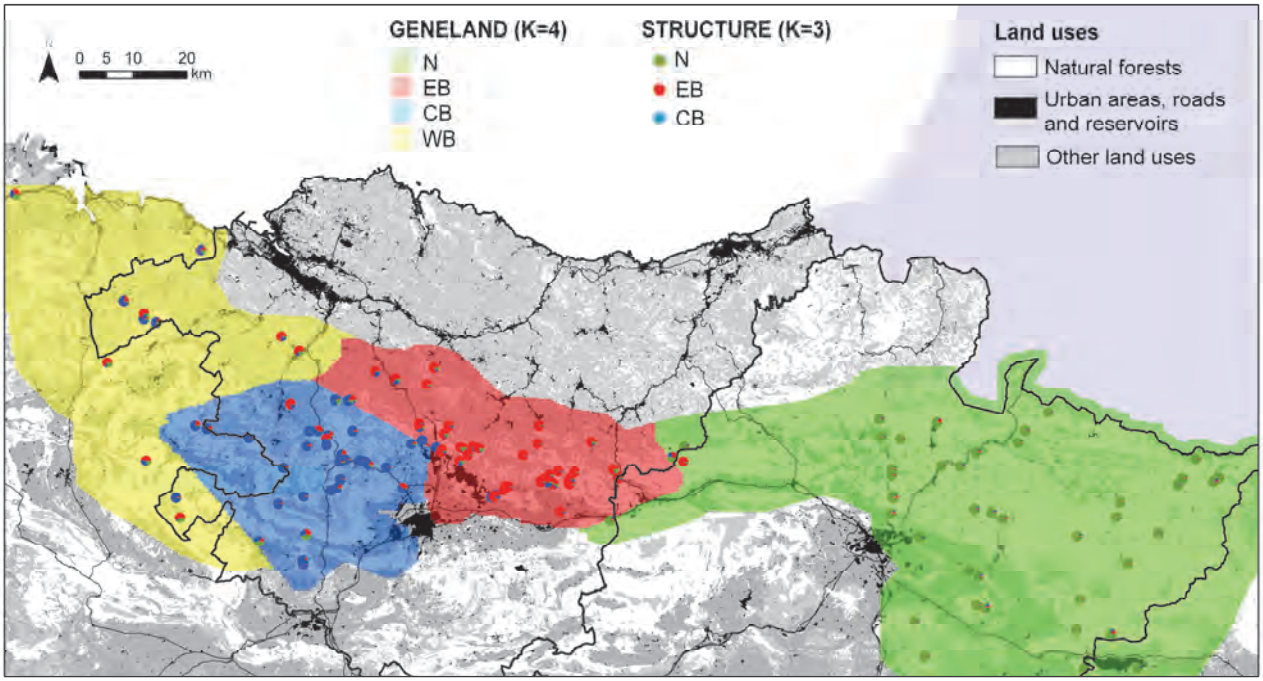

Fig 2. Modal population for each pixel of the study area according to GENELAND (K=4) and spatial distribution of the cluster membership coefficients according to STRUCTURE (K=3) for each individual. The four genetic clusters are represented by different colours: Navarre (N) cluster-green, Eastern Basque (EB) cluster-red, Central Basque (CB) cluster-blue, Western Basque (WB) cluster-yellow. Structure results: Each individual is represented by pie charts at the location where they were sampled (n = 140). The colours indicate the average membership coefficients for each individual to each of the three clusters uncovered by STRUCTURE (green, red and blue for cluster N, EB and CB, respectively).

The modal assignments by STRUCTURE roughly corresponded to those identified with spatial information in the GENELAND analyses (Fig 3b and Fid 2), with the exception of the WB cluster, which was not identified in STRUCTURE analysis (Fig 3b and Fig 2). However this group consisted of only a few (n=12), highly admixed individuals located in the western part of the study area (Fig 3b and Fig 2). This pattern was clearly evident when we interpolated on the map of the study area the cluster membership coefficients obtained with STRUCTURE and the modal population for each pixel of the study area obtained by GENELAND (Fig. 2). Although GENELAND suggested the presence of an extra genetic cluster, there was a clear convergence between the assignments of the individuals to each cluster provided by both Bayesian methods. STRUCTURE inferred basically the same clusters (i.e. N, EB and CB) with the exception of the WB cluster, which was only detected by GENELAND. 85.71% of the individuals (120/140) were assigned probabilistically to the same genetic cluster i.e. N, EB and CB by both methods (taking into account the assignment of the individuals by STRUCTURE to the population for which the estimated membership was the highest). In the case of the 12 individuals (8.57%) identified

as a different cluster by GENELAND (WB), these corresponded to individuals assigned by STRUCTURE to the EB or CB cluster and/or clearly admixed individuals (q<0.7) and all of them were located in the western part of the study area (Fig 3b and Fig 2). The 8 individuals (5.71%) for which each method provided different population assignments were located between contact zones of different clusters (Fig 3b and Fig 2).

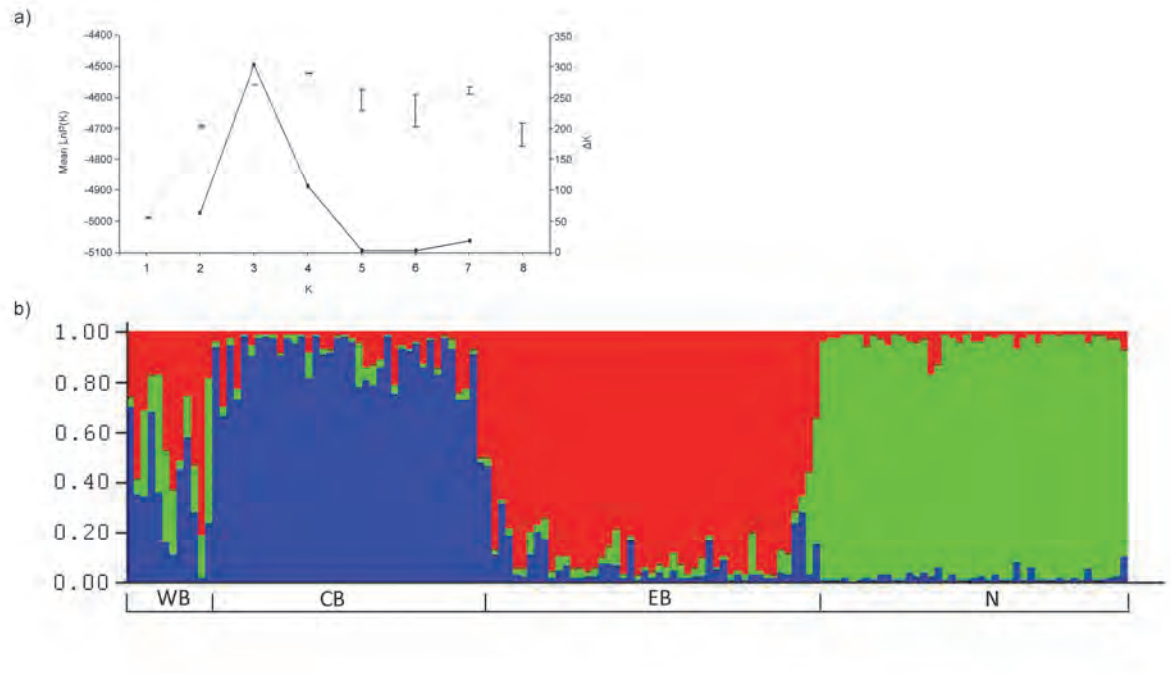

Fig. 3 Structure analysis results. a) Estimation of the number of pine marten clusters (K) from 20 independent runs for K = 1–8. Dotted line is the mean Ln probability of the data (Ln P(K)) (Pritchard et al. 2000) and the solid line is the second-order rate of change (ΔK)(Evanno et al. 2005), inferring that K=3. (b) Distribution of the three genetic clusters generated by STRUCTURE. The vertical lines are broken into coloured segments showing the proportion of each individual assigned to each of the inferred K. The three genetic clusters are presented by different coloured columns and are displayed according to East-West spatial location of individuals: Navarre (N) cluster-green, Eastern Basque (EB) cluster-red, Central Basque (CB) cluster-blue. Letters at the bottom of the figure correspond to Geneland inferred cluster composition for comparison. The Western Basque (WB) Geneland inferred cluster corresponds to 12 admixed individuals.

Overall, both Bayesian methods indicate that the entire *M. martes* population is separated into at least three subpopulations with a strong spatial pattern (Fig 3b and Fig 2) and with sharp borders between populations. Thus, there is a clear spatial genetic pattern confirmed by spatially explicit (GENELAND) and non-explicit (STRUCTURE) Bayesian methods. Comparing landscape data with population boundaries (Fig 2), it is clear that gene-flow barriers revealed by both Bayesian methods coincided with

spatial distribution of anthropogenic habitat gaps (boundary between CB and EB cluster) and the presence of the sympatric stone marten (boundary between N and EB cluster) (Fig 1 and Fig 2).

Genetic diversity and HWE

The overall pine marten dataset (n=140) is not at HWE (p=0.0003) (Table 1). These results were consistent with the existence of population structure, which is to be expected at this broad geographic level. On the other hand, we observed no deviation from HWE in each of the inferred populations (Table 1). These results confirmed the validity of the clusters inferred by Bayesian means. The clusters range in sample size from 12 to 47, with an average size of 35 individuals.

Table 1 Genetic diversity indices of pine marten samples genotyped at 15 microsatellite loci.

Cluster	n	A	Ho	He	HWE P value(SE)	Overall FIS
EB	47	4.200	0.5292	0.5621	0.0891 (0.0056)	0.0692
WB	12	3.8677	0.5878	0.5699	0.3991 (0.0192)	0.01298
CB	38	3.7333	0.5191	0.5338	0.3976 (0.0098)	0.04092
N	43	5.1333	0.5608	0.5845	0.0559 (0.0047)	0.05245
Total	**140**	**5.7333**	**0.5412**	**0.6191**	**0.0003 * (0.0002)**	**0.05130**

Genetic clusters identified by Geneland: EB, Eastern Basque; WB, Western Basque; CB, Central Basque; N, Navarre. n, number of individuals; A, mean number of alleles per locus, Ho, the mean observed heterozygosity, He, expected heterozygosity, HWE, Hardy-Weinberg equilibrium P value and standard error and overall Fis. Significant values are marked with asterisk (p<0,001)

Linkage disequilibrium was not apparent for any pair of loci within any of the subpopulations after performing Bonferroni corrections. The results of MICROCHECKER indicated that null alleles were apparently present at two loci: MP0188 and Ma19. However, the estimated frequency of the null allele at these loci occurred at a relatively low frequency (0.054-0.060). As such, it is valid to utilise the data generated using these markers to assess the levels of genetic diversity within, and the structure between, populations.

For all 140 pine martens, the average observed (HO) an expected (HE) heterozygosity with values at 0.542 and 0.6191, respectively (Table 1). All 15 *loci* were variable with the total numbers of alleles ranging

between 3 and 12 per locus. The mean number of alleles per locus in the 4 subpopulations ranged from 3.73 (WB) to 5.13 (N). Similar levels of genetic diversity were observed within each of the 4 subpopulations (Table 1). Observed heterozygosities ranged from 0.511 in the CB subpopulation to 0.5878 in the WB subpopulation. FIS values were positive but not significantly different from zero in each of the inferred clusters.

Genetic differentiation, migration rates and population bottleneck

Genetic differentiation among clusters was also revealed by FST values and migration rates. All inter-cluster pair-wise comparisons summarized by mean FST were significant ($P < 0.05$, Table 2). FST values ranged from 0.056 to 0.170 (Table 2) and suggested low migration rates. The N cluster was the most differentiated subpopulation with the highest FST value.

Table 2 F-statistic (FST) tests for pairwise population differentiation (GENELAND groups) based on microsatellite *loci* frequencies.

Cluster	EB	WB	CB	N
EB	-	0.05684*	0.07981*	0.11379*
WB	-	-	0.07480*	0.07753*
CB	-	-		0.17262*

*P < 0.05.

This result is in agreement with AMOVA analysis showing a high percentage of variation (11.07) when considering Navarre and the Basque country as the main groups in comparison to the 4.27% of variation among individuals within inferred clusters. Considering all the individuals the percentage of variations was 84.66 supporting the evidence of population substructure. Migration rate analysis also indicated that the migration rates between each genetic cluster were low, with bi-directional migration rates ranging between 2.5 and 3.6 individuals per generation (Table 3). However, our results indicated higher emigration from the EB cluster to adjacent areas (i.e. CB and N clusters) than from adjacent areas to the EB cluster. BOTTLENECK analysis found significant support ($P < 0.05$) for historical reductions in effective population size in each of the inferred clusters under all three microsatellite mutational models (i.e IAM, SMM, TPM).

Table 3 Bi-directional estimates of gene flow (Nm) between pine marten clusters as calculated with MIGRATE.

Migration from					
		EB	WB	CB	N
Migration to	EB	-	2.51806	2.5973	2.86636
	WB	2.84265	-	3.62889	2.85025
	CB	3.09339	2.52047	-	2.65878
	N	3.17622	2.51639	2.6798	-

Spatial autocorrelation

Spatial autocorrelation analysis also suggested local genetic structure within the study area. The negative regression slope (b=-0.0437 SE=0.00955) between kinship coefficient and logarithmic distance between individuals was significant (P < 0.001). There was significant deviation from the population mean kinship estimate in the closest and most distant distance classes (Fig. 3). Positive values of kinship coefficient were found at short distances, meaning that neighboring individuals had a higher genetic relatedness than random pairs of individuals, whereas negative values of kinship occurred at larger distances, indicating isolation by distance within the whole study area. The intercept of the correlogram with the x-axis was approximately 70 km (Fig. 3), suggesting that within this distance individual pine martens are more related than on average in the population.

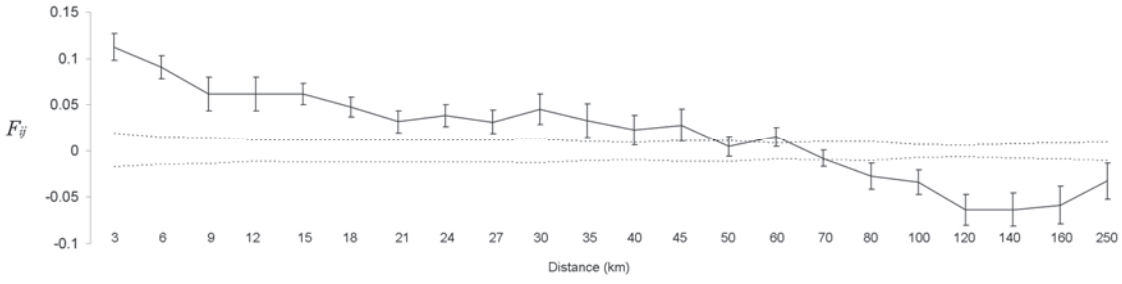

Figure 4. Average kinship coefficients, Fij, between pairs of pine martens individuals plotted against the Ln geographical distance. Dashed lines represent 95% confidence intervals for Fij under the null hypothesis that genotypes are randomly distributed. Significant deviation of all distance classes from the population mean (P<0.001) was observed with the exception of distance classes of 50 and 70km.

DISCUSSION

In this study we used non-invasive genetic sampling of faeces to assess the distribution of sympatric martens and to elucidate spatial genetic structure of the forest dwelling pine marten. First, as pine martens are elusive and stress-sensitive (Barja 2007), and inhabit sympatrically with the stone marten we applied a species identification method to asses spatial distribution of sympatric martens and to isolate the pine marten DNA samples. This DNA source is regularly used for non-invasive genetic studies of carnivores (Schwartz & Monfort 2008; Beja-Pereira et al. 2009). However, to our knowledge this is the first time that faecal DNA has been used to asses spatial genetic structure of the European pine marten. This allowed us to gather a higher number of samples for this study without any physical contact with individuals.

The application of a sufficiently variable panel of microsatellites for individual identification and population characterization through Bayesian analyses have allowed us to detect the presence of at least 3 genetic clusters distributed across the study area with non-overlapping distribution, but also of physical barriers to gene flow.

Spatial distribution of sympatric marten species

Our results indicated that the PCR-RFLP method (Ruiz-González et al. 2008) proposed for use on non-invasive faecal samples is a reliable, efficient, time-saving and cost-effective procedure for improving our knowledge of the spatial distributions of sympatric marten species, and, subsequently, to isolate pine marten samples for further microsatellite analyses. An overall high species identification rate was obtained (670 out of 798 analyzed samples; mean 84%). Species identification success was rather high with respect to other similar approaches (e.g.: 58%, Livia et al. 2007; 53.4%, Pilot et al. 2007) and similar to those obtained, by the same method, in the northern Iberian Peninsula (Rosellini et al. 2008; Ruiz-González et al. 2008) and Italy (Balestrieri et al. 2009).

The results obtained in the present study improved our previous knowledge about the spatial distribution patterns of sympatric marten species (Proulx et al. 2004). Both species showed a notorious spatial segregation in the study region, but they also co-exist locally in some forested areas. As a typical stenotopic species (Storch et al. 1990; Brainerd & Rolstad 2002, Zalewski & Jedrzejewski 2006), the results of our study confirmed that the pine marten is a species strongly associated with forest habitats, wherein its distribution is restricted to forested areas. This distribution pattern leaves this species particularly vulnerable to forest fragmentation (Proulx et al. 2004). Indeed, it was almost absent in the northern Basque country area, where the natural forests are scarce and human occupancy and anthropic

derangements of natural habitats are more intense. It was also absent from wide intensive crop fields in the Ebro Valley. Moreover, as a typical cold adapted Eurosiberian species, the pine marten was not present in the southern part of the study area, characterized by being more influenced by Mediterranean climate, although wide and well preserved natural deciduous forests are still present. In the absence of the pine marten, the stone marten was widely distributed in this area. This is in agreement with previous studies that indicated that in areas of the Iberian Peninsula without pine martens, stone martens are known to occupy the higher quality forest habitat (Virgós et al. 2000; Virgós & Garcia 2002;). These results suggested that climatic conditions are playing an important role in pine marten distribution, having a considerable influence on the bioecological relationships with the stone marten as the study area is located at the south-western limit of a typical Eurosiberian species (Proulx et al. 2004). The spatial distribution limit of pine martens outlined in this study is a step towards understanding the factors affecting the occurrence of the pine marten at the southern limit of its European distribution. Moreover, the more exact knowledge of pine marten distribution derived from this study has an important conservation value for the revision of the protective status of the species currently listed as "Rare" on the Basque Catalogue of Threatened species (Decree 167/1996 and Order of July 8, 1997). By contrast, the stone marten seems to be very adaptable and it was found not only in forests but also in a wide variety of different habitats including open areas, as well as a number of places markedly transformed and intensively exploited by humans. The latter species is generally regarded as generalistic in habitat with synanthropic behaviours in Europe (Reig 1992; Broekhuizen & Müskens, 2000; Lanszki 2003;) and has even invaded urban areas (Herr et al. 2009) but it can also establish home ranges in extensive agricultural areas near to rural villages and also in woodlands (e.g. Delibes 1983; Genovesi et al. 1997; Virgós & Casanovas 1998; Broekhuizen & Müskens, 2000; Virgós et al. 2000; Virgos & Garcia 2002; Santos & Santos-Reis 2010) as has been documented in this study. The stone marten has a clear dominance over the pine marten in the southern study area (i.e. Mediterranean area) and is the only *Martes* species found towards the south in the Iberian Peninsula, suggesting that its more thermophile condition (López-Martín 2007) provides a comparative advantage facing current climate change.

Wherever the two species occur sympatrically, the stone marten is often associated with rural and suburban areas (Delibes 1983; Reig 1992), while the pine marten occupies mainly forested areas. In one of the earliest studies about interspecific competition between martens, Delibes (1983) suggested that in altered environments the stone marten is displaced to more urbanised areas, but would tend to become less synanthropic wherever the density of the pine marten decreased. This compression of the spatial niche of the stone marten has been explained as a consequence of interspecific competition, favouring the slightly

bigger pine marten (Delibes 1983). In the study area, the rarity of the pine marten could be associated with events of direct persecution during recent decades and the increased effects of forest loss and fragmentation (López-Martín, 2007). This situation provides a better competitive framework to the stone marten, which shows more behavioural plasticity than the pine marten, proving capable of adaptation to different habitats and climatic conditions, and being less vulnerable to anthropogenic changes and human presence (Herr et al. 2009). Consequently, the colonization of new areas by the pine marten will be significantly reduced in areas where the stone marten is present, thus reducing gene flow between populations.

Recent studies have shown contrasting results regarding the relationships between the two species on a local scale. The pine marten was the only *Martes* in a mountainous and well conserved forest area in north-western Spain (Rosellini et al. 2008), while the pine marten is expanding its range into an agricultural landscape matrix in NW Italy which was previously considered a prerogative of the more synanthropic stone marten. In a forest-field mosaic of central Poland the two martens exploited the same micro-habitats (Pilot et al. 2007; Goszczyński et al. 2007), but they substantially differed in their ways in area searching and foraging behaviour (Posluszny et al. 2007) as well as in their attitude towards open areas and human settlements (Goszczyński et al. 2007).

Our results agreed with previous studies highlighting that sympatric relationships differ depending on several factors. The ecological displacement (e.g. Rosellini et al. 2008) or co-existence (Pilot et al. 2007; Goszczynsky et al. 2007) that occurs when both species overlap in the same area probably depends on a combination of several factors, including the relative abundance of each predator species within the local carnivore guild, the availability of trophic resources, climate conditions (cold adapted *Martes martes* vs more thermophile *Martes foina*) and landscape effects of forest fragmentation and human derangement of the surrounding habitats. Both species have inverse attitudes towards these factors (stenotopic *Martes martes* vs more synanthrophic *Martes foina*). Competition for these resources and the adaptability to new environmental conditions can play a fundamental role in the interactions between these species (Schoener 1974; Linnell & Strand 2000).

In summary, the results provided by the combined use of non-invasive genetic analysis of faecal samples and GIS technology suggest that our protocol would serve to assess the landscape effects on distribution patterns, identify and resolve information gaps and design effective research and management programmes. Moreover, an ongoing work on habitat suitability modelling will allow us to assess the interspecific relationships of martens in relation to environmental and landscape variables (Ruiz-González et al. in prep.).

Faecal DNA genotype reliability

Research on non-invasive genetics of mustelids are limited and biased towards species identification methods (e.g. Gómez-Moliner et al. 2004; Livia et al. 2007; Ruiz-González et al. 2008). In this study, species identification of the field-collected scats ensured that only target pine marten samples were used for further microsatellite analysis. Initial steps for species identification are thus crucial in landscapes where both species overlap extensively (Ruiz-González et al. 2008) allowing reliable spatial distribution assessment. Microsatellite genotyping of faecal samples has also been carried out on a small number of mustelid species and used mainly for individual identification and population size estimation (e.g. European otter, Arrendal et al. 2007; Hajkova et al. 2009; wolverine, Hedmark & Ellegren 2007; badger, Wilson et al. 2003; Frantz et al. 2006). To our knowledge, this study is the first to investigate and evaluate the spatial genetic structure of the European pine marten from the focus of faecal DNA.

Reliable amplification of microsatellite markers is a prerequisite for estimating the size and structure of populations within the landscape (Taberlet et al. 1999; Valière et al. 2007). The most conservative estimate of PID (i.e. PID_{-Sib} statistics) suggested that DNA profiles consisting of the 15 *loci* used in our study would be sufficient to distinguish between individuals of the study species, including siblings, with 99% certainty ($PID_{-Sib}=2{,}09x10^{-5}$). The success rate of pine marten microsatellite genotyping (presented here as a percentage of faecal samples that provided consensus genotypes, for all loci needed for reliable individual identification) in our study was 36.56%. This result is approximately around average in comparison with many other non-invasive studies undertaken on other carnivore species (e.g Frantz et al. 2003; Hedmark & Ellegren 2007; Hajkova et al. 2009; Marucco et al. 2009; Lanszki et al. 2010; Stenglein et al. 2010). Similarly, our error rates (ADO = 17.8%, FA = 2.9%) were comparable to those obtained in other non-invasive genetic studies of mustelids (e.g Frantz et al. 2003; Hajkova et al. 2006; Ferrando et al. 2008).

Genetic variability of the European pine marten

Previous studies focused on the pine marten have given information about the genetic variability and structure of several European populations (Kyle et al. 2003; Mergey 2007; Pertoldi et al. 2008; Mullins et al. 2010). So far, however, information about Iberian populations was unavailable. Moreover, these studies were mainly focused on tissue (Kyle et al. 2003, Mergey 2007; Pertoldi et al. 2008) or remotely plucked hair (Mullins et al. 2010) so this study marks an improvement in genetic surveys of pine marten through faecal sampling.

Studying pine marten populations across Europe, Kyle et al. (2003) found microsatellite HE values in the range of 0.56–0.64 for continental and 0.34-0.66 for insular populations (8 microsatellite *loci*). In the same study, the pine marten populations have been shown to have an overall FST value of 0.18 (range: 0.016–0.330) which comprise comparisons between geographically distant populations (e.g. FST value between Italy and Germany 0.044). Mergey (2007) obtained similar values for different French populations (11 *loci*, HE=0.518) and values of FST ranging between 0.047-0.052 for populations separated by more than 130 km. Pertoldi et al. (2008) found HE and FST values in the range of 0.67-0.79 and 0.044-0.097, respectively for Danish populations (11 *loci*). Mullins et al. (2010) found slightly lower values of HE for the reduced and isolated Irish populations (0.35, 17 loci). In this study, using 15 variable *loci*, we detected HE values of 0.586. So, taking into account the higher number of variable loci used in this study we can estimate that, compared with continental European populations, Iberian populations of pine marten have lower levels of genetic variability. Interestingly, the FST values reported in this study, which range from 0.056 to 0.113, were surprisingly high taking into account the regional scale of the study area and the contiguous nature of the inferred clusters. However, caution is called for when trying to interpret differences in genetic variability and FST values between different studies as these differences can also be attributed to the different number of microsatellites used.

Spatial genetic structure and putative barriers to geneflow

Here we utilised individual-based Bayesian approaches for inferring the location of potential barriers to gene flow and identifying population structure (Manel et al. 2003; Guillot et al. 2005a).

Our data did not support the hypothesis that pine martens in the study area exist as a single, panmictic population. When we considered all the pine martens as a whole, we detected a significant deviation from random mating, indicating that *M. martes* were not distributed in a random fashion and were instead associated in genetically defined subpopulations. Subpopulation structuring of pine martens at regional level is confirmed when looking for genetic partitions using Bayesian methods. The results generated independently by STRUCTURE and GENELAND suggested the presence of at least three genetic units. Levels of genetic differentiation among the clusters represented by pair-wise FST, were greater than similar measures estimated among other pine marten populations sampled across much wider geographic areas (Kyle et al. 2003; Mergey 2007; Pertoldi et al. 2008). Even though the number of clusters varied between approaches (GENELAND, K=4 and STRCTURE k=3), the location of, and membership within, each of the inferred clusters was largely concordant between methods. Both spatial and aspatial Bayesian clustering methods inferred mainly the same three genetic clusters (i.e. N, EB and CB) and have a clear convergence

between the assignments of the individuals to each cluster (120 out of 140 individuals analysed were assigned probabilistically to the same genetic cluster). Accordingly, it seems that pine martens did not form a single reproductive unit, with a clear spatial subdivision within the study area.

A certain amount of non-convergence between different Bayesian clustering methods thus appears to be relatively frequent (Frantz et al. 2009; Francois & Durand 2010). In recent applications of spatially explicit and implicit models together to the same dataset, when more than one model is used, the studies often report consensual results (Fontaine et al. 2007; Latch et al. 2008; Liu et al. 2009), but there are interesting exceptions (Coulon et al. 2006; Rowe & Beebee 2007; Ball et al. 2010; Francois & Durand 2010). The detection of four clusters by GENELAND *versus* the three clusters identified by STRUCTURE can be interpreted as an example of increased power in spatially structured populations as has been previously documented (e.g. Coulon et al. 2006). However, recently Francois & Durand (2010) showed that models without admixture (i.e. GENELAND) are not sufficiently robust to the inclusion of admixed individuals in the sample, thus leading to an incorrect assessment of population genetic structure in many generic cases. Consequently, taking into account the high proportion of admixed individuals encompassed within the WB cluster, the validity of the fourth inferred GENELAND cluster should be interpreted with caution. Moreover, this group is composed of a reduced number of individuals (n=12). Therefore, the validity of the results regarding this cluster should be further evaluated with an increased sample size.

As noted by the authors of the Bayesian algorithms used here (Pritchard et al. 2000; Guillot et al. 2005b) and recently shown by simulation (Frantz et al. 2009; Schwartz & McKelvey, 2009), deviations from random mating not caused by barriers to gene flow (i.e. spatial autocorrelation and isolation by distance) and the sampling scheme may have impacts on the detection and interpretation of genetic structure. In this study, spatial autocorrelation analysis strongly hinted an isolation by distance pattern. However, the conformation of the inferred subpopulations to HWE expectations, and given the fact that the inferred clusters showed significant differentiations as indicated by estimates of pairwise FST values, suggests that these subpopulations have been appropriately defined (Guillot & Santos 2009). The low migration rates detected between identified clusters also gave strong support to the genetic structure detected. Besides, agreement between the results of similar models and algorithms (i.e. GENELAND and STRUCTURE) give a high degree of confidence to the proposed clustering solution (Guillot et al. 2010).

Finally, our results clearly demonstrated the existence of a neat spatial pattern, with no overlapping distribution between subpopulations and with sharp discontinuities between contiguous areas that are related to different environmental features, while this was in no way included in the algorithm itself.

The N, EB, and CB clusters were delineated with similar boundaries in STRUCTURE to those identified with spatial information in the GENELAND analysis, both confirming that the main genetic discontinuities found are spatially coherent. Even though the analysis with STRUCTURE did not use any prior geographical information, it inferred the same genetic discontinuities between groups, with the only exception of the boundary with the WB cluster, which was only corroborated by GENELAND. The location of their boundaries suggested the existence of some putative barriers to geneflow in the study area, but as has been previously documented for other forested species, the combination of several landscape features with low permeability can lead to population differentiation (Coulon et al 2006; Broquet et al. 2006).

Different and non-exclusive hypotheses can explain the significant genetic structure observed in pine marten populations at the regional scale. Firstly, over-harvesting processes (Helldin 2000) and direct persecution due to their valuable fur greatly depleted pine marten populations in recent decades, also in North Spain (López-Martín, 2007). The three primary genetic consequences of hunting are the alteration of population structure, loss of genetic variation, and evolution resulting from selection (Harris et al. 2002; Allendorf et al. 2008; Coltman 2008, Allendorf & Hard 2009). Hunting activities, which stopped only a few decades ago in North Spain, and its consequences could be responsible for the recent decline of pine marten populations in the study area and thus, an important factor in the origin of the recurrent population bottlenecks detected in all the inferred clusters. Thus, hunting is probably one of the factors involved in the origin of the present genetic structure.

Secondly, habitat fragmentation may have important consequences for population genetic structure, which may be even greater in habitat specialists that show a high dependence on particular resources and strong sensitivity to habitat changes and loss (Coulon et al. 2006; Broquet et al. 2006; Devictor et al. 2008). Habitat fragmentation may be due to the presence of (non-linear) areas of non-suitable landscape elements that act as 'moderators of gene flow' and lead to the differentiation of genetic units (e.g. Coulon et al. 2006), as also to the existence of narrow linear elements (e.g. roads, rivers) that divide otherwise continuous patches of habitat, or both. Indeed, depending on the abilities of organisms to move through the matrix separating habitat patches, gene flow directions and quantities can be deeply modified, which can in turn affect the genetic structure of the species (e.g. Coulon et al. 2006; Broquet et al. 2006; Wasserman et al. 2010), even in the case of highly mobile carnivores (e.g. Latch et al. 2008, Zalewski et al. 2009). Certainly, deforestation and forest fragmentation have been reported to affect the distribution and density of pine martens (Brainerd et al. 1995; Kurki et al. 1998), which are believed to need a minimum woodland area to survive (Zalewski & Jędrzejewski 2006) and tend to avoid treeless areas (Storch et al.

1990; Brainerd and Rolstad 2002, Pereboom et al. 2008). As a result of habitat specialization this species is particularly sensitive to changes in its original habitat, including habitat loss, and landscape-scale effects of habitat fragmentation (Brainerd 1990; Bright 1993; Proulx et al. 2004; Pereeboom et al. 2008). This specific habitat requirement is probably the cause of very limited gene flow between contiguous subpopulations and the strong genetic structure observed between geographically close localities in the highly fragmented forests in Basque country area, as demonstrated by the Bayesian clustering of groups of individuals. On the other hand Navarre, formed by a largely continuous forested area, belongs to a single panmicitic population. These results are in agreement with previous studies suggesting that, for typical forest dwelling species, one of the main factors structuring present-day genetic diversity could be attributed to forest fragmentation processes (e.g. Coulon et al. 2006; Broquet et al. 2006; Olivieri et al. 2008; Segelbacher et al. 2008; Craul et al. 2009; Liu et al. 2009; Pavlacky et al. 2009; Bruggeman et al. 2010).

When comparing the gene-flow discontinuities revealed by Bayesian means and the spatial pattern of all landscape features, some evidences of geographical barriers could be identified. The genetic discontinuity found between CB and EB clearly overlapped with the geographic location of an area characterized by a high density of roads, the presence of large man-made reservoirs and the high presence of human urbanization. Indeed, this area has been previously identified as one of the critical connectivity areas in the framework of the Basque connectivity network (Gurrutxaga et al. 2010a, b). The reduced migration across this boundary may be rooted in species behaviour (i.e. aversion to move across treeless areas; Storch et al. 1990, Brainerd & Rolstad 2002, Pereboom et al. 2008) and direct road mortality (Balkenhol & Waits 2009). The high differentiation detected between CB and EB subpopulations suggested that this area strongly affected gene flow between these spatially contiguous areas. The genetic discontinuity located between the EB and N clusters corresponded to an area where the pine marten distribution shows a clear gap in spite of the presence of suitable forest habitat. Indeed, the distribution gap matched with the presence of the more synathropic stone marten. There is no previous genetic evidence about the effect of interspecific competition on dispersal and genetic structure on mustelids. However our results suggested that interspecific competition between sympatric marten species could be playing an important role in structuring pine marten populations. Spatial distribution data showed that each marten species does not utilize the landscape uniformly. Thus, contrasting distribution patterns, differences in habitat utilization and ecological traits in both stone and pine marten could also be playing an important role in structuring pine marten populations. Consequently, the colonization of new areas by the pine marten will be significantly reduced in areas where the stone marten is well established, thus becoming a barrier to gene

flow between pine marten populations. These results provide an interesting insight into the effects of interspecific competition on dispersal and genetic structure in sympatric carnivores.

Altogether, our results have shown that pine martens inhabiting a fragmented landscape in the Basque country and Navarre may not be considered to belong to a single panmicitic population, comprising at least three different genetic units with strong genetic differentiation despite a rather small distribution area. The data reported in this study suggested that habitat loss and fragmentation could be the main factors responsible for the spatial genetic structure found in a typical forest dwelling species. Several landscape features (i.e. unforested areas, urbanized areas, roads and man-made reservoirs) acted as moderators of gene flow because of their high resistance to pine marten migrations, and hence their cumulative effect led to the differentiation of the inferred genetic units. Moreover, past over-harvesting processes, together with the probable interspecific competition with the stone marten added to the fact that the study area is placed at the southern limit of pine marten distribution, could be acting synergically with forest fragmentation as factors shaping the current spatial genetic structure.

Implications for conservation and connectivity restoration

Understanding the spatial genetic structure of populations can provide insight into the ecological or evolutionary processes of the species, and enable wise conservation decisions (Segelbacher et al. 2010). Anthropogenic habitat degradation and fragmentation not only leads to distribution range contractions and population extinctions, but may also have significant genetic, and thus evolutionary, consequences for populations (Fischer & Lindenmayer 2007; Nabe-Nielsen et al. 2010). For the conservation of species inhabiting fragmented habitats, securing dispersal between local habitat patches appears to be the major challenge (Taylor et al. 1993; Cushman et al. 2009). Pine martens have experienced a large range contraction in the last few decades in the study area, and, we found some landscape features that already constrain connectivity among existing habitat patches. Further conservation measures thus need to consider population processes operating at the landscape scale ensuring population connectivity (Crooks 2006). Improving habitat quality and connectivity in the study area will not only secure the long-term persistence of the pine marten but also favour connectivity for other forest associated species (Gurrutxaga et al. 2010b). The future coexistence and relative abundances of the two martens in forest habitats will thus depend on the mode of forest management as well as on the existence of effective migratory corridors connecting neighbouring forest patches (Goszczynski et al. 2007; Gurrutxaga et al. 2010b).

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PAPER VI

LANDSCAPE GENETICS AS A TOOL FOR THE EMPIRICAL ASSESSMENT OF A REGIONAL ECOLOGICAL NETWORK: THE EUROPEAN PINE MARTEN (*Martes martes*) AS A TARGET-SPECIES

ABSTRACT

Coherent ecological networks (ENs) composed of core areas linked by ecological corridors are being developed worldwide with a view on the maintenance of landscape connectivity and biodiversity conservation. Although these ENs are developed in accordance with the precautionary principle and ecological theory, they have been subject to criticisms based on the lack of empirical evidence regarding their efficiency in achieving their goals. Landscape genetics in combination with non-invasive genetic sampling provides a valuable framework for the study of rare and elusive species in order to understand how landscape features influence gene flow, by correlating inter-individual pairwise genetic distances with least-cost modelling. Here we analysed the accuracy of the parameters used in a resistance map which was drawn up in the design of the regional EN in the Basque Country (North Spain) with regards to the geneflow of a target species: the European pine marten. Moreover, different binary resistance maps which covered a gradient from greater to lesser preference of the focal species in relation to forest environments have been evaluated to determine the land uses which restrict gene flow of the pine marten. Our results confirmed the utility of the corridors designed and the need to keep taking them into account in land use planning. Also, we identified areas of intensive agriculture and the major road networks as landscape features which limit pine marten gene flow. To the best of our knowledge, this is the first time that a GIS modelled EN has been evaluated through a landscape genetics approach.

Key words: Landscape genetics, non-invasive genetic sampling, *Martes martes*, landscape connectivity, least-cost modelling, land use planning

INTRODUCTION

Long-term biodiversity conservation requires the preservation of ecological and evolutionary processes, such as gene flow, dispersal movements and change shifts in biota (Opdam and Washer 2004). The ability of individuals to move across changing landscapes is crucial for maintaining regional populations (Fahrig 2007; Cushman et al. 2009). The preservation of these processes requires in turn that landscape connectivity be preserved, especially when we take into account the synergetic effects of habitat fragmentation and climate change (Opdam & Washer 2004). Connectivity is defined as the degree to which landscape facilitates or impedes movement of organisms among resource patches (Taylor et al. 1993). Connectivity is species-specific and describes the response of individuals to landscape features and the patterns of dispersal and gene flow that result from these individual responses (Brooks 2003). Landscape connectivity depends to a large extent on the spatial configuration of the habitat and on land use (Opdam & Washer 2004).

Ecological networks have been promoted in the last few decades as coherent systems composed of core areas linked by ecological corridors capable of facilitating the dispersal, migration and gene flow of wild species in landscapes and regions (Bennett & Wit 2001; Jongman & Pungetti 2004). They are configured and managed with the objective of maintaining ecological functions and conserving biodiversity (Bennett & Witt 2001). Although the development of ecological networks is based on the precautionary principle and on ecological theory (Jongman & Pungetti 2004), the absence of empirical evidence regarding their effectiveness and the difficulty in obtaining this evidence has been a focus of criticism regarding the extent to which they have in fact ensured landscape connectivity and increased biodiversity conservation (Boitani et al. 2007).

In the design of ecological networks there is a need to predict regional ecological corridors and to quantify the degree of expected landscape connectivity between specific areas (Segelbacher et al. 2010). 'Least-cost modelling' is one commonly employed approach for designing ecological corridors (Adriaensen et al. 2003), in which resistance values are assigned to distinct habitat or land use types and the least-cost paths (LCP) between specific locations are calculated using a geographical information system (GIS). How landscape influences effective distances between locations is calculated as the accumulated cost through the least cost paths (Adriaensen et al. 2003; Ray 2005). For most organisms, setting the resistance values is a difficult process in which expert judgement and data available in the literature play an important role (Adriaensen et al. 2003; Beier et al. 2008; Spear et al. 2010).

Reliable mapping of corridors must be based on a correct representation of the local resistance relative to movement of the organism in focus (Beier et al. 2008; Spear et al. 2010). Landscape genetics, a research area that integrates landscape ecology, population genetics and spatial statistics, provides a valuable framework for testing the influence of landscape structure and composition on dispersal and gene flow (Manel et al. 2003; Balkenhol et al. 2009). It facilitates detection of the ways in which the resistance of given landscape patches affects dispersal movements and, in consequence, gene flow (Holderegger & Wagner 2006; Segelbacher et al. 2010). Thus, one of the principal applications of landscape genetics in landscape planning and conservation biology is precisely to empirically test resistance maps or friction surfaces (Epps et al. 2007; Shirk et al. 2010). This facilitates the optimal design of ecological corridors (Epps et al. 2007; Beier et al. 2008; Cushman et al. 2009), the detection of barriers to gene flow and the identification of the landscape features which favour dispersal (Coulon et al. 2004; Broquet et al. 2006; Wang et al. 2008; Schwartz et al. 2009; Shirk et al. 2010).

Gene flow is a function of the degree to which a landscape resists dispersal, which, in turn, is highly dependent on both the species and the landscape in question (Hastings & Harrison 1994; Spear et al. 2010). Thus, gene flow estimated through highly polymorphic genetic markers such as microsatellites helps to validate landscape connectivity modelled by LCP (Coulon et al. 2004; Broquet et al. 2006; Epps et al. 2007). The most common methodology adopted for landscape genetics studies aiming to relate landscape patterns with gene flow processes is to correlate inter-individual genetic distances with effective distances as derived from GIS LCP models and Euclidean distances with methods such as simple (Mantel 1967; Coulon et al. 2004, Broquet et al. 2006; Wang et al. 2008; Zhu et al. 2010) and partial Mantel tests (Smouse et al. 1986; Cushman et al. 2006; Schwartz et al 2009; Wasserman et al. 2010). Thus, testing the departure of genetic distances from isolation by distance pattern (IBD) (Wright 1943), through the inclusion of effective distances, provides evidence of the specific effect of landscape features on genetic structure.

Landscape genetics has shifted towards individual-based sampling and analysis, especially when organisms are continuously distributed (Segelbacher et al. 2010). However, sufficient sample collection for this purpose is a difficult task, especially in rare and elusive species in which sampling is a limiting factor (Beja-Pereira et al. 2009). In this context, molecular methods incorporating non-invasive sampling via the collection of scats or hairs have become common for population monitoring of carnivores, providing a valuable DNA source for genetic surveys (Taberlet et al. 1999; Piggott & Taylor 2003; Waits & Paetkau 2005). Noninvasive genetic sampling allows us to address studies of wildlife species without the need to

capture or even observe them (Waits & Paetkau 2005). Thus this study has the added advantage of permitting us to infer landscape connectivity without needing to intervene directly in the species in focus.

In 2005 a regional ecological network was drawn up in the Basque Country (North Spain). This was achieved by delimiting the ecological corridors linking protected areas (Gurrutxaga et al. 2010a). A functional group of forest mammal species was selected so as to obtain parameters for a generic resistance map which would serve as a basis for the design of the ecological corridors by least-cost modelling (Adriaensen et al. 2003). These mammals were considered suitable target-species due to their sensitivity to the most important recent fragmentation and homogenization dynamics in the regional landscape, such as road construction, urbanization and agrarian intensification (Jongman 2002). The resistance of the landscape matrix was parameterized through bibliographical review and expert opinion (Gurrutxaga et al. 2010a). The design of the resistance map was based on the assignation of different resistance levels to different land uses; this was especially so given that the ecological network was drawn up specifically for use in the framework of land use planning. The regional government of the Basque country incorporated that coherent ecological network as a reference for the environmental assessment of plans, programmes and projects in 2005 (Gurrutxaga et al. 2010a). In addition to its intrinsic internal relevance, the Basque country has been chosen for its crucial role in the regulation of relevant biotic flows. This is because of its strategic location between two important biodiversity reservoirs in south-western Europe, the mountain chains of the Pyrenees and the Cantabrian Range (Jongman et al. 2006; Worboys et al. 2010). Consequently, the preservation and restoration of connectivity in this transitional area between mountain ranges requires adequate land use planning (Workboys et al. 2010).

In the set of functional forest mammals which was taken into account in the design of the coherent ecological network in the Basque Country the European pine marten (*Martes martes*) is the most woodland-dwelling species (Proulx et al. 2004). The pine marten is generally associated with forest habitats, mainly mature forests (Zalewski & Jędrzejewski 2006; Proulx et al. 2004). Nonetheless, they have also been recently reported in fragmented landscapes where woods consist of isolated, small fragments within an agricultural landscape matrix (Pereboom et al. 2008; Balestrieri et al. 2010). Deforestation and forest fragmentation have been reported to affect the distribution and density of pine martens (Brainerd et al. 1995; Kurki et al. 1998), which are believed to need a minimum woodland area to survive (Zalewski & Jędrzejewski 2006) and tend to avoid treeless areas (Storch et al. 1990, Brainerd & Rolstad 2002, Pereboom et al. 2008, Ruiz-González et al. 2008). Given their strong associations with structural complexity in forests, the species is particularly sensitive to human influences on their habitats, including habitat loss, and landscape-scale effects of habitat fragmentation (Bright 2000, Pereboom et al. 2008).

Consequently, the pine marten is a species which is well suited to studies focused on the effects of forest fragmentation on genetic structure and gene flow.

In a recent study (Ruiz-Gonzalez et al. in prep) Bayesian analysis methods suggested that the pine marten in the study area was divided into at least two populations where several human-induced landscape transformations (i.e. roads, forest fragmentation) could well be mainly responsible for the observed genetic discontinuities. However, individual-based analyses relating landscape structure to genetic distance across heterogeneous landscapes permit a more accurate assessment of multiple alternative hypotheses relating landscape pattern to gene flow, and thus indentifying the landscape variables responsible for the inferred genetic structure.

The main objective of this research is to develop an individual-based non-invasive landscape genetic approach in order to evaluate alternative resistance maps relating landscape structure to gene flow Specifically, we aim to determine firstly i) whether or not the resistance map with which the regional ecological network was originally designed in the Basque Country was correctly parametrized regarding the European pine marten gene flow. This is done with a view to obtaining empirical validation of the EN from the ecology of a target species which is sensitive to relevant landscape dynamics; and additionally test ii) different binary resistance maps which covered a gradient from greater to lesser preference of the focal species in relation to forest environments in order to identify which land uses favour or impede genetic interchange of the European pine marten in the study area.

METHODS

Study area and spatial data

The region of the Basque Country is located to the north of the Iberian Peninsula (Fig. 1) and belongs to the Atlantic and Mediterranean biogeographical regions. It comprises an area of 7235 km^2 and has a population density of 298 inhabitants per square kilometre. There are three NUTS-3 provinces: Alava; Biscay; and Guipuzcoa. Approximately, forests cover 28% of the area, forestry plantations 29%, non-wooded mountains 24%, cultivated land 14%, and urban land and infrastructures 5.7%.

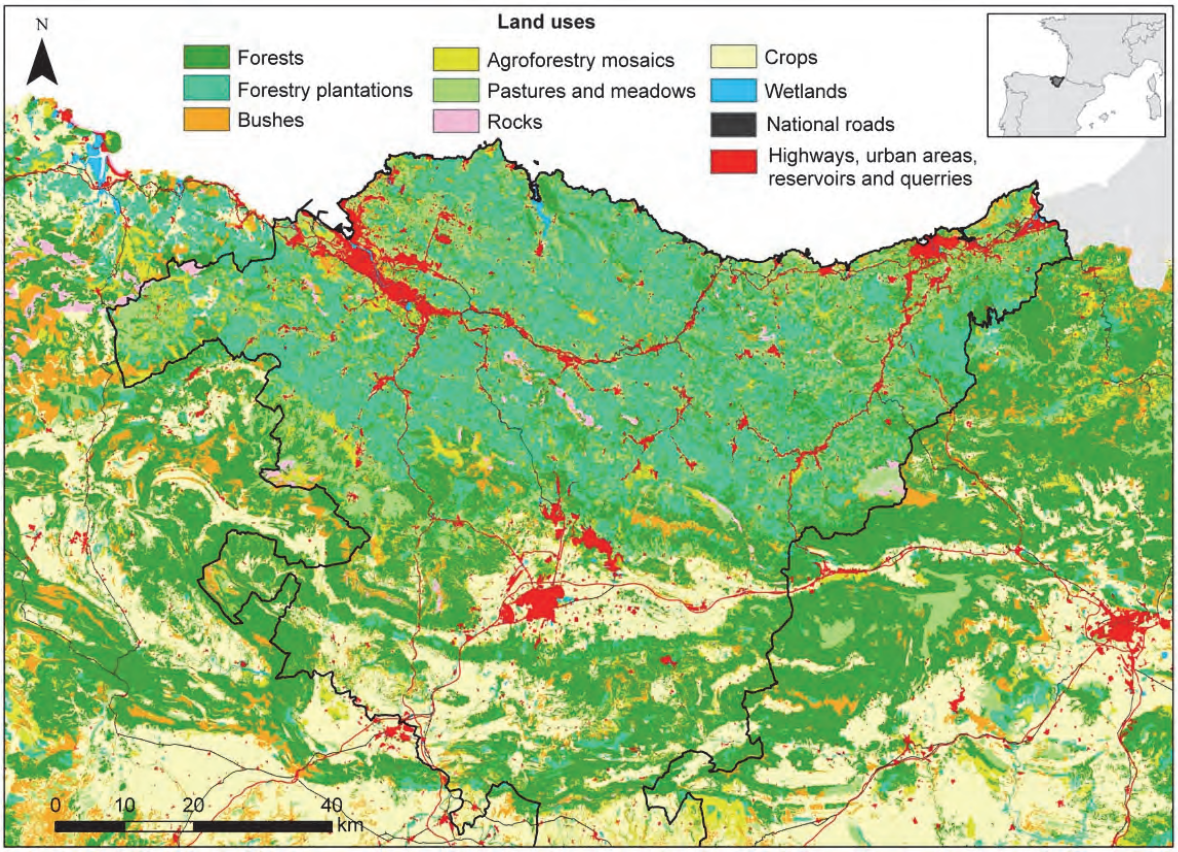

Figure 1. Distribution map of land uses in the study area.

Land use information was obtained in vector format from the most recent forest map of Spain (Spanish Ministry for the Environment, 2006) and from national road network maps (Spanish National Geographic Institute, 2008).

Non-invasive genetic sampling and species identification

We used non-invasive scat sampling to collect genetic samples from the *Martes* sp. (*Martes martes* and *Martes foina*) in the study area between 2005 and 2009. We conducted a multi-stage sampling scheme, in which samples from a pilot study were used to assess the appropriateness of the sampling with respect to the research questions. Thus, two scat-based surveys were conducted between 2004 and 2009 all over the sympatric range of both marten species in the study area. The first one, conducted in the 2004-2005 period was used to initially estimate the distribution range of the two sympatric species of the genus *Martes* in the study area and isolate genetic samples of the focal species (*M. marrtes*). The second, conducted between 2006 and 2009 was used to refine species distribution assessment and to obtain a higher number of *M. martes* fresh samples for microsatellite genotyping after a PCR-RFLP species identification process. Additionally, fresh tissue specimens from road-kill pine martens were included in the study, when

possible. Universal Transversal Mercator (UTM) coordinates were recorded for all the samples collected using a global positioning system (Garmin eTtrex). The faecal samples were stored in autoclaved tubes containing ethanol 96% and frozen at –20°C until processed (Ruiz González et al. 2008). DNA was isolated from tissues and scat using the Qiagen DNeasy Tissue DNA (Qiagen, Hombrechtikon, Switzerland) and DNA Stool MiniKit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions, respectively. As pine marten faeces cannot be distinguished from those of the sympatric stone marten (*Martes foina*), which is widespread in the study area, and can also be easily confused with those of other carnivores (Davison et al. 2002), a molecular technique was applied for the identification of faecal samples. Species identification was accomplished by a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method, providing for an effective genetic identification of sympatric marten species according to Ruiz-González et al. (2008).

Microsatellite analyses and individual identification

Identification of individual pine martens used nuclear DNA following methods in Ruiz-González et al. (in prep.) All the faecal samples identified by the PCR-RFLP method as pine marten were genotyped at 15 variable microsatellite *loci* using a multiplex protocol according to Ruiz-González et al. (in prep.) (Table S1). DNA quality was initially screened by PCR-amplifying each DNA sample four times at four *loci* (Multiplex 1: MP0188; MP0059; Gg-7; Ma-1). Only samples showing > 50% positive PCRs were further amplified four times at the remaining 11 *loci*. Samples with ambiguous results after four amplifications per locus or with <50% successful amplifications across loci were removed from further analysis as they were not considered reliable genotypes. Multiplex PCR products were run on an ABI (Foster City, CA) 3130XL automated sequencer (Applied Biosystems), with the internal size standard GS500 LIZ™ (Applied Biosystems). Fragment analyses were conducted using the ABI software Genemapper 4.0.

GIMLET software v 1.3.4 (Valière 2002) was used to calculate the probabilities of identity (PID and PID-sibs) so as to quantify the efficacy in discriminating the fifteen *loci* in combination. Consensus genotypes from four replicates were reconstructed using GIMLET, accepting heterozygotes if the two alleles were seen at least in two replicates and homozygotes if a single allele was seen at least in three replicates. GIMLET was also used to estimate genotyping errors: allelic dropout (ADO) and false alleles (FA) (Taberlet et al. 1996; Pompanon et al. 2005)

Genetic diversity and pairwise individual genetic distances

We summarized genetic variation through the number of alleles per locus (A) and expected (HE) and observed (HO) heterozygosities using GENETIX v 4.05.2 software (Belkhir et al. 2004). Estimates of pairwise linkage disequilibria for each pair of loci and deviation from HWE were tested using the exact test implemented in GenePop version 4.0 (Raymond & Rousset 1995; Rousset, 2008) in each previously defined subpopulation in the study area, according to Ruiz-González et al (in prep), and for the whole dataset. Statistical significance was evaluated by running a Markov Chain Monte Carlo (MCMC) consisting of 10,000 batches of 10,000 iterations each, with the first 10,000 iterations discarded before sampling (Guo & Thompson 1992). Significance levels were adjusted with sequential Bonferroni correction in order to correct for the effect of multiple tests (Rice 1989). MICRO-CHECKER software (Van Oosterhout et al. 2004) was used to check for potential scoring errors, the presence of null alleles and linkage disequilibrium.

The a_r inter-individual genetic distance (Rousset 2000) were computed using the program SPAGeDI (Hardy & Vekemans 2002) since this parameter of relatedness has been successfully applied to infer the effect of landscape on genetic structure in other vertebrates (Coulon et al. 2004; Broquet et al. 2006).

Resistance maps and effective distances calculation

Ecological network resistance map

Different resistance maps were used with a raster cell resolution set to 50 m. In the first place a resistance map was used which was analogous to that used in the design of the regional ecological network of the Basque Country (EN resistance map). The friction surface outlined in 2005 (Gurrutxaga et al. 2010a), was updated with the available vectorial information regarding land uses in the study area (Spanish Ministry for the Environment 2006; Spanish National Geographic Institute 2008). Raster breaks in linear barrier elements were avoided by the reinforcement of the size of national roads and highways (Adriaensen et al. 2003). Sections of highways which run through viaducts or tunnels were assigned the resistance value corresponding to the land use of the surrounding area. Equally, a friction map was used which was a variant of that used previously, with a view to testing the effect of noticeably decreasing the resistance attributed to national roads, highways, urban areas, reservoirs and quarries (ENnb map) (Table 1).

Binary resistance maps

Different binary resistance maps were used for the purpose of determining the land uses which were favourable and unfavourable to the dispersal movements of the martens. Broquet et al.'s (2006) methodology was adopted for the assignment of friction values. In this way, preferential land uses for

dispersal were assigned a value of 1, while non-favourable to dispersal habitat sites were assigned a value of 50. As pine martens are believed to need a minimum woodland area to survive (Zalewski & Jędrzejewski 2006) and tend to avoid treeless areas (Brainerd and Rolstad 2002; Pereboom et al. 2008; Ruiz-González et al. 2008), the different binary resistance maps (A to G) covered a gradient from greater to lesser preference of the focal species in relation to forest environments, ranging from strictly forest land up to and including open spaces (Table 1, Fig. 2). Different land uses were combined to create a single predictor variable (each resistance map). Additionally, in some resistance maps we included information regarding the river network as a means of dispersal (Table 1). Raster breaks in fluvial linear elements were avoided by the reinforcement of the size of rivers (Adriaensen et al. 2003). Friction surfaces were also used which included as barrier features the national roads (resistance 200), highways, urban areas, reservoirs and quarries (resistance 1000).

Table 1. Friction values corresponding to the resistance maps taken evaluated: *EN*) a resistance map analogous to that used in the design of the ecological network of the Basque country; *ENnb*) a variant of the latter which suppresses the barrier effect of national roads, highways, urban areas, water reservoirs and quarries; *A* to *G*) binary resistance maps, on a gradient from greater to lesser preference of the focal species in relation to forest environment. Maps with letter "r" include the river network with resistance 1. Maps with letter "b" include the barrier effect of national roads, highways, urban areas, reservoirs and quarries.

Land uses	A	B	Br	C	D	Dr	Db	E	Er	Eb	F	G	EN	ENnb
Forests	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Forestry plantations	50	1	1	1	1	1	1	1	1	1	1	1	10	10
Scrubland	50	50	50	1	1	1	1	1	1	1	1	1	5	5
Agroforestry mosaics	50	50	50	50	1	1	1	1	1	1	1	1	20	20
Pastures and meadows	50	50	50	50	50	50	50	1	1	1	1	1	30	30
Rocks	50	50	50	50	50	50	50	50	50	50	1	1	40	40
Crops	50	50	50	50	50	50	50	50	50	50	50	1	60	60
Wetlands	50	50	50	50	50	50	50	50	50	50	50	50	100	100
National roads	50	50	50	50	50	50	200	50	50	200	50	50	200	50
Highways, urban areas, reservoirs and quarries	50	50	50	50	50	50	1000	50	50	1000	50	50	1000	50
Rivers	-	-	1	-	-	1	-	-	1	-	-	-	-	-

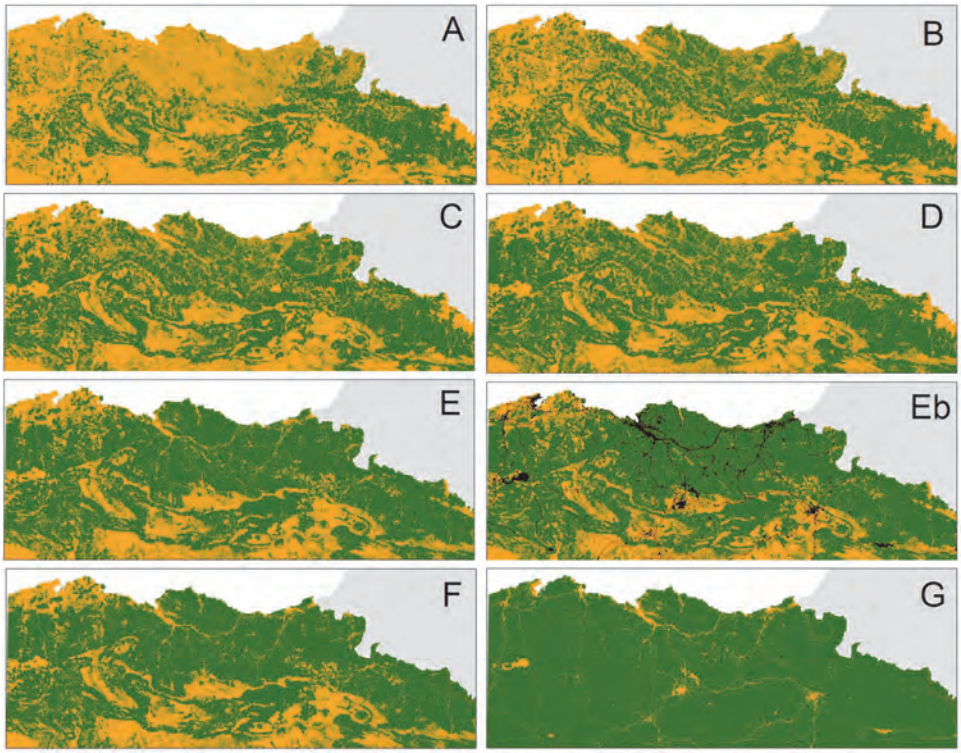

Figure 2. Resistance maps A, B, C, D, E, Eb, F y G. Green-coloured cells represent areas with resistance value 1, orange-coloured cells those with resistance value 50.

The effective and Euclidean distances between each pair of individuals were calculated with PATHMATRIX 1.1. (Ray 2005). Pair-wise effective distances between individuals were calculated as the accumulated cost through the least cost paths (LCP) throughout each friction surface (Adriaensen et al 2003; Ray 2005) (Fig. 3).

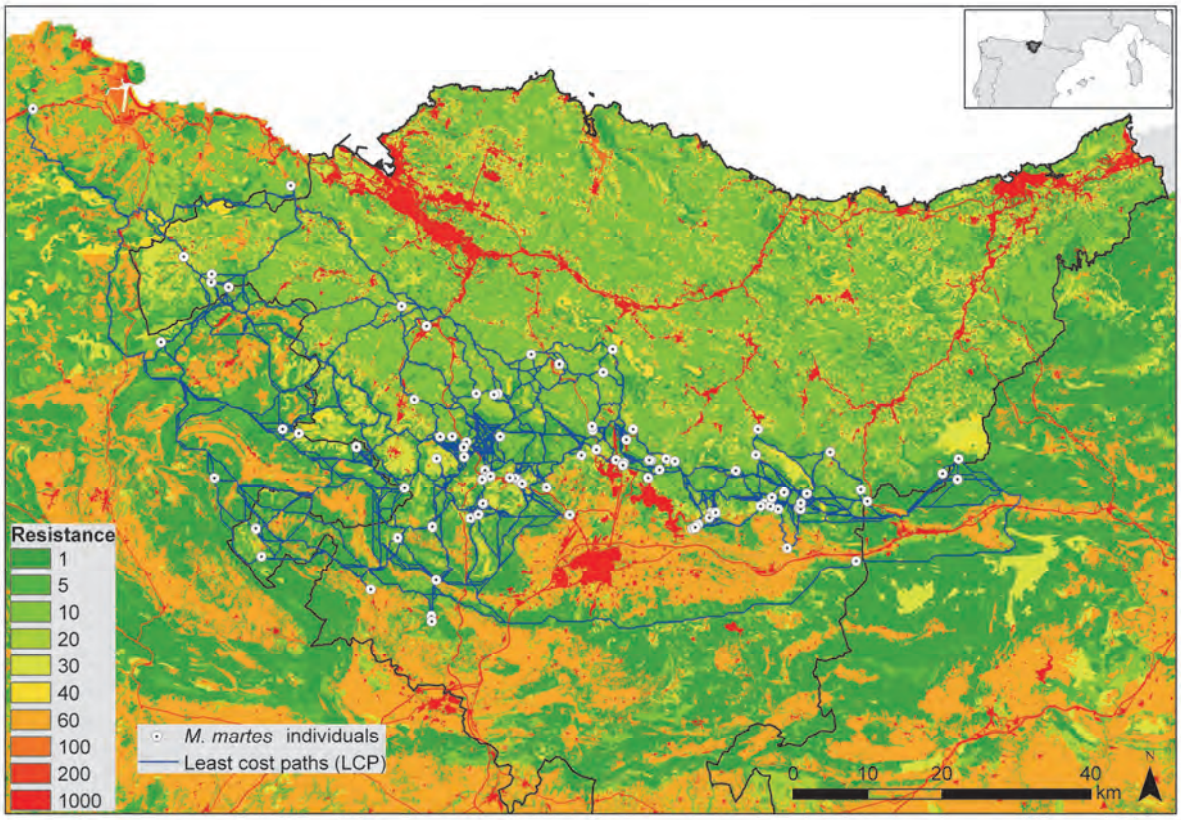

Figure 3. Least cost paths (LCP) obtained between the 101 pine marten individuals in accordance with the EN resistance map, analogous to that used in the design of the corridors in the ecological network of the Basque Country.

Relationship between genetic and geographical distances: effective vs Euclidean

The pairwise genetic distances matrix (a_r) was correlated with different matrices of geographical distances encompassing a total number of 5151 pairwise comparisons i) Euclidean distance, to determine whether the patterns of differentiation follow an isolation by distance pattern (null hypothesis) and ii) the effective distances calculated for each of the resistance maps, to infer landscape structure effects on gene flow. We also computed Partial Mantel tests (Smouse et al. 1986) between genetic distance and effective distance, factoring out the effects of Euclidean distance. The correlation between distance matrixes was calculated by means of the Mantel test (Mantel 1967) and partial Mantel tests (Smouse et al. 1986) as implemented in Xlstat software (Addinsoft, New York, USA) using genetic distances and the log of the effective and Euclidean distances, as recommended by Rousset (1997) for tests performed on species using a two dimensional landscape. P-values were obtained using a permutation procedure (10,000 permutations). We identified the model most supported as the one with the highest significant correlation value.

RESULTS

Non-invasive sample collection and species identification

Out of 733 faecal samples collected from the entire study area, 141 were discarded because they were not fresh samples or because they presumably belong to the same individual (samples separated by <1km). 494 out of 592 analyzed samples were classified as one of the target species (*M. martes* and *M. foina*) by our PCR-RFLP method. Thus, unequivocal species identification was possible in 83.45% of the samples. We effectively identified 232 faecal samples as stone marten and 262 as pine marten. Additionally, we obtained 57 tissue samples from road-killed pine martens.

Out of 262 faecal samples identified as pine marten, 108 were not included to the microsatellite genotyping procedure. These samples correspond to the sampling period from 2004-2005, which was used for a first distribution assessment of sympatric martens in the study area. Thus, 213 pine marten samples (154 faecal samples and 59 tissue samples) were used for microsatellite genotyping.

Individual identification, genotype checking and genetic diversity,

The first quality-screening test, based on 4 replicates of four *loci*, was not passed by 73 non-invasive samples (47.40%), which were immediately discarded. The remaining 81 samples (52.59%) were amplified at the other 11 *loci*. After multiple-tubes genotyping 27 samples from this sub-set (17.53% from the total analyzed samples) were then discarded because they showed < 50% PCR success, or because of high failure rates. Full multilocus microsatellite genotypes were obtained for the remaining 54 samples. (66.67% from the samples that passed the screening and 35.06 from the total samples analyzed). The observed average error rates across loci were: ADO = 0.218 and FA = 0.017. PID analysis showed that the set of 15 *loci* would produce an identical genotype with a probability of 1.69×10^{-10}, and with a probability of 4.45×10^{-5} for a full-sib. Thus, only 4.45 pine martens in 100,000 siblings are expected to share by chance an identical genotype, suggesting no "shadow effect" (i.e. all the genotypes identify distinct individuals; Mills et al. 2000), and that matching genotypes are recaptures of the same individual.

After a regrouping procedure we identified 42 individual genotypes from faecal samples. The 59 tissue samples were correctly genotyped at 15 *loci* and provided new individuals. In total we identified 113 genotypes that correspond with 101 different individuals. The number of times each individual was detected in the survey varied from 1 to 3, with a total number of 12 re-samplings.

The overall pine marten dataset (n=101) is not at HWE ($p<0.001$). These results were consistent with the existence of population structure, as has previously been demonstrated by Ruiz-González et al. (in prep).

In contrast, we observed no deviation from HWE in each of the previously inferred populations according to Ruiz-González et al (in prep). Linkage disequilibrium was not apparent for any pair of loci within any of the subpopulations after performing Bonferroni corrections. The results of MICROCHECKER indicated that null alleles were apparently present at two loci: Mp0188 and Ma19. However, the estimated frequency of the null allele at these *loci* occurred at a relatively low frequency (0.041-0.065).

For all 101 pine martens, the average observed (HO) and expected (HE) heterozygosity values were 0.53 and 0.58, respectively (Table S1). All 15 *loci* were variable with total numbers of alleles ranging between 3 and 8 per locus.

Correlations between genetic and geographical distances

Ecological network resistance map

A significant positive correlation was obtained between the genetic distances and Euclidean distances ($r=0.214$; $p<0.0001$), bearing clear witness to the existence of a pattern of isolation by distance (IBD) (Fig. 4). The correlation between genetic and effective distance calculated on the basis of the EN map was noticeably greater ($r=0.256$; $p<0.0001$) (Fig. 4). The degree of correlation when using the ENnb map was less than that which obtained with EN, though still greater than that obtained using Euclidean distance ($r=0.236$; $p<0.0001$) (Fig 4.). Both EN and ENnb models appeared better supported than the null model of IBD as this latter retained a significant positive relationship with a_r-based genetic distance even after factoring out the effects of Euclidean distance (Table 2). However, the original model used in the design of the ecological network (EN), which included a higher barrier effect for national roads, highways, urban areas, reservoirs and quarries was better supported than the alternative model (ENnb).

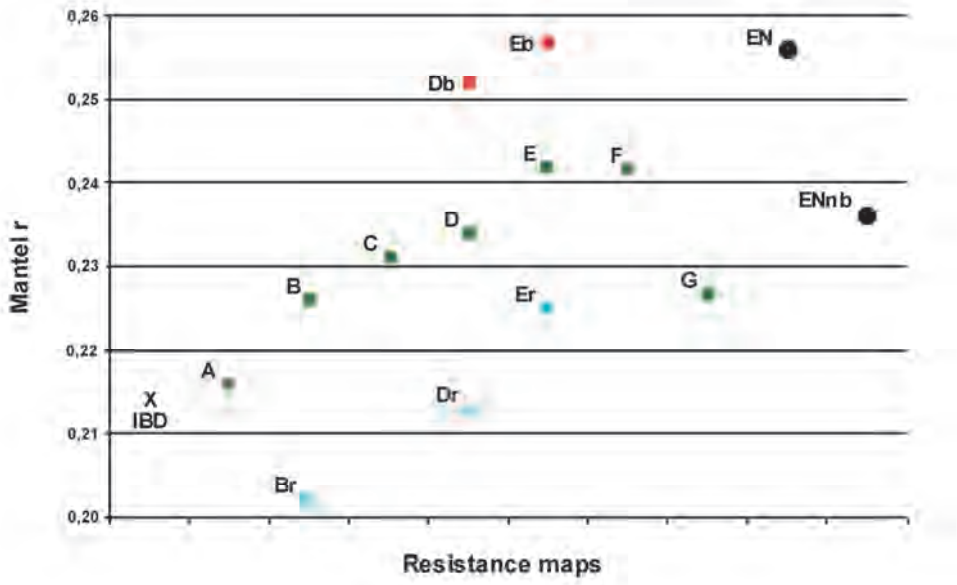

Figure 4. Pearson correlation coefficients (Mantel r) between genetic distance and effective distance in the different resistance maps examined. The correlation coefficient (Mantel r) between genetic and Euclidean distance (isolation by distance, *IBD*) was 0.214. In all cases the correlation was significant ($p<0.001$).

Table 2. Partial Mantel's test correlation with genetic distance (ar) for each resistance map after factoring out the effects of the null model of isolation by distance (IBD).

Correlation	Partial r	P value
a_r x EN	0.145	<0.0001
a_r x ENnb	0.103	<0.0001
a_r x A	0.072	<0.0001
a_r x B	0.079	<0.0001
a_r x Br	0.021	0.04
a_r x C	0.089	<0.0001
a_r x D	0.098	<0.0001
a_r x Dr	0.033	0.021
a_r x Db	0.130	<0.0001
a_r x E	0.136	<0.0001
a_r x Er	0.101	<0.0001
a_r x Eb	0.174	<0.0001
a_r x F	0.136	<0.0001
a_r x G	0.103	<0.0001

Binary resistance maps

When using binary resistance maps, the correlation between genetic and effective distance gradually increased on including, in addition to natural forest (Map A), forestry plantations (B), scrublands (C), agroforestry mosaics (D), and pastures and meadows (E) as environments favouring dispersal (Fig. 4). This correlation did not vary on including rocky areas as dispersal environments (F), while it decreased on including cultivated land (G). The correlation reached its maximum value on including in resistance map E the barrier effect of highways, urban areas, reservoirs and quarries (friction map Eb) (Fig. 4). The correlation decreased on including in the resistance maps the total river network as an environment favouring dispersal (Br, Dr, Er). In all models, the relationship between genetic distance and effective distance was always significant when Euclidean distance was factored out of the relationship (Table 2). The correlation values after factoring out the effect of Euclidean distance showed the same pattern as the correlation values obtained by means of a simple Mantel test. The best two supported models are Eb and EN according to simple and partial Mantel r values.

DISCUSSION

Empirical evaluation of an ecological network through landscape genetics

Maps of corridors are commonly used by land management groups, but unfortunately are more often the product of expert opinion rather than empirical data (Boitani et al. 2007). To our knowledge, this is the first time that a GIS modelled ecological network (Gurrutxaga et al. 2010a) was evaluated through a landscape genetics approach. Moreover, the use of non invasive genetic sampling has the added advantage of permitting us to infer landscape connectivity without needing to intervene directly in the species in focus.

In this study, even though contemporary gene flow follows a pattern of isolation by distance, the resistances of the landscape matrix which were used in the design of the ecological network of the Basque Country (EN friction map) (Gurrutxaga et al., 2010a) provide a much better explanation for the genetic pattern observed. Oftentimes, geographical distance and landscape resistance will simultaneously influence gene flow (Trizio et al. 2005). Individuals that are geographically distant are often also separated by high-cost distances, making the distinction between purely spatial and landscape effects particularly challenging in practice (Cushman et al. 2006). However, in this study while Euclidean distance was significantly correlated with genetic distance the correlation was better using the effective distances. Moreover, partial

Mantel tests suggested that IBD it was not the main factor that strongly influenced pine marten gene flow as the landscape structure had a significant independent effect on genetic distance when we controlled for Euclidean distance.

The comparison with the correlation coefficients obtained in other similar studies based on the genetic distance between individuals (e.g. Coulon et al. 2004; Broquet et al. 2006; Wang et al. 2008; Schwartz et al. 2009), shows that the improvement obtained with respect to the pattern of isolation by distance is relatively high. Even though some authors have used the length of LCPs as an indicator of landscape connectivity between pairs of individuals (Broquet et al. 2006; Coulon et al. 2004), this study has used the accumulated cost of LCPs since this constitutes a more exact indicator of the effect of landscape structure on dispersal ability and gene flow. This can be attributed to the fact that individuals who move along different LCPs of equal length are affected by the differing efforts, and thus they must exert due to the differing resistance they meet with according to the land uses of the trajectories they move across (Stevens et al. 2006). A number of studies have used binary surfaces to investigate landscape influences on genetic connectivity (Broquet et al. 2006; Coulon et al. 2004). In this study, the resistance map used (EN) parameterizes with different resistance values varying along a fairly wide range. Complex resistance maps like this are more realistic than simple binary models which divide the habitat into favourable and non-favourable, a tendency which has predominated in previous studies (Coulon et al. 2004; Broquet et al. 2006; Schwartz et al. 2009). These models assume that the surrounding matrix is uniformly inhospitable, which really constitutes an important simplification of the reality (Holderegger & Wagner 2008). Models reflecting a mosaic, such as EN resistance map, on the other hand provide with a heterogeneous model which more closely reflect the differing patch-like nature of landscapes.

Influence of land uses on gene flow: insights into pine marten ecology

The most consistent marten-habitat relation appears to be a general association with forest habitats, and avoidance of open, non-forested habitats (Proulx et al. 2004; Pereeboon et al. 2009). Thus, the marten's unwillingness to cross open habitats may restrict the species' ability to disperse and colonise new forested areas (Caryl 2009). Many previous landscape genetic studies have evaluated a single landscape resistance hypothesis relative to a null model of isolation by distance (e.g. Coulon et al. 2004; Broquet et al. 2006; Schwartz et al. 2009). To avoid the probable risk of inferential error due to spurious correlations with alternative but untested hypotheses (Cushman & Landguth 2010), we evaluated several landscape models independently. Here, different binary resistance maps, with different hypotheses concerning the friction to pine marten dispersal arising from land uses, were used to infer landscape effects on gene flow. In order to

obviate the limitations associate with binary resistance maps, which reflect an oversimplification of the landscape matrix, in this study we combined different land uses so as to create a single predictor variable (each resistance map from A-G), including step by step land uses ranging from strict forest to open spaces. This methodology has allowed us to determine which land uses favour or impede dispersal and, in consequence, the gene flow of a species. The correlation results obtained with the binary models suggest that it is not alone forest masses which serve as favourable environments for dispersal. Scrubland, agroforest mosaic, and grassland habitats also favour dispersal, since the correlation increases as, step by step, these environments are included as predictor variables of pine marten geneflow. These results are in consonance with recent studies, based on radio tracking, carried out on European pine martens, which provide new data which differ substantially from traditional descriptions in the scientific literature (Caryl, 2008; Pereboom et al. 2008). These studies show that martens are not exclusively confined to extensive forest patches but that they also use other patches including scrubland and agroforest mosaics (Mergey 2007; Caryl 2008; Pereboom et al. 2008; Balestrieri et al. 2010). Indeed, the inclusion of scrub habitat in marten home ranges is likely to be related to its role in the connectivity of forest habitats (Caryl 2008; Pereboom et al. 2008). In the same way, the improvement in correlation on including pastures and meadows seems to indicate that the species does not always renounce to cross these open spaces when there is forest habitat in the immediate vicinity as has been previously suggested by radiotracking data (Pereboom et al. 2008). This is precisely the case in the area under study, where pastures and meadows are inserted in areas in the immediate vicinity of forest. The inclusion of homogeneous croplands reduces the correlation between genetic distance and effective distance, suggesting that zones with intensive agriculture impede species dispersal. This could be due to the scarcity of natural vegetation in these zones and the distance separating them from forested areas. When we include the total river network as a medium favourable to dispersal, the correlation diminishes. This can be attributed to the excess in connectivity between different favourable habitats without taking into account the ecological quality of the medium itself and its various crossings.

The inclusion of the barrier effect of major roads and urban areas leads to a substantial improvement in understanding the genetic pattern which was detected, since the correlation with models Eb and EN was greater than that obtained with models E and ENnb, respectively. A previous study based on Bayesian analysis detected these landscape features as one of the principal factors in explaining the genetic discontinuity between the two inferred marten populations in the study area (Ruiz-González et al. in prep). Both LCP and Bayesian analyses confirm the importance of these variables as barriers or moderators of gene flow. Moreover, these results reaffirm the need to guarantee the permeability of infrastructures by

means of adequate wildlife crossings (Gurrutxaga et al. 2010b). Also adequate urban planning is required, so as to avoid urban sprawl and the conversion of the remaining natural or seminatural areas especially along valley bottoms.

Since our ability to detect the effects of landscape structure on genetic differentiation depends on both the landscape features used and the relative costs of each feature, different resistance values could provide different results (Spear et al. 2010). In this study, due to computational constraints we evaluated binary models with only 1 and 50 friction values. Consequently, a more detailed insight into the effect of a range of different resistance values would be desirable. However, similar landscape genetics studies have used friction values similar to those used in the present study in order to infer landscape effect on gene flow (Coulon et al. 2004; Broquet et al. 2006; Wang et al. 2008; Schwartz et al. 2009). Moreover, in order to reduce these limitations we have used both binary and complex resistance maps so as to parameterize the resistances inherent to each land use in different ways. Taking into account partial Mantel r values, the best supported models were EN, Eb, and E. In spite of they differ in the friction assignment procedure and in the relative values assigned to each land use, yet each model provide similar results and highlights similar landscape effects on gene flow, even after factoring out the effects of Euclidean distance. The agreement between binary and complex models, in spite of the difference in the friction values assigned to each land use, provides support for the results obtained. Additionally, the concordance between landscape genetics model inferences and species ecology provided by radio tracking (Pereboom et al. 2008), reinforce the validity of the conclusions arrived at.

Ruiz-González et al. (2008) found that pine marten occurrence in the study area is highly dependent on the presence of forest and consequently sensitive to forest fragmentation as has been previously suggested in other studies (Brainerd et al. 1995, Kurki et al. 1998). Nevertheless, the presence of forest habitats is not the only factor which explains the gene flow, indicating that the habitat selection and gene flow of pine martens may be driven by different factors (Spear et al. 2009). This may be because gene flow is driven by mating and dispersal events and habitat selection reflects the behaviour of individual organisms to maximize fitness within home ranges.

Similar least cost path modeling has also been conducted on American marten in Ontario (Canada) (Broquet et al. 2006) and on European pine marten in Ardennes, La Bresse, and L'Isere (France) (Mergey 2007) both showing the importance of forest structure for dispersal across large landscapes. In addition, more recently Wasserman et al. (2010) has extended the least cost path approach by incorporating multi-scale modeling and improved model selection approach for the study of American pine marten gene flow

in Idaho (USA). In this study, showed that gene flow in the Northern Idaho (USA) American marten population is driven by a gradient of landscape resistance that is a function of elevation, which was a proxy for snowpack, with marten avoiding lower elevations and dispersing in mid to high elevation montane forests.

Even though human-induced changes to the landscape have been quite recent in the study area, the genetic structure nonetheless reflects current landscape structure. Landguth et al. (2010) suggested that individual-based landscape genetic approaches are accurate enough to detect the effects of existing landscape features on genetic structure and connectivity, as the lag time to barrier detection is short (1-15 generations), at least for animals with high dispersal abilities. According to the relationship between home range and dispersal distance outlined in Bowman et al. (2002) median and maximal dispersal distance for the pine marten are approximately 8km and 46km for males and 7km and 40km for females, if we consider the mean size of the home range to be 1,3km^2 and 1km^2 respectively for pine marten males and females in the study area (O. Berdión unpublished data). Thus, taking into account the relatively high dispersal abilities of the pine marten, we assume that the strong correlation found in this study between contemporary landscape patterns and pine marten gene flow reflects current landscape effects more than past events. However, we cannot discard the influence of other historical events and landscapes which could have long-term effects that confound inferences about the impacts of current landscape features on gene flow (Landguth et al. 2010). Consequently, controlling for the influence of the historic landscape configuration on genetic structure may be necessary if we are to disentangle historical and contemporary processes affecting genetic variation (Zellmer & Knowles 2009)

CONCLUSION

Individual-based analyses correlating the genetic distances between individuals with the cost distances between them obtained from multiple landscape resistance hypotheses is useful approach to understand the effect of landscape matrix on gene flow. Overall, this study suggests that a standard isolation-by-distance model is not sufficient to explain the observed genetic pattern, and including landscape variables through a resistance map significantly improves the prediction power of this standard model. Moreover, the results obtained suggest that the population connectivity of pine martens in the study area may be vulnerable to habitat loss and fragmentation processes, as the optimal resistance map identified suggest that pine marten gene flow is predominately driven by the presence of natural and semi-natural land uses (i.e forests, forestry plantations, scrublands, agroforestry mosaics, pastures and meadows) and the avoidance of intensively human altered land uses (i.e. crops, reservoirs, roads, highways and urban areas).

Our results confirm also that landscape genetic studies are useful tools for the empirical assessment of a regional ecological network. The parameterization found in the resistance map which was used to design the regional corridors in the Basque Country (north Spain) was adequate to explain pine marten gene flow. Given the importance of this species as bio-indicator of relevant landscape dynamics, the results obtained underline the importance of continuing to incorporate regional corridors as a reference for land use planning and management in the study area in order to preserve landscape connectivity.

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Table S1. Properties of the 15 microsatellite loci multiplexed used in this study and summary of the genetic variability. The table includes: number of alleles (N_A) and observed (H_O) and expected (H_E) heterozygosities for each locus and for whole data set.

MULTIPLEX	Locus	N_A	H_E	H_O
MULT_1	Gg-7	5	0.66	0.59
	Ma-1	4	0.56	0.45
	MP0059	5	0.65	0.49
	MP0188	3	0.46	0.35
MULT_2	Lut-453	3	0.50	0.57
	Mel-1	5	0.65	0.69
	Mel-10	5	0.66	0.57
MULT_3	Lut-435	7	0.42	0.28
	Ma-19	4	0.54	0.55
	Mvi-57	6	0.37	0.36
	Mvi072	8	0.71	0.62
MULT_4	Lut-615	2	0.47	0.53
	Ma-2	5	0.68	0.62
	Mer41	6	0.77	0.68
	Mlut-27	4	0.66	0.62
Mean		**4.13**	**0.58**	**0.53**

CONCLUDING REMARKS

From the studies performed in this PhD thesis the following conclusions can be drawn:

1. There is an incomplete taxonomic and evolutionary framework for a significant portion of the *Martes* complex. The current knowledge about intraspecific genetics, population genetics and landscape genetics of genus *Martes* is limited and devoted to only a few species (i.e *Martes martes*; *Martes americana*; *Martes pennanti*).

2. The mtDNA groups inferred for *Martes martes* in this study shows a strict phylogeographic pattern throughout the species range with the presence of three major phylogroups, each of them related to specific biogeographic regions which could probably represent different ecotypes. Overall, our study indicates a complex phylogeographic history for *M. martes* indicating a mixed pattern of recolonization of central and northern Europe from both Mediterranean and non-Mediterranean refugia, providing new insights into the cryptic northern glacial refugia. Pleistocene climatic conditions have played an important role in initiating phylogeographic differentiation as well as sculpting this pre-existing phylogeographic variety into today's sister species *M. martes* and *M. zibellina*.

3. The PCR-RFLP method developed to identify samples of *M. martes* and *M. foina* is a reliable, efficient, time-saving and cost-effective procedure for improving our knowledge of the spatial distributions and bio-ecological traits of these two sympatric species. The application of this method to non-invasively collected samples allowed us to: i) infer sympatric distribution areas of both marten species in the northern Iberian Peninsula, ii) monitor the small-scale distribution of sympatric martens in combination with camera-traps (NW Spain), iii) asses the range expansion of the pine marten in an agricultural landscape matrix (NW Italy) and iv) reliably investigate pine marten food habits.

4. The novel multiplex panel of 15 microsatellites *loci* designed is reliable and effective for faecal DNA genotyping of sympatric marten species (*M. martes* and *M. foina*), in order to i) identify individuals, ii) infer population structure, and iii) determine genetic variability. The application of this panel facilitated species distinction discarding the presence of putative hybrids between both marten species. Our results showed that the differences in the level of expertise between sample collectors significantly influence the

success rate of microsatellite genotyping of pine marten faecal samples but not the species identification success rate.

5. Non-invasive genetic sampling of faeces in combination with landscape genetics represent a valuable framework for the study of rare and elusive species in order to understand how landscape and environmental features influence population genetic structure.

5.1 Bayesian clustering methods (Geneland and Stucture), supported by HWE analysis, Fst values, and the low migration rates between groups showed that the pine marten have a clear spatial genetic structure with the existence of different genetic clusters showing non-overlapping distribution. Our data suggested that habitat loss and fragmentation could be one of the main causes of the spatial genetic structure found in a typical forest dwelling species. Other factors, such as, direct persecution, probable interspecific competition with the stone marten and the circumstance that the study area is situated at the limit of the pine marten distribution, could be acting synergically to shape its current spatial genetic structure.

5.2 Individual-based least cost distance analysis suggested that the population connectivity of pine martens in the study area may be vulnerable to habitat loss and fragmentation processes, as the optimal resistance map identified put forward that pine marten gene flow is predominately driven by the presence of natural and semi-natural land uses (i.e forests, forestry plantations, scrublands, agroforestry mosaics, pastures and meadows) and the avoidance of intensively human altered landscapes (i.e. crops, reservoirs, roads, highways and urban areas).

6. Our results confirm that landscape genetic studies are useful tools for the empirical assessment of regional ecological networks. The parameterization found in the resistance map which was used to design the regional corridors in the Basque Country (north Spain) was adequate to explain pine marten gene flow. Given the importance of this species as bio-indicator of relevant landscape dynamics, the results obtained underline the importance of continuing to incorporate regional corridors as a reference tool for land use planning and management in order to preserve landscape connectivity.

Appendix A

(PaperIIIa)

Rosellini S, Osorio E, **Ruiz-González A**, Piñeiro A, Barja I (2008)

Monitoring the small-scale distribution of sympatric European pine martens (*Martes martes*) and stone martens (*Martes foina*): a multievidence approach using faecal DNA analysis and camera-traps.

Wildlife Research, 35: 434–440

Appendix B

(PaperIIIb)

Balestrieri A, Remonti L, **Ruiz-González A**, Gómez-Moliner BJ, Vergara M, Prigioni C (2010)

Range expansion of the pine marten (*Martes martes*) in an agricultural landscape matrix (NWItaly).

Mammalian Biology, 75: 412–419

Appendix C

(PaperIIIc)

Balestrieri A, Remonti L, **Ruiz-González A**, Capelli E, Vergara M, Gómez-Moliner BJ, Prigioni C.

Food habits of genetically identified pine marten (*Martes martes*) expanding in agricultural lowlands (NW Italy).

Acta Theriologica. In press

APPENDIX A

PAPER IIIa

ROSELLINI S, OSORIO E, **RUIZ-GONZÁLEZ A**, PIÑEIRO A, BARJA I (2008)

MONITORING THE SMALL-SCALE DISTRIBUTION OF SYMPATRIC EUROPEAN PINE MARTENS (*Martes martes*) AND STONE MARTENS (*Martes foina*): A MULTIEVIDENCE APPROACH USING FAECAL DNA ANALYSIS AND CAMERA-TRAPS

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Monitoring the small-scale distribution of sympatric European pine martens (*Martes martes*) and stone martens (*Martes foina*): a multievidence approach using faecal DNA analysis and camera-traps

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Abstract. The European pine marten (*Martes martes*) and stone marten (*Martes foina*) are two closely related mustelids that live sympatrically over a large area of Europe. In the northern Iberian Peninsula, the distribution ranges of both species overlap extensively. The objectives of this study were (1) to verify whether, on a small scale, both species also live sympatrically and (2) to compare camera traps and scat DNA as methods for detecting marten species. The study was conducted in a protected area (province of Ourense, north-west Spain), which covers 6700 ha. To test the sympatry hypothesis, 90 fresh faecal samples, identified as faeces of genus *Martes* on the basis of their morphology, were collected from June 2004 to August 2006. The specific identification of faecal samples was conducted using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) techniques. In addition, 20 camera-traps (916 camera-trap-nights) were in operation during the study period. Of the faecal samples collected, 88.8% were attributed to the European pine marten, while the remaining 11.2% were not amplified by PCR and thus could not be assigned. The European pine marten was identified in 57.9% of the photos of carnivores and the stone marten was not detected in any. The faecal DNA analysis and camera-trap results supported previous conclusions about habitat preferences and the distribution of the two species obtained using other methods. The two non-invasive methods that were used in this study were shown to be reliable techniques that can be employed simultaneously, because each method has advantages and disadvantages that are influenced by the size of the area inventoried, sampling effort, and cost and efficiency of the method. The data gathered using these methods provided important information on the understanding of trophic and competitive interactions between the species.

Introduction

The European pine marten (*Martes martes* Linnaeus, 1758) and stone marten (*Martes foina* Erxleben, 1777), species similar in both morphology and feeding habits, are closely related mustelids that live broadly sympatrically over an extensive area of Europe. Limited knowledge of their ecological niches in sympatric areas, their extremely elusive behaviour and the high degree of difficulty in distinguishing their faeces on the basis of morphology alone, contributes to the difficulty of studying and monitoring their populations.

The European pine marten occupies most of Europe, from Mediterranean biotopes to Fennoscandia taiga, and western Siberia and Iran (Proulx *et al*. 2004). The stone marten is present throughout continental Europe, but it is absent from Great Britain and Ireland as well as most of the Mediterranean islands except Crete (Proulx *et al*. 2004). On the Iberian Peninsula, the European pine marten has a Eurosiberian distribution and is also present on the islands of Majorca and Minorca (Mitchell-Jones *et al*. 1999; Palomo and Gisbert 2002); the stone marten is present throughout the Iberian Peninsula (Fig. 1) (Palomo and Gisbert 2002).

The European pine marten is associated primarily with Eurosiberian deciduous and coniferous forests (Delibes 1983; Mitchell-Jones *et al*. 1999) and prefers mature forests and areas with permanent watercourses (Barja 2005*a*). In contrast, the stone marten can survive in a variety of habitats across its distribution range, including fragmented woodlands, villages and their periphery, and areas near farms (Waechter 1975; Delibes 1983; Sacchi and Meriggi 1995; Blanco 1998; Mitchell-Jones

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et al. 1999; Virgós and Garcia 2002; Lanszki 2003). Libois and Waechter (1991) also observed that the stone marten tends to principally occupy deforested and altered areas when the European pine marten is present. In addition, its predilection for rocky habitats allows it to use buildings and other man-made structures (Delibes 1983). However, this species appears to be highly flexible as it is also found in typical forest habitats in Russia and is therefore not exclusively dependent on the presence of rocky terrain (Waechter 1975). The stone marten is also more thermophilic than the European pine marten, allowing it to occupy urban environments (Waechter 1975). The ecological requirements of both species can sometimes overlap; in these circumstances, the European pine marten stays in forested landscapes and the stone marten seems to be displaced to more urbanised areas (Delibes 1983).

Accurate species identification is a key step in conservation biology and a necessary component of wildlife management and conservation studies. However, determining presence and absence of elusive or cryptic species can be logistically difficult, particularly when relying solely on field signs such as faeces, hair or tracks (Piggott and Taylor 2003). Traditionally, this kind of information is subjected to a variety of morphological analyses for definitive species identification (Kohn and Wayne 1997). Nevertheless, there are situations in which such samples, deposited by sympatrically occurring carnivores of similar body size, cannot be assigned at the species level on the basis of morphology alone (Farrell *et al*. 2000; Davison *et al*. 2002; Birks *et al*. 2004; Kurose *et al*. 2005). Misidentification of species from faeces is probably common, and has been indicated for different sympatric carnivore species (Paxinos *et al*. 1997; Ernest *et al*. 2000; Farrell *et al*. 2000; Davison *et al*. 2002; Palomares *et al*. 2002; Dalén *et al*. 2004; Sugimoto *et al*. 2006; Pilot *et al*. 2007). Traditionally, the presence of martens has been determined from data obtained from road kills or hunting information, live-trapping surveys, sighting surveys (Messenger and Birks 2000), track plates and camera-traps (Zielinski and Kucera 1995), fur-snagging devices (Messenger and Birks 2000; Lynch *et al*. 2006) and scat-based surveys, which have several limitations for detecting and identifying different marten species (Birks *et al*. 2004). Each method has advantages and disadvantages that are influenced by the size of the area inventoried, sampling effort, and cost and efficiency of the method (Birks *et al*. 2004). Methods of non-invasive genetic sampling of animal populations and camera-trapping are becoming more common for identification of mammalian species (Karanth 1995; Zielinski and Kucera 1995; Paxinos *et al*. 1997; Murakami 2002; Palomares *et al*. 2002; Azlan and Sharma 2003; Gómez-Moliner *et al*. 2004; Swann *et al*. 2004; Kurose *et al*. 2005; Trolle and Kéry 2005). Because the faeces of the European pine and stone marten cannot be reliably separated on the basis of morphology alone (Marchesi *et al*. 1989; Pilot *et al*. 2007), the application of methods that will positively distinguish the two could be an invaluable tool for conducting non-invasive surveys to determine the presence and distribution, trophic and habitat requirements, niche overlap and competitive interactions of the mustelids in sympatric areas.

Although several studies on the distribution of mammals have been conducted on the Iberian Peninsula (Blanco *et al*. 1992; Rodriguez and Delibes 1992; Virgós 2001; Palomo and Gisbert 2002), there is a remarkable absence of information on the distribution of the European pine marten and the bio-ecological relationship between it and the stone marten. Consequently, the goal of this work was to apply a multi-evidence approach incorporating two different survey techniques (faecal DNA analysis and camera-traps) to monitor the small-scale distribution of the two marten species in the northern Iberian Peninsula. This study compared the effectiveness, logistics and monetary efficacy of both techniques for this type of research.

Materials and methods

Study area

The study was performed in the Montes do Invernadeiro Natural Park, a mountainous area located in the north-west Iberian Peninsula (Universal Transverse Mercator (UTM) coordinates: 29T 064633–643 and 467462–472) (Fig. 1). The study area, which covers 6700 ha, varies in altitude from 880 to 1707 m. The climate is continental, with hot summers and cold winters

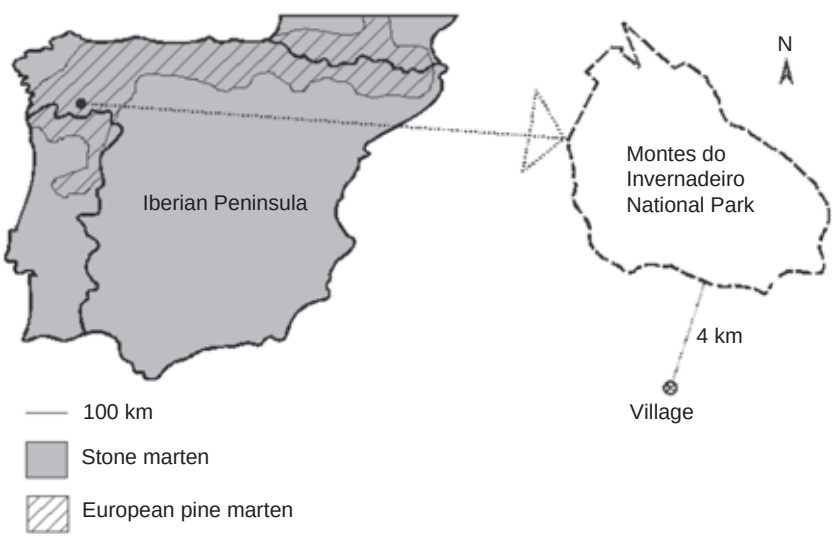

Fig. 1. Broad-scale distribution of the stone marten and the European pine marten over the Iberian Peninsula (modified from Mitchell-Jones *et al.* 1999; and Palomo and Gisbert 2002) and location of the study area.

(Barja 2001). The flora is diverse, including Mediterranean plant communities and Atlantic relic forests (Castroviejo 1977; Barja 2001). The plant community is scrubland, dominated by heather (*Erica australis*), prickled broom (*Pterospartum tridentatum*) and sandling (*Halimium lasianthum*). The original forests remain in valleys and along watercourses and are formed principally by associations of oak (*Quercus robur*), birch (*Betula celtiberica*), holly (*Ilex aquifolium*) and rowanberry (*Sorbus aucuparia*). Large tracts of extended forestlands are formed by Scot pine (*Pinus sylvestris*), which has repopulated deforested areas (Barja 2001). The park is occupied by carnivores such as the Iberian wolf (*Canis lupus*), badger (*Meles meles*), European polecat (*Mustela putorius*), stoat (*Mustela erminea*), European common weasel (*Mustela nivalis*), genet (*Genetta genetta*), wildcat (*Felis silvestris*), red fox (*Vulpes vulpes*) and otter (*Lutra lutra*). The study area appears to have suitable resources to support both the European pine marten and the stone marten. However, the presence of both species in the study area has not previously been examined. There are no human populations in the study area and the nearest village is 4 km from the park.

Collection of faecal samples

The European pine marten, the stone marten and other carnivores use forest roads and frequently defaecate on them as a way of visual-scent marking (Robinson and Delibes 1988; Barja *et al*. 2004; Barja 2005*b*; Barja *et al*. 2005). Initially, two scat-based surveys were conducted to estimate the distribution range of the genus *Martes* in the study area. The study area comprised 67 cells (UTM) of 1 km^2 each. In the initial survey, two 400-m transects in each cell were examined and 26 yielded *Martes* scats. Later, in order to collect fresh *Martes* faecal samples, transects 300 m long were surveyed on foot along forest roads monthly from June 2004 to August 2006 in these 26 cells alone. These surveys were conducted only in the 26 cells where signs of the presence of *Martes* species (scats, footprints, sightings) had previously been detected. We found fresh faeces in 17 of the 26 cells and old faeces in the remaining nine cells. Fresh faeces were characterised by their strong smell, presence of a mucus layer and lack of any sign of dehydration. Morphological characteristics, such as size and shape, were used to distinguish faeces of the genus *Martes* from those of medium-sized carnivores such as red fox and wildcat. The territory of the European pine marten is 1.3 km^2 for males and 1.0 km^2 for females (O. Berdión, Department of Zoology and Animal Cell Biology, Universidad País Vasco, Spain, pers. comm.). The mean territory size of the stone marten is similar to that of the European pine marten, being about 0.96 km^2 (López-Martín 2003). Thus, in order to increase the likelihood of obtaining faecal samples from separate individuals, the transects were placed 700 m apart. The transects were uniformly distributed throughout the study area and 218.7 km were surveyed. When fresh faeces of the genus *Martes* were detected, the date, time and UTM cell were recorded. A sample was collected from the fresh scat using a gloved hand. A new glove was used for each sample to avoid cross-contamination. All collected faecal samples were stored in hermetic and sterilised tubes, preserved with ethanol (96%) and maintained at –20°C until assayed (Piggott and Taylor 2003; Gómez-Moliner *et al*. 2004).

Genetic analysis of faecal samples

The specific identification of faecal samples was accomplished by the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). The faecal DNA extraction procedure was based on the protocol described in Gómez-Moliner *et al*. (2004). Extraction blanks were included to monitor for contamination and were processed as separate samples in the subsequent amplifications. A small fragment (276 bp) of mitochondrial DNA (mtDNA), D-loop region, was amplified with specific primers developed for *Martes* and *Mustela* by PCR. The primers were designed to amplify small fragments to maximise the probability of amplification of degraded DNA. Following DNA amplification, PCR products were digested with the restriction enzymes *Hae*III and *Rsa*I. The combined use of both enzymes produced a species-specific banding pattern allowing the scats of *M. martes* and *M. foina* to be discriminated. This method was recently developed and provides a reliable and effective molecular technique for unequivocal genetic identification of sympatric marten species through the use of species-specific differential haplotypes (Ruiz-González *et al*. 2008).

Camera trapping

Every three months, from June 2004 to October 2006, 10–20 automatic line-triggered cameras (models: Moultrie, Canon Prima AF 9s, Stealth Cam) were set in place and baited with chicken placed 70 cm from the ground and 2 m from the cameras. The cameras were equipped with different activation systems. Sixteen used passive and active infrared activation and four employed a plate system triggered by the pressure of the animal's weight. The cameras were placed in those cells where *Martes* faeces had been detected during the surveys and in optimal areas for both marten species even if signs of the animals were not detected. The optimal areas were selected on the basis of the known habitat preferences of both marten species (Delibes 1983; Sacchi and Meriggi 1995; Virgós and Garcia 2002; Barja 2005*a*). The cameras remained in the field for 3–12 days (916 camera-trap-nights). The cameras were placed in a total of 22 UTM cells (32.8% of cells had cameras in them). Each camera was revisited every third day.

The European pine marten and the stone marten are similar morphologically. Both species have a spot on the neck called a 'bib', which is yellow in the European pine marten and white in the stone marten (Cabrera 1914). Therefore, bib colour was used to distinguish between the two species. Spot patterns, size and symmetry of the bibs differ between individuals of the same species (Cabrera 1914). In the present study, the differences in the bib morphology and distance between cameras allowed us to identify individuals. To increase the probability of photographing the bibs, the cameras were baited with chicken placed 70 cm from the ground and 2 m from the cameras. The morphology of the bibs was analysed in the photographs obtained and when the spot pattern, size and symmetry coincided, the photographs were assigned to a single individual. Also, when the distance between cameras was greater than 1.3 km, we considered that the photographs obtained were of different individuals of the same species. This assumption was supported by the morphological differences of the bibs in the photographs.

Results

The faecal DNA analysis allowed us to detect the European pine marten in 17 UTM cells and the camera-trapping in 9 cells (in 6 cells the species was detected by both methods). Therefore, the presence of the European pine marten was confirmed in 20 cells (29.9% of cells) (Fig. 2). However, neither method detected the stone marten. Of 90 analysed faecal samples from throughout the study area, after applying the PCR-RFLP method, we successfully identified 80 as being from the European pine marten and none from the stone marten. Consequently, 88.8% of the collected samples could be assigned to a species; in the remaining 11.2% the DNA extracted was not amplified by the primers used. The mean number of fresh faecal samples analysed per cell was 5.3; the mean number of scats detected was 1.97 per month per kilometre surveyed. In 65.4% of the study cells (17 of 26), fresh scats were collected. The faecal DNA analysis confirmed the presence of the European pine marten in 100% of the cells in which fresh faeces were collected.

The camera-traps captured 126 photographs of four different terrestrial carnivores (Fig. 3). The European pine marten was the most photographed species (57.9%), followed by red fox (33.3%), wildcat (5.6%) and Iberian wolf (3.2%). This sampling method confirmed the presence of the European pine marten in 40.9% of camera-equipped cells, red fox in 36.4% of the cells, wildcat in 27.3% and Iberian wolf in only 18.2%. The inter-camera distance (>1.3 km) allowed for the identification of nine different individuals of European pine marten. Also, seven different individuals were identified according to the morphology of the bibs. The cells where the presence of the European pine marten was confirmed (using faecal DNA analysis and camera-trapping) included a high percentage of tree cover (coniferous and deciduous forests), permanent watercourses and a high percentage of rocky substrate and outcroppings. In contrast, the cells in which pine martens were not detected were dominated by scrubland.

Discussion

In this study, both research methods indicated that, while the European pine marten was present in the study area, the stone marten was not. The PCR-RFLP technique allowed us to achieve a high rate of species identification (88.8%). These genetic analyses open the possibility for studying population genetics of the species, recognising that nuclear marker genotyping from scats is more difficult than mtDNA amplification. The remaining 11.2% of samples did not amplify. The possibility that some scat samples had been incorrectly identified in the

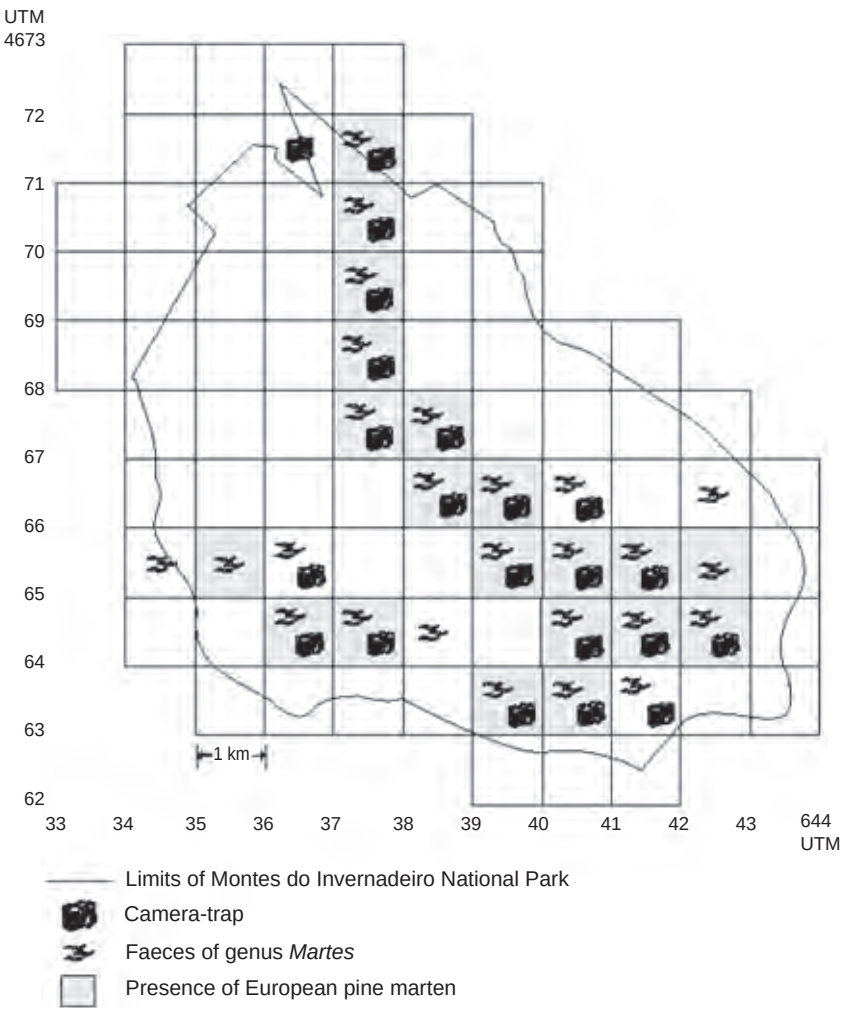

Fig. 2. Placement of the camera-traps, location of the faeces of the genus *Martes* and distribution of the European pine marten obtained from two different methods (faecal DNA analysis and camera-traps).

field as belonging to the marten would mean an even higher amplification success. Misidentifying fox scat as marten scat is a particularly common error (Davison *et al*. 2002). However, the primers developed for *Martes* and *Mustela* by PCR did not amplify in fox scats.

Camera-traps detected martens in only 40.9% of the cells (9 of 22), whereas faecal DNA detected them in 100% of the cells in which fresh faeces were collected (17 of 17). However, both methods have advantages and disadvantages that are influenced by the size of the area inventoried, sampling effort, detectability and cost and efficiency of the method. Although a large number of faecal samples can be collected, the faecal DNA is highly degraded (Taberlet *et al*. 1999) and the faeces collected can be from other carnivore species for which the primers used do not amplify, implying important economic costs. Also, the collection of fresh faeces requires a high field effort. However, this method is advantageous for both large- and small-scale investigations. An advantage of camera-trapping is the possibility of obtaining a broad range of significant information regarding the carnivore's community. The absence of specific attractants does not allow the species of interest to be selectively photographed. Also, the greater effort required for instrument placement, physical monitoring of the cameras and revisits to the study area are a disadvantage for large-scale research efforts. In the current study, camera-traps detected the European pine marten in cells in which the faecal DNA analysis did not detect its presence. Also, the use of camera-traps allowed us to visually detect the European pine marten (and identify individuals) and other carnivores (red fox, wildcat and Iberian wolf), which are difficult to study due to their elusive and nocturnal habits. Camera-trapping allows the collection of valuable information about multiple species, including herbivores and carnivores, within any given community (Karanth 1995; Peterson and Thomas 1998; Azlan and Sharma 2003; Swann *et al*. 2004; Trolle and Kéry 2005). Nevertheless, the simultaneous use of both methods in a small-scale marten survey can enhance the accuracy of data and result in a more ecologically relevant study.

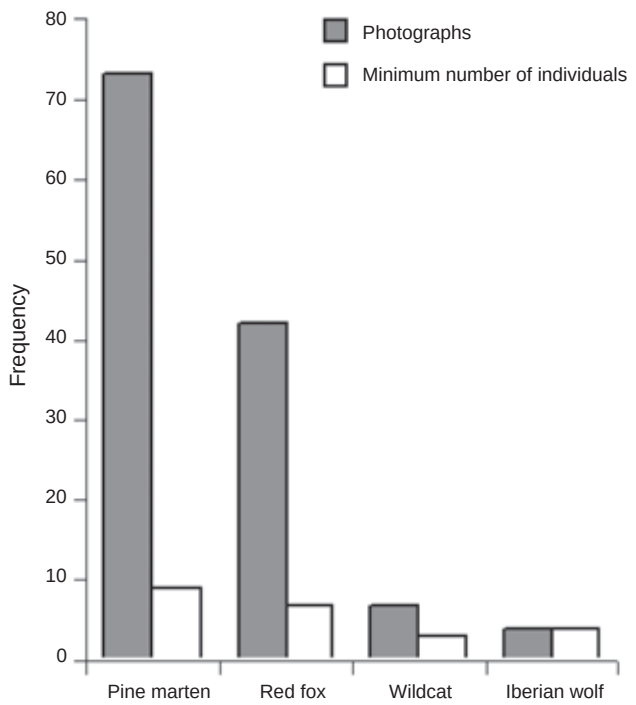

Fig. 3. Photographs of carnivores obtained using camera-traps, and an estimate of the minimum number of individuals present.

Small mammals are relatively abundant in the study area (64.4 animals ha^{-1}) (I. Barja, S. Rosellini and A. Piñeiro, unpubl. data) and are the primary prey of the European pine marten all year round (Rosellini *et al*. 2007). In some areas of the northern Iberian Peninsula small mammals have also been found to constitute the largest proportion of the stone marten diet (Delibes 1978). Nevertheless, despite the apparent presence of adequate food resources for the survival of both species within the study area, only the European pine marten is present. The ecological displacement that occurs when both species overlap in the same area is likely related to their relative densities and to the availability of trophic resources, even though prey items appear to be abundant. Competition for these resources can play a fundamental role in the interactions between individuals and species (Schoener 1983; Begon *et al*. 1986; Keddy 1989). Interspecific and intraspecific competition can cause a decrease in individual fitness (Begon *et al*. 1986). Since reduced fitness has significant negative consequences from an evolutionary standpoint, some kind of differentiation in the utilisation of trophic resources and habitat must occur in order for both species to coexist (Schoener 1974). Such differentiation can allow both species and individuals to avoid depressed fitness, that is, a decrease in fecundity and/or survival (Schoener 1974; Barrientos and Virgós 2006). In sympatric carnivores, there is ample evidence that ecological competition determines size and stability of populations (Palomares *et al*. 1996; Barrientos and Virgós 2006). Similar carnivores that coexist in the same area, therefore, normally differ in their trophic strategies (Schoener 1974; Clevenger 1994; Barrientos and Virgós 2006). In mustelids, the sympatry among species has been explained by differences in body size, which reduces interspecific competition (Erlinge 1986). While the high degree of trophic overlap between the two martens studied here has been reported by some authors (Marchesi *et al*. 1989; Clevenger 1994), the mechanisms of coexistence have been proposed only by Clevenger (1994).

The results of this work suggest a functional ecological mechanism for competition between the two studied species. On the Iberian Peninsula, the distribution ranges of the European pine marten and the stone marten overlap within the study area, providing a situation for ecological competition, and species segregation, on both a large and small scale. The exclusive detection of the European pine marten within the study area is likely associated with its preference for uniform and well conserved forests (Barja 2005*a*), resulting in the displacement of the stone marten to disturbed, deforested and urban areas (Delibes 1983; Libois and Waechter 1991).

On a smaller scale, habitat fragmentation can allow for sympatric distribution of very similar species (Pilot *et al*. 2007). The observed spatial segregation of the two mustelids is probably due to both physical size, the European pine marten being the larger of the two (Delibes 1983; Barea and Ballesteros 1999; Barja 2005*a*), and the more general and flexible habits of the

stone marten (Waechter 1975; Delibes 1983; Sacchi and Meriggi 1995; Mitchell-Jones *et al*. 1999; Virgós and Garcia 2002; Lanszki 2003). Interspecific competition is, therefore, avoided and the stone marten, due to its more generalist habits, shows a higher level of adaptability to human-altered environments, which may have implications for long-term viability of the species (Clevenger 1994).

The genetic analysis of four faecal samples collected in the periphery of the closest village to the study area, and the location of several dead mustelids, indicated that the European pine marten was also present there. This information and the other results obtained during this study seem to indicate that the scarce human-caused environmental alterations might influence in the absence of stone marten. In the study area and surrounding areas the density of humans is very low and the forest habitats are well conserved, with a progressive abandonment of agricultural activities, including crops and animal husbandry. We suggest, therefore, that further studies are needed to analyse the sympatry hypothesis on a small scale, considering different degrees of urbanisation and environmental disturbance. The possible competition and habitat segregation between the two marten species in the forests of the northern Iberian Peninsula is a basis for future studies regarding the animal community of forest habitats. These investigations provide fundamental information relative to habitat management directed principally at the conservation of the European pine marten since it apparently depends a great deal, if not exclusively, on unimpacted forest resources.

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Appendix B

PAPER IIIb

Balestrieri A, Remonti L, **Ruiz-González A**, Gómez-Moliner BJ, Vergara M, Prigioni C (2010)

Range expansion of the pine marten (*Martes martes*) in an agricultural landscape matrix (NW Italy).

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ORIGINAL INVESTIGATION

Range expansion of the pine marten (*Martes martes*) in an agricultural landscape matrix (NW Italy)

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Abstract

Habitat segregation is considered to favour the coexistence of sympatric pine martens *Martes martes* and stone martens *M. foina*, the latter being displaced to agricultural and urbanised areas. Subsequent to the report of pine martens in cultivated areas of the western River Po plain (NW Italy), we reviewed all available information on the presence of this species in plain areas of northern Italy and, for two study areas, applied a non-invasive PCR-RFLP method for the identification of *Martes* species from faecal mtDNA.

A total of 24 pine marten records were collected, grouped in the western part of the River Po plain. The number of records showed an exponential increase from 1988 to 2007, the percentage of woods in a 10 km^2 wide circular plot surrounding the location of records being inversely correlated to its distance from the 300 m a.s.l. contour line. In the two study areas, 36 out of 119 "marten-like" faeces were assigned to the pine marten, whilst none belonged to the stone marten. In the best monitored area, the pine marten was present almost constantly.

Our results suggest that the pine marten is expanding its range to include cultivated areas which were previously considered a prerogative of the more synanthropic stone marten.

Keywords: Non-invasive genetic sampling; Road-kills; Distribution; Interspecific competition

Introduction

The range of mammals contracts and expands over time, according to changes in climate (Walther et al. 2002; Parmesan and Yohe 2003) and habitat (Frey 1992; Benedict et al. 2000; Albert et al. 2004; Geluso 2004; Geluso et al. 2005) often caused by human influence (Kerr and Currie 1995; Brashares et al. 2001). In particular, despite their high mobility, the expansion of mammalian carnivores has been shown to be influenced by both food availability (e.g., on mustelids: King 1989; Jedrzejewski et al. 1995) and habitat fragmentation (Carroll et al. 2001; Crooks 2002).

In the last twenty years, as a consequence of a combination of factors – including legal protection, pollution control, habitat restoration and increasing prey availability -, the demographic trend of several mammalian predators has reversed, resulting in either the recolonisation of areas where they had disappeared during the 20th century (e.g., for Italy, the otter *Lutra lutra*,

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Prigioni et al. 2007, and the wolf *Canis lupus*, Boitani 2003) or the occupancy of novel environments (e.g. urban red foxes *Vulpes vulpes* in Switzerland; Gloor 2002).

The effects of range expansion on interspecific relationships can potentially vary from coexistence to the geographic replacement of native species by invaders, the latter having been reported in consequence of the introduction of alien species (IUCN 2000).

While the top-down effects that carnivores have on prey populations have been the subject of much research (e.g., Skogland 1991; Palomares et al. 1995; Smedshaug et al. 1999), those that predator species have on each other have been far less studied (Linnell and Strand 2000).

Competitive interactions among predators include intraguild predation (Palomares and Caro 1999) and may induce weaker species to seek habitats avoided by their competitors (“refuges”) (Durant 1998; Prigioni et al. 2008), sharply limiting their population density (Lindstrom et al. 1995). Competition is more likely to occur between interacting related species of similar size (see Powell and Zielinski 1983, about *Martes* and *Mustela* species).

Some endangered carnivore species are effectively limited by predator species that have recently expanded their range owing to protection policies (Creel and Creel 1996; Gorman et al. 1998; Kelly et al. 1998).

The pine marten *Martes martes* and the closely related stone marten *Martes foina* are quite similar in terms of body size, morphology and feeding habits (Goszczynski 1976; Marchesi et al. 1989). Their distribution range overlaps across a large part of continental Europe (Proulx et al. 2004), the two species showing different habitat preferences. Habitat segregation has been regarded as a mechanism favouring their coexistence (Waechter 1975; Delibes 1983).

The pine marten occurs primarily in well structured deciduous and coniferous forests (Delibes 1983; Buskirk 1992), but in southern Europe it has shown great ecological plasticity, having been reported in coppices, the Mediterranean maquis and cultivated land with woodland fragments (De Marinis and Massetti 1993; Pittiglio 1996). Nonetheless, pine martens strongly avoid open habitats (Storch et al. 1990; Brainerd and Rolstad 2002) and are believed to need a minimum woodland area to survive, equal to about 2 km^2 for the temperate zone (Zalewski and Jedrzejewski 2006). The stone marten can live in a variety of habitats, from boreal woods (Novikov 1962) to agricultural areas (Sacchi and Meriggi 1995; Lanszki 2003) and also occurs in villages and towns (Broekhuizen 1983; Prigioni and Sommariva 1997). Wherever the two species occur sympatrically, the stone marten is often associated with rural and suburban areas (Jensen and Jensen 1970; Waechter 1975), whilst the pine marten occupies forested areas. This compression of the spatial niche of the stone marten has been explained as a consequence of interspecific competition, favouring the slightly bigger pine marten (Delibes 1983). Nonetheless, the two species have also been reported to be syntopic (Kruger 1990; Genovesi 1993; Pilot et al. 2007; Ruiz-González et al. 2008).

Both martens occur in the Italian peninsula (Genovesi and De Marinis 2003). In northern Italy, the stone marten occurs from Alpine meadows to cultivated plains, whilst the pine marten is associated with deciduous and coniferous forests between 1000 and 2000 m a.s.l. (Martinoli 2001; Bon et al. 1995). However, road-killed pine martens have been recently reported also for the western plain of the River Po (Sindaco 2006; Savoldelli and Sindaco 2008), an intensively cultivated area, deeply altered by production activities.

Road kills are unpredictable events which can help to draw a large-scale picture of species distribution, but are ineffective when trying to examine their actual range at a local scale. In contrast, the systematic survey of field signs can yield reliable results on the distribution, abundance and habitat requirements of carnivores (Sadlier et al. 2004).

The monitoring of marten populations is hindered by our inability to distinguish the faeces of the two species, making indirect survey methods unreliable. Moreover marten faeces can be confused with those of other carnivores, such as, for northern Italy, the red fox and the polecat *Mustela putorius* (Davison et al. 2002).

Nonetheless, recent progress in molecular techniques has supplied several non-invasive genetic methods for the identification of martens (review in Ruiz-González et al. 2008), which can be applied to field monitoring.

Thus, with the aim of assessing the spread of the pine marten in plain areas of northern Italy, we used two different approaches: *i*) we reviewed available road kill records of the pine marten in plain areas of northern Italy (Piedmont, Lombardy, Veneto and Friuli-Venezia Giulia regions); *ii*) to confirm the presence of the species in two sampling areas of the middle western plain of the River Po we applied a non-invasive genetic method based on the analysis of DNA extracted from faecal samples.

Material and methods

Available road kill records for northern Italy were considered below the 300 m a.s.l. contour line, which broadly marks the upper limit of the plain (T.C.I. 1957). For Piedmont, we consulted the Data Banks of Piedmont Region, whilst for Lombardy and Veneto regions we referred to the data collected for the regional Atlases of Mammals from, respectively, 1980 to 2001

(Prigioni et al. 2001) and 1970 to 1995 (Bon et al. 1995). The thirty-year monitoring of road kills provided useful information about marten distribution in the eastern part of the study area (L. Lapini, unpublished data). Papers on Italian martens and the proceedings of several national congresses, published between 1985 and 2008, were also consulted. Moreover unpublished, original records, reported by both field naturalists and game-keepers, were collected. All road killed martens were examined by expert zoologists, minimizing the risk of misidentification. In three cases, genetic analyses were performed on tissue samples to confirm the identifications.

All records were digitalised in a Geographic Information System (ArcView 3.1, ESRI, California, USA) and grouped in four five-year long periods (1988–1992, 1993–1997, 1998–2002 and 2003–2007). A regression analysis was used to find the best equation describing the trend of pine marten records between 1988 and 2007.

To highlight the progressive penetration of the pine marten into the River Po plain, the smallest distance between each record and the 300 m a.s.l. contour line was measured. For each five-year period, the mean and maximum distances from this contour line were then calculated.

Considering the minimum woodland area of 2 km^2 needed by pine martens according to Zalewski and Jedrzejewski (2006), we assessed the relative percentage of woods in the areas of marten presence by overlaying 10 km^2 wide circular plots, centred on each record location, to a land-cover digitalised map. Plot area was chosen with reference to the mean home range size of pine martens in neighbouring Switzerland (660 ± 220 ha; Marchesi 1989) and Germany (765-1500 ha; Kruger 1990). The percentage of woods was related to the smallest distance from the 300 m contour line by Pearson's correlation test (r).

Surveys for "fresh" marten scats were conducted in 2007/08, in two different plain areas – the Natural Reserve "Garzaia di Valenza" (SE Piedmont, 02/2007–04/2008, no 1 in Fig. 1) and the Natural Reserve "S. Massimo" (SW Lombardy, 08/2008–10/2008, no 2 in Fig. 1).

The first Reserve covers 12.3 km^2 on the left bank of the River Po. The whole territory is flat, extensively covered by cultivated fields and poplar (*Populus* sp.) plantations. Woods consist of willows (*Salix cinerea*, *S. alba*), oak (*Quercus robur*), poplars (*Populus alba* and various hybrids) and alder (*Alnus glutinosa*), bordering an abandoned river meander and three naturalized artificial lakes. The black locust (*Robinia pseudoacacia*) is widespread along roads and man-made embankments. Mean altitude is 90 m a.s.l. Here, two pine martens were killed by cars in February 2003 and March 2004, respectively.

The second area covers 4.9 km^2 on the right bank of the River Ticino, which flows about 5 km away. Alder (*A. glutinosa*) woods and mixed hygrophilous assemblages, where alders are joined by willows (*S. alba*) and poplars (*P. alba* and *P. nigra*) are merged into an agricultural matrix (mainly rice and maize). Mean altitude is 70 m a.s.l. Here, pine marten presence was reported for the first time in October 2005.

For both study areas the climate is sub-continental temperate, with an average temperature of 12.4 °C and an annual average precipitation of 1000–1040 mm.

Sampling was conducted along linear features, such as wood/field margins, paths and country roads so as to cover both open and forested habitats. In 2007, surveys were carried out once a week, whilst in 2008 their frequency was doubled so as to reduce the risk of faecal DNA degradation.

Faecal samples were initially assigned to the genus *Martes* if less than 10 mm large and to the red fox if larger than 15 mm. Samples with intermediate width were attributed on the basis of their overall appearance.

A portion (about 30%) of each "marten-like" faecal sample was picked up with sticks and preserved in 96% ethanol and by freezing until DNA extraction, the rest was retained for dietary analysis. The specific identification of faecal samples was accomplished by a recently developed polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method, providing for an effective genetic identification of sympatric marten species (Ruiz-González et al. 2008).

The faecal mtDNA extraction procedure was based on the protocol described by Gómez-Moliner et al. (2004). Two specifically designed primers were used, which generate 276 bp long amplicons. These primers amplify the DNA from the two *Martes* species and from four *Mustela* species. The simultaneous use of the restriction enzymes RsaI and HaeIII differentiated *M. martes* from *M. foina* and both of them from the other carnivore species whose mtDNA is amplified by the selected primers (see Ruiz-González et al. 2008 for further details).

For the study area Garzaia di Valenza, which was continuously monitored through 14 months, the percentage of positive surveys for the pine marten (%P) was expressed as the per cent ratio between the number of months with at least one positive survey and the total number of surveyed months.

Results

A total of 24 road kill records was collected, most (58.3%) referring to the last five years (Table 1). The mean altitude of records was 183.7 ± 76.5 (S.D.) m a.s.l. (min = 70 m a.s.l.). Two out of six individuals for which the sex had been recorded were females. The number of pine marten records showed an exponential increase

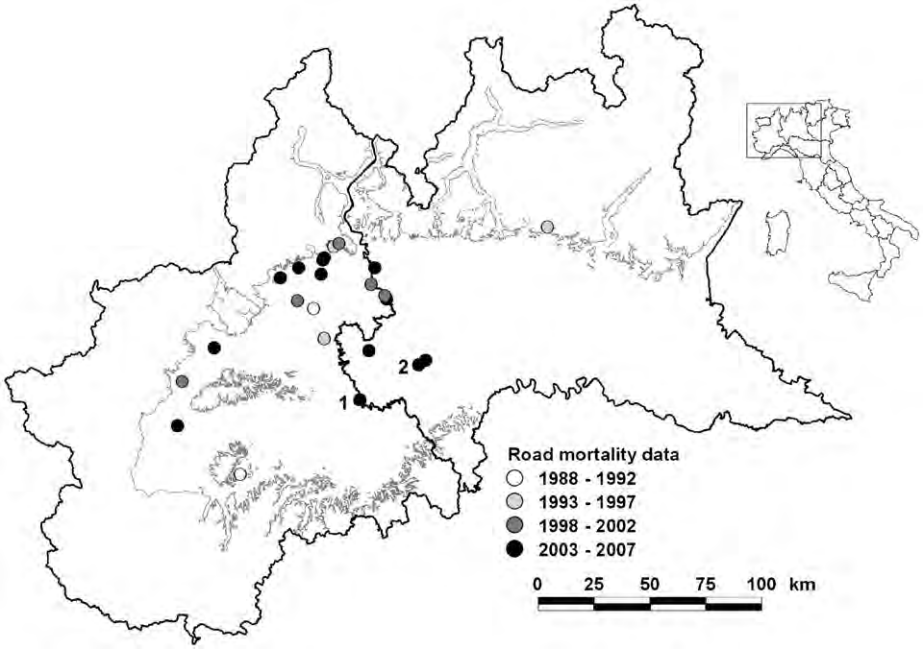

Fig. 1. Road mortality and genetic reports of pine martens in the western River Po plain, relative to the 300 m a.s.l. contour line (1: "Garzaia di Valenza" and 2: "S. Massimo" are the two areas of genetic survey).

Table 1. Number of road mortality reports of the pine marten and their mean and maximum distance (km) from the 300 m a.s.l. contour line.

Years	N	Mean D	Max D
1988–1992	2	10.7	20.5
1993–1997	3	10.4	28.5
1998–2002	5	10.9	22.6
2003–2007	14	14.3	41.1

(Fig. 2), the distance of recorded localities from the 300 m a.s.l. contour line being higher in the last five-year period (Table 1).

The pine marten is actually reported from plain, mainly cultivated areas of two regions (Piedmont and Lombardy) of north-western Italy (Fig. 1). Records are grouped in the western part of the River Po plain, whilst in its eastern part only one dubious record, dating back to 1981, was found (province of Udine, about 300 m a.s.l.).

The percentage of woods in the area surrounding pine marten records was inversely correlated ($r = -0.57$, $p = 0.0033$, $N = 24$) to its distance from the 300 m a.s.l. contour line (Fig. 3). For 48.5% of records the percentage of woods inside the 10 km^2 circular plots was less than 20%; in the central plain localities, woods were almost totally absent.

Almost all records could be easily assigned to a river valley, 75% of records occurring on four main rivers (Ticino, Sesia, Po and Agogna).

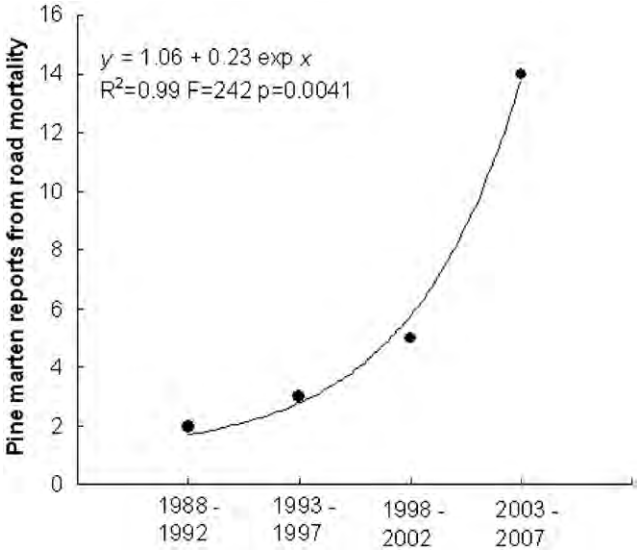

Fig. 2. Exponential increase of the number of pine marten records below 300 m a.s.l., from 1988 to 2007.

A total of 36 out 119 (30.3%) "marten-like" faeces was successfully identified by the PCR-RFLP method. All faeces were assigned to the pine marten, respectively 19/99 (19.2%) for the Garzaia di Valenza (11.3% in 2007 and 52.6% in 2008) and 17/20 (85%) for S. Massimo, whilst none belonged to the stone marten. The remaining samples did not amplify.

For these two areas, the percentage of woods inside the 10 km^2 circular plots was, respectively, 2.4% and 16.2%.

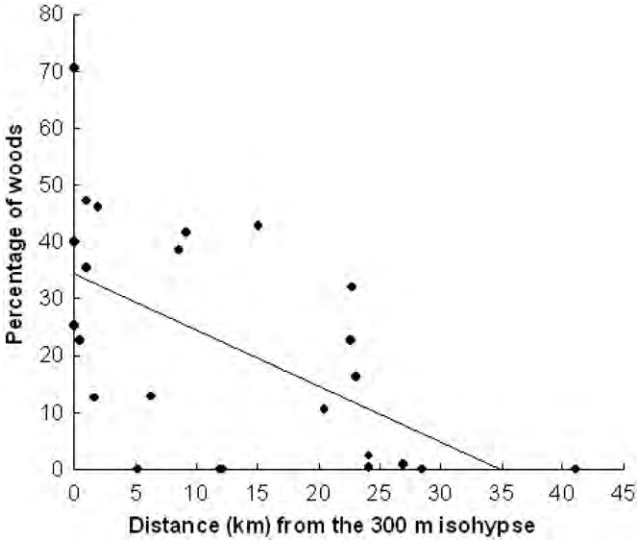

Fig. 3. Percentage of woods in the 10 km^2 circular plots centred on pine marten records, plotted against the distance of the records from the 300 m a.s.l. contour line ($r = -0.57$, $p = 0.0033$, $N = 24$).

In the Garzaia di Valenza, for which marten monitoring was almost constant for the whole study period, %P was 78.6 (N = 14).

Discussion

Although the lack of effective sampling may limit the accuracy of our method for some areas of the River Po plain (e.g. eastern Lombardy), the expansion of the pine marten seems to be a recent phenomenon occurring only in the western part of the River Po plain.

The penetration of the pine marten in arable lands probably follows the main watercourses, which offer patches of semi-natural woodland. On the basis of record distribution, the colonisation follows a north-south direction, the rivers Sesia, Agogna and Ticino representing the main dispersal routes (see also Canova and Rossi 2008, about raccoons *Procyon lotor* from Switzerland).

In the Republic of Ireland, the increasing range of the pine marten has been imputed to reduced persecution and increased rates of afforestation, favouring habitat connectivity (O' Mahony et al. 2006). In northern Italy, marten expansion in the plain may depend on demographic pressure, inducing dispersers to come down from the Italian Pre-Alps, together with increasing food availability in agricultural areas, as a consequence of the use, starting from the 1980s, of more sustainable farming techniques (Stout 1986; Robson 1997). A positive demographic trend favours immigration and range expansion (e.g., about Mustelids: Blandford 1987; Strachan and Jefferies 1996) and high population pressure has been imputed to trigger the colonization of Swiss cities by foxes (Gloor 2002). Unfortunately, information on numbers and trend of pine marten populations in Italy is inadequate to support any hypothesis (Genovesi and De Marinis 2003).

In the middle part of the River Po plain, which seems to have been reached by martens only in the last five-year period, woods are generally absent or consist of relatively isolated, small fragments within an agricultural landscape matrix. Our results contrast with those obtained in both Scottish (Balharry 1993a; Halliwell 1997) and Polish (Zalewski and Jedrzejewski 2006) study areas, where a minimum wooded area was reported to be needed by adult pine martens for survival and reproduction.

Considering the sharp aversion of martens for open areas, the colonization of such an unsuitable environment implies that pine martens are flexible enough to adapt rapidly to novel environmental conditions (Pigliucci 2001; West-Eberhard 2003). Behavioural flexibility enhances colonization success, enabling the persistence of initial populations before adaptive evolution can occur (Sax et al. 2005). Plasticity in both territorial (Balharry 1993b) and feeding behaviour (Marchesi 1989; Jedrzejewski et al. 1993) probably allows the pine marten to face a variety of environmental conditions, as suggested by its distribution in Mediterranean Italy. Accordingly, in the British Isles, where the pine marten is the only autochthonous *Martes*, martens have been able to survive in spite of extensive deforestation and currently exploit also open and rocky landscapes (Birks et al. 2004).

Nonetheless, agricultural areas might be suboptimal habitats for pine martens, acting as dispersal sinks (Kawecki 1995; Kirkpatrick and Barton 1997); in these terms, marten presence (rather than colonisation) in the plain would be occasional, depending on the immigration of non-resident floating male martens from surrounding areas, for lack of vacant wooded territories.

In our study areas, genetic sampling does not seem to support this hypothesis. For the Garzaia di Valenza, the presence of the pine marten was stable during the 15-month study period; considering the first records for this area, the species has probably been living there at least from 2003. For S. Massimo, three years have passed between the first record and the genetic confirmation of pine marten presence.

Moreover, the few road-killed individuals for which the sex was recorded included both males and females, pregnant females with male offspring representing the minimal viable propagule for the creation of new populations (Fitch et al. 1952).

Variation in scat abundance has been used to distinguish between resident and temporary populations of pine martens (Balharry et al. 1996), but no empirical

evidence supports the assumption that low scat densities correspond to non-breeding martens, marking activity being less intense in low density populations (Birks et al. 2004). As suggested by the increasing rate of success of DNA amplification in 2008, the small number of identified faeces probably depended largely on the overlong interval between consecutive surveys carried out in 2007 and, secondarily, the need for a "training" period to reduce the rate of faeces misidentification (Davison et al. 2002).

Discussing his competition hypothesis, Delibes (1983) suggested that in altered environments the stone marten is displaced to more urbanised areas, but would tend to become less synanthropic wherever the density of the pine marten decreased. The expansion of pine martens into an agricultural landscape offers the opportunity of analysing the effects of the opposite process on the distribution of the stone marten.

Recent studies have shown contrasting results about the relationship between the two species at a local scale. The pine marten was the only *Martes* in a mountainous area of north-western Spain (Rosellini et al. 2008), whilst in a forest-field mosaic of central Poland the two martens exploited the same micro-habitats (Posluszny et al. 2007).

The possibility of the two species coexisting probably depends on a combination of several factors, including the relative abundance of each predator species within the local carnivore guild and food availability. Our results for the best monitored study area (Garzaia di Valenza) suggest that the pine marten could be dominant over the stone marten, displacing the latter from the residual wooded areas of the River Po plain.

The PCR-RFLP analysis of faecal DNA was shown to be a powerful method for distinguishing the species of the genus *Martes* and is suitable, together with traditional survey methods, for assessing the spatial distribution of these elusive mustelids. Particularly, it represents a useful tool for investigating the ecological relationship between sympatric martens at a small scale. An ongoing work on microsatellite genotyping with a set of 14 loci useful for providing individual-specific genotypes of pine marten from faecal DNA, will allow to assess also the number of individuals occurring within the study areas.

Further field work is needed to confirm the stable presence of the pine marten in other areas of the western River Po plain and to test the interspecific relationships of martens across a wider range of environmental conditions.

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Appendix C

PAPER IIIc

Balestrieri A, Remonti L, **Ruiz-González A**, Capelli E, Vergara M, Gómez-Moliner BJ, Prigioni C.

Food habits of genetically identified pine marten (*Martes martes*) expanding in agricultural lowlands (NW Italy).

Acta Theriologica. In press

PAPER IIIc

FOOD HABITS OF GENETICALLY IDENTIFIED PINE MARTEN (*Martes martes*) EXPANDING IN AGRICULTURAL LOWLANDS (NW ITALY).

ABSTRACT

We assessed the diet of pine marten (*Martes martes*) expanding in the heavily human-altered agricultural plain of the River Po, northern Italy. Between February 2008 and November 2009, surveys were carried out twice a week during seasonal sampling sessions of 3-4 consecutive weeks. To distinguish the faecal samples from those of sympatric carnivores, a PCR-RFLP method was applied. The availability of small mammals was assessed by the analysis of 59 barn owl (*Tyto alba*) pellets. A total of 109 pine marten faeces was analysed. Its diet consisted of fruit, rodents, lagomorphs and birds. Seasonal variation occurred for fruit, which prevailed in summer, and rodents, which were more preyed upon in autumn. In winter, the diet of the pine marten was almost totally based on vertebrates, lagomorphs were the main source of protein in summer. Use of small mammals differed significantly from their availability, voles, particularly bank vole *Myodes glareolus*, being preferred to mice (*Apodemus* sp.) and rats (*Rattus* sp.). Medium-size mammals formed about 18% of pine marten diet, a value generally reported for high latitude habitats. In agricultural areas the pine marten proved to be an opportunistic predator, able to face the reduced availability of small mammals by preying upon medium-size prey and fruit.

Key words: PCR-RFLP method, non-invasive sampling, rodent availability, diet analysis, range expansion

INTRODUCTION

The pine marten (*Martes martes*) occurs throughout much of Europe and northern and central Asia, from northern Portugal to western Siberia (Stubbe 1993, Proulx et al. 2004). Although in the Mediterranean area the species has been reported in insular maquis and coppices (De Marinis and Masseti 1993a, Murgia et al. 1995) and cultivated land with woodland fragments (Pittiglio 1996, Pereboom et al. 2008), the pine marten is generally associated with forest habitats, mainly mature coniferous and mixed forests (Delibes 1983, Buskirk 1992, Proulx et al. 2000). Deforestation and forest fragmentation have been reported to affect the distribution and density of pine marten (Brainerd et al. 1994, Kurki et al. 1998), which are believed to need a minimum woodland area to survive (Zalewski and Jędrzejewski 2006) and tend to avoid treeless areas (Storch et al. 1990, Brainerd and Rolstad 2002, Pereboom et al. 2008). Accordingly, in the Republic of Ireland, afforestation and increasing habitat connectivity have been suggested as the main factors favouring the ongoing expansion of the pine marten (O'Mahony et al. 2006).

Nonetheless, in the last years the pine marten has progressively expanded in the western part of the intensively cultivated plain of the River Po (NW Italy). In the core of this area, woods consist of isolated, small fragments within an agricultural landscape matrix (Balestrieri et al. 2010).

Food availability is considered a main environmental factor influencing the population dynamics of small mustelids (King 1989, Jędrzejewski et al. 1995). Like other *Martes* species, the pine marten is a generalist predator, consuming a wide range of resources according to their local and seasonal availability (reviews in: De Marinis and Masseti 1995, Zalewski 2004). Small mammals are its main prey throughout Europe, representing up to 81% of all prey (on average ca. 45%; Zalewski 2004). Fruit (Marchesi and Mermod 1989), birds (Helldin 2000) and invertebrates (Clevenger 1993a) may sometimes represent the most frequent food item, while medium- and large-size mammals (lagomorphs and ungulate carrion) generally constitute secondary prey at high latitudes (Zalewski 2004). Amphibians (Reig and Jędrzejewski 1988), fish (Lockie 1961) and crustaceans (Ruiz-Olmo and Nadal 1991) are eaten only occasionally.

In agricultural landscapes, the reduction of both habitat quality and diversity have been claimed as the main causes of biodiversity loss and affect the availability of food resources for predators (Benton et al. 2003, Firbank 2005). In particular, small mammals are confined to field margins and non-cropped areas scattered in cultivated fields (Fitzgibbon 1997) and in agricultural areas rodent communities show lower diversity (Millán de la Peña et al. 2003) and biomass (Michel et al. 2006) than those in forested habitats.

The plasticity of pine marten food habits should allow the mustelid to cope with the shortage of its main prey in sub-optimal habitats. Nonetheless, while pine marten diet has been largely studied in boreal and

deciduous forests (Marchesi and Mermod 1989, Jędrzejewski et al. 1993, Helldin 2000), in field-forest mosaics it has still been poorly studied (Posluszny et al. 2007). The recent expansion of the pine marten in north-western Italy offers the opportunity to investigate the feeding adaptability of a typical forest-dwelling predator in heavily human-altered agricultural lowlands. The main aim of our study was to draw a first picture of the food habits of the pine marten in the River Po plain by means of scat analysis. As pine marten faeces cannot be distinguished from those of the stone marten (*Martes foina*), which is widespread in the whole plain (Genovesi and De Marinis 2003), and can be also easily confused with those of other carnivores (Davison et al. 2002, Harrington et al. 2010), molecular techniques were applied for the identification of faecal samples.

To examine seasonal variation in the species diet, sampling for faeces was carried out over a 20-month period. Small mammals being considered their preferred prey, use by pine marten of this resource was compared to its availability in the study area.

We predicted that pine marten would compensate for the possible reduced availability of small mammals with respect to forested habitats by relying on alternative food items according to their seasonal availability.

Study area

Surveys were carried out in two sites of the western plain of the River Po (Fig. 1), each covering about 4 km^2 and consisting of small patches of hygrophilous woods merged into an agricultural landscape matrix. For both study sites mean altitude is 90 m a.s.l. and climate is sub-continental temperate, with an annual average temperature of 12.4 °C and average rainfall of about 1000 mm.

The first site partly coincided with the Natural Reserve "Garzaia di Valenza" (45° 01’ N, 8° 64’ W; hereafter: NRGV), on the left bank of the River Po. The whole territory is flat, extensively covered by cultivated rice and maize fields (ca. 57%) and poplar *(Populus* sp.) plantations (ca. 19%). Woods (ca. 13%) consist of willows (*Salix cinerea*, *S. alba*), oak (*Quercus robur*), poplars (*Populus alba* and various hybrids), alder (*Alnus glutinosa*) and black locust (*Robinia pseudoacacia*), bordering an abandoned river meander and three naturalized artificial lakes.

The second site was included in the Natural Reserve “San Massimo” (45° 18’ N, 8° 99’ W; hereafter: NRSM) 5 km to the west of the River Ticino. Alder woods and mixed hygrophilous assemblages (ca. 30%), where alders are joined by willows (*S. alba*) and poplars (*P. alba* and *P. nigra*), are surrounded by rice and maize fields (ca. 54%). There are a few farms scattered throughout the site.

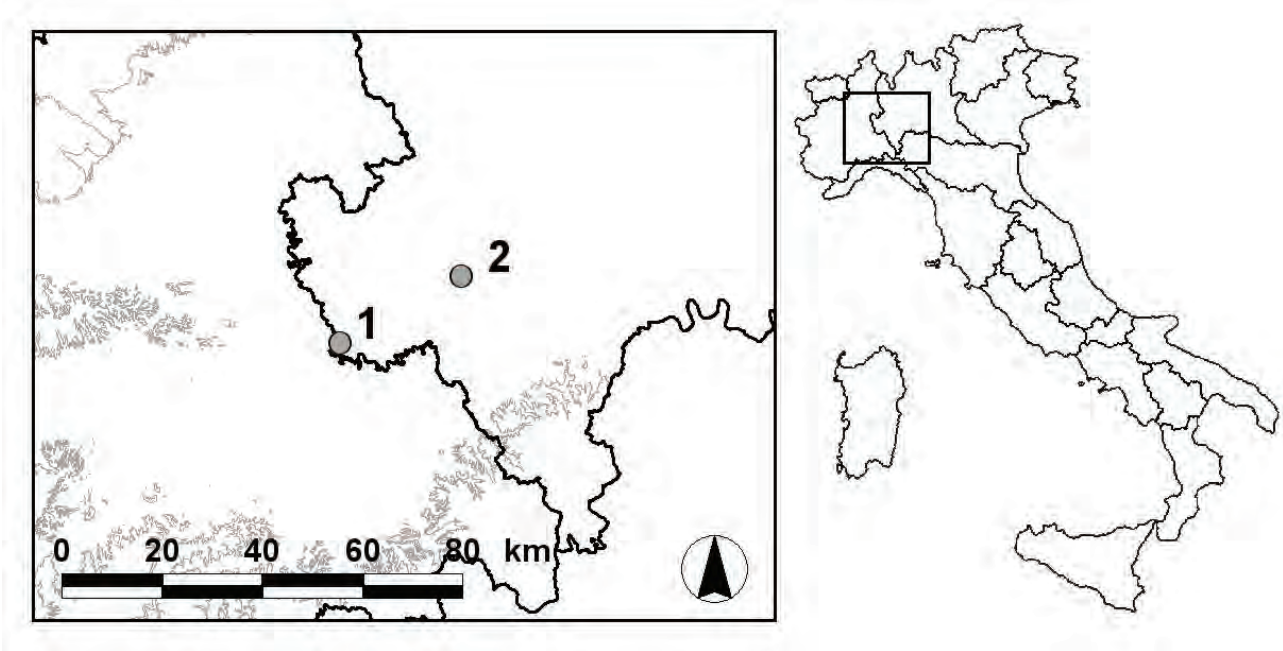

Figure 1. Study area. 1: Natural Reserve "Garzaia di Valenza"; 2: Natural Reserve "S. Massimo".

MATERIALS AND METHODS

Field surveys

Sampling was carried out between February 2008 and November 2009, along two linear transects in each study site (2.2 and 2.3 km in NRGV and 2.6 and 1.8 km in NRSM), coinciding with paths, country roads and wood/field margins and covering both open and forested habitats.

As climate, vegetation cover and land use are very similar in the two study sites, we assumed that both ecological conditions and food availability for pine marten were the same. Thus diet data were merged as to provide an overall picture of the species food habits in the agricultural landscape.

To reduce the risk for faecal DNA degradation, surveys were carried out twice a week during seasonal sampling sessions of 3-4 consecutive weeks (N = 56).

Faecal samples were initially assigned to the genus *Martes* according to their size (diameter < 10 mm) and shape (Bang and Dahlström 1974). A portion (about 20%) of each faecal sample was picked up with sticks and preserved in 96% ethanol and by freezing for DNA extraction, while the rest was retained for dietary analysis.

Genetic analyses

DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. The specific identification of faecal samples was accomplished by a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method, providing for an effective genetic identification of sympatric marten species (Ruiz-González et al. 2008). The procedure consists of PCR amplifying a mitochondrial D-loop region by two specifically designed primers - Mm_L1 (5'-CCCAAAGCTGACATTCTAAC-3') and Mm_H1 (5'-ATGGGCCCGGAGCGAGAAGAGGTACAC-3'). These primers amplify the mtDNA from *Martes martes*, *M. foina* and four *Mustela* species, of which only *M. putorius* had been previously reported for our study area. Other sympatric carnivore species, in particular the red fox, whose scats can be easily mistaken for those of martens (Davison et al. 2002), render no amplicons. The resulting 276-bp-long amplicons were digested by two restriction enzymes simultaneously - HaeIII and RsaI – allowing the differentiation of the two *Martes* species and both of them from the other mustelids whose mtDNA is amplified by the selected primers (see Ruiz-González et al. 2008 for further details).

Diet analysis

Faecal samples were washed through three sieves of 1.5, 0.3 and 0.1 mm mesh and food remains inspected to count or estimate the total numbers of each kind of food.

Mammal hairs were compared at 20x and 40x magnifications with the keys of Debrot et al. (1982), Teerink (1991) and a personal collection of hair photos. Bird feathers were identified with reference to Day (1966). The undigested remains of insects (wings, legs and cuticle parts) and wild or cultivated fruits (seeds) were identified using personal collections. Since marten are known to consume earthworms (Lynch and McCann 2007), sediment remaining in the sieve with the smallest mesh was examined under a binocular microscope to detect the presence of *chaetae*.

Although conversion factors (e.g.: Zielinski 1986, Jędrzejewska and Jędrzejewski 1998) make it possible to assess biomass consumed from the dry weight of prey remains, we agree with Zielinski and Duncan (2004) on the difficulty of separating the undigested remains effectively. As a consequence, the contribution of each food item in terms of volume was assessed according to Kruuk and Parish's method (1981), which provides for quantifications of food volumes as reliable as those obtained by the analysis of stomach contents (Balestrieri et al. in press). For each faecal sample, the method entails the estimate by eye of the bulk of each item 'as ingested'. This estimate presents little difficulty for small prey, which are ingested in

their entirety, whilst for larger prey the relative volume is computed as 100 minus the score given to the other remains. To reduce the equating of occurrence bias (Kelly 1991, Ciucci et al. 1996), undigested remains occurring in negligible proportions were not considered in the analysis. Prey were identified to the lowest possible level.

Results were expressed as per cent mean volume (%mV = total estimated volume of each food item as ingested / total number of examined spraints), which represents the proportional contribution of each food item to the overall diet (Kruuk and Parish 1981). To allow the comparison with previous studies, data were also expressed as per cent frequency of occurrence (%FO: number of faecal samples containing a specific food item / total number of faecal samples x 100) and per cent relative frequency of occurrence (%RFO = number of occurrences of each item / total number of items x 100).

Seasonal trophic niche breadth was estimated by Levins' index $B = 1/\left(\sum p_i^2\right)$ (Levins 1968), using the proportions of occurrence (p_i = %mV/100) of five main food categories (fruit, insects, birds, lagomorphs and rodents). The index ranges between 1, when only one food item is used, and 5, when all items are consumed in equal proportions.

Rodent availability

The diet of the barn owl is considered an accurate reflection of the relative abundance of small mammals (Libois 1984, Taberlet 1986, Clark and Bunck 1991, Taberlet and Fumagalli 1995, Love et al. 2000). To assess rodent availability for pine marten in the study area, the relative abundance of rodent species in barn owl *Tyto alba* diet was investigated through the analysis of 59 pellets collected in winter (N = 39) and summer (N = 20) 2009 under two barn owl shelters (one in NRGV and one in NRSM). Pellets were stored individually until analysis. Each pellet was then soaked in water and then teased apart using tweezers and a needle. Prey remains were identified on the basis of reference keys (Chaline et al. 1974, Amori et al. 1986) and personal collections of rodent skulls. Data were expressed as %RFO (= number of occurrences of each species / total number of small mammals preyed x 100).

Statistical analysis

Spearman's rank correlation test (r_s) was used to check for any relationship between the proportion of use (%mV) of the main food categories.

To analyse seasonal variation in pine marten diet, data were split as follows: winter: Jan-Mar; spring: Apr-Jun; summer: Jul-Sep; autumn: Oct-Dec. As the proportions (%mV/100) describing diet composition sum to one, to overcome the 'unit sum constraint' seasonal variation was assessed by Compositional

Analysis (Aitchison 1986, Aebischer et al. 1993). Ratios were calculated using the proportional data for the five main food items. As a first step, raw seasonal volumetric data were compared by Kruskal-Wallis' test and the group for which the null hypotheses had the lowest probability of being correctly rejected (insects: χ^2 = 2.1, 3 d.f., P = 0.54) was used as the denominator in the transformation. Logarithms (ln) of the resulting four ratios were then calculated to normalize their distribution (Aitchison 1986). As in the log-ratio transformation zero proportions cannot be computed, zeros were replaced with 0.01 (Aebischer et al. 1993). Data were analysed using Multivariate Analysis of Variance (MANOVA) and Games-Howell post-hoc test, which does not rely on homogeneity of variance. Variance being significantly different, Welch and Brown-Forsythe statistics were used to test the results of separate univariate ANOVAs before rejecting the null hypothesis. To account for multiple tests on repeated data the level of significance of post-hoc tests was calculated by Bonferroni's sequential technique (Rice 1989).

The chi-squared (χ^2) test and Bonferroni's confidence intervals for the proportion of use were used to compare the use by pine marten of six species/groups of small mammals (insectivores, Gliridae, *Myodes glareolus*, *Microtus* sp., *Rattus* sp. and *Apodemus* sp.) to their availability as assessed by the analysis of barn owl pellets (p = number of occurrences of each group / total number of small mammals in the diet of both the pine marten and barn owl).

RESULTS

Eighty-one per cent of faecal samples was successfully genotyped, yielding 109 pine marten faeces. The diet of the pine marten included fruit (%mV = 32.5), small rodents (%mV = 28.3), medium-size mammals (lagomorphs; %mV = 17.7) and birds (%mV = 15.5; Tab. 1). Wild cherries (*Prunus* sp.), blackberries (*Rubus* sp.) and figs (*Ficus carica*) were the main fruit, while voles, particularly bank voles (*Myodes glareolus*), were a more frequent prey than both mice (*Apodemus* sp.) and other voles (*Microtus savii*). Coypu (*Myocastor coypu*) were found in 2.7% of faecal samples. Birds included mainly small passerines and pigeons (*Columba* sp.), while lagomorphs consisted of nearly equal proportions of hares (*Lepus europaeus*) and introduced Eastern cottontails (*Sylvilagus floridanus*). Invertebrates, mainly imagos and larvae of Coleoptera, were eaten in negligible proportions and did not include earthworms (Tab. 1). The %mV of fruit was inversely correlated with those of rodents (r_s = -0.42, P < 0.0001) and lagomorphs (r_s = -0.29, P < 0.01), while the %mV of rodents was inversely correlated with that of lagomorphs (r_s = -0.32, P < 0.001)

Table 1. Diet of the pine marten as assessed by the analysis of 109 faeces (%mV = per cent mean volume; %FO = per cent frequency of occurrence; %RFO = per cent relative frequency of occurrence; N = number of identified items).

Food items	N	%mV	%FO	%RFO
Fruit	46	32.5	41.3	30.1
Undetermined fruit	8	5.6	7.3	3.4
Phytolaccaceae	1	0.2	0.9	0.7
Phytolacca americana	1	0.2	0.9	0.7
Moraceae	10	6.5	9.2	6.8
Ficus carica	10	6.5	9.2	6.8
Rosaceae	29	20.1	26.6	19.9
Rubus sp.	12	9.1	11.0	8.2
Prunus sp.	14	8.6	12.8	9.6
Pirus communis	4	2.4	3.7	2.7
Vitaceae	1	0.1	0.9	0.7
Vitis vinifera	1	0.1	0.9	0.7
Insects	6	2.7	5.5	4.1
Coleoptera imagos	2	1.4	1.8	1.4
Coleoptera larvae	2	1.0	1.8	1.4
Hymenoptera	1	0.3	0.9	0.7
Orthoptera	1	0.1	0.9	0.7
Birds	25	15.5	22.9	17.1
Undetermined birds	1	0.9	0.9	0.7
Galliformes	2	1.2	1.8	1.3
Passeriformes	16	8.6	14.7	11.0
Columbiformes	6	4.8	5.5	4.1
Mammals	61	48.4	56.0	41. 8
Small mammals	39	28.7	31.2	26.6
Sorex araneus	1	0.4	0.9	0.7
Glis glis	1	0.9	0.9	0.7
Muscardinus avellanarius	4	1.8	3.7	2.7
Myodes glareolus	19	15.3	17.4	13.0
Microtus savii	4	3.7	3.7	2.7
Rattus sp.	1	0.9	0.9	0.7
Apodemus sp.	4	2.8	3.7	2.7
Undetermined rodents	5	2.9	3.4	3.4
Medium-size mammals	22	17.7	20.2	15.1
Sylvilagus floridanus	11	9.0	7.5	7.5
Lepus europaeus	9	7.1	6.2	6.2
Undetermined lagomorphs	2	1.6	1.4	1.4
Large mammals	4	2.9	2.7	2.7
Myocastor coypus	4	2.9	2.7	2.7

The proportion of use of the main food categories varied among seasons (Wilks' Lambda = 0.525, F = 6.2, 12 d.f., P < 0.0001; Tab. 2). Significant variation occurred for fruit (F = 12.2, 3 d.f., P < 0.0001) and rodents (F = 8.4, 3 d.f., P < 0.0001). Games-Howell post-hoc tests showed that fruit was eaten more in summer than in autumn and winter (P < 0.01 for both comparisons), while rodents were more preyed upon in autumn than in summer (P < 0.01). On the whole, fruit formed the bulk of marten diet in summer and rodents in autumn (mV% ≈ 70 for both items), while in the other two seasons the overall share of lagomorphs and birds ranged between 36% and 57% of the overall diet. Accordingly, niche breadth was the lowest in summer-autumn (B = 2.0 for both seasons) and increased progressively in winter (B = 3.2) and spring (B = 3.9). In winter, the diet of the pine marten was almost totally based on vertebrates, whilst lagomorphs were the main source of protein in summer.

Table 2. Seasonal variation in both per cent relative frequency of occurrence (%RFO) and per cent mean volume (%mV) of the main food items in pine marten diet. Number of faecal samples analysed in brackets.

Food items	Spring (32)		Summer (31)		Autumn (21)		Winter (25)	
	%RFO	%mV	%RFO	%mV	%RFO	%mV	%RFO	%mV
Fruit	31.1	33.9	59.5	67.7	13.8	12.1	3.3	4.0
Insects	4.4	3.34	4.8	4.8	6.9	1.9	0.0	0.0
Birds	15.6	13.9	9.5	7.4	13.8	12.1	33.3	30.4
Lagomorphs	17.8	22.0	11.9	14.2	3.4	4.8	26.7	27.2
Rodents	24.4	26.9	7.1	5.8	58.6	69.0	33.3	38.4

Remains of 95 small mammals were recovered from barn owl pellets. Wood mice (*Apodemus sylvaticus*) were the most common prey, followed by rats (Tab. 3). Use of small mammals by the pine marten differed significantly from their availability (χ^2 = 73.8, 4 d.f., P < 0.001). Pine marten preyed upon bank voles more than expected, while the opposite was true for mice and rats (Fig. 2).

Table 3. Per cent relative frequency of occurrence (%RFO) of small mammal species in barn owl pellets.

Small mammals	N	%RFO
Sorex araneus	1	1.0
Crocidura suavolens	2	2.1
Crocidura leucodon	2	2.1
Muscardinus avellanarius	1	1.0
Rattus sp.	14	14.6
Rattus norvegicus	12	12.5
Apodemus sylvaticus	38	39.6
Myodes glareolus	14	14.6
Microtus arvalis	1	1.0
Microtus savii	10	10.4
Arvicola terrestris	1	1.0

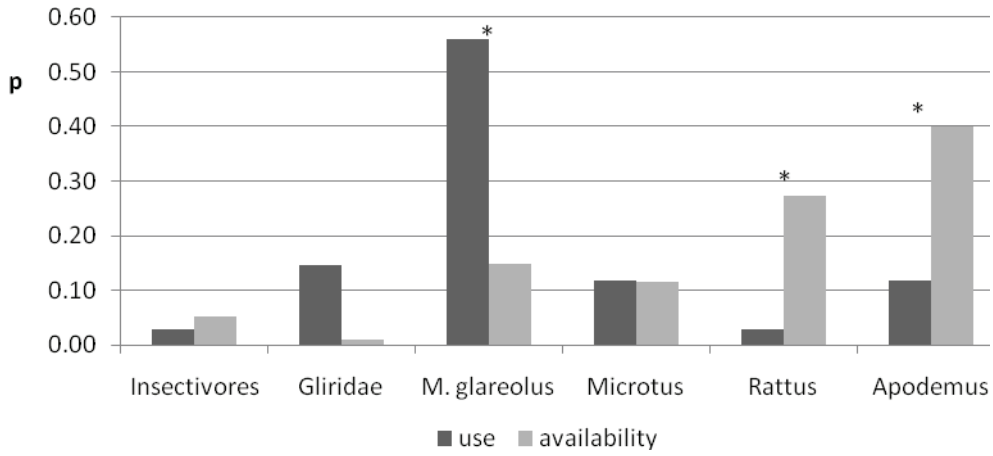

Figure 2. Use of small mammals by the pine marten *vs*. their availability (*: P < 0.001, Bonferroni's confidence intervals; p = number of occurrences of each group / total number of small mammals in the diet of both the pine marten - use -, and barn owl - availability).

DISCUSSION

The misidentification of scats can lead to the misrepresentation of the diet of the species being investigated (Reed et al. 2004). The PCR-RFLP method used, having been specifically designed for faecal material (Ruiz-González et al. 2008), allowed us to distinguish pine marten samples from those of sympatric carnivore species and, consequently, to draw a reliable picture of the diet of this mustelid in agricultural areas. Genotyping success was rather high with respect to other approaches (e.g.: 58%, Lucentini et al. 2007; 53.4%, Pilot et al 2006) and similar to those obtained, by the same method, in the Iberian peninsula (Ruiz-González et al. 2008, Rosellini et al. 2008), suggesting that the method is robust. The shorter time interval to scat collection (3-4 *vs.* 7-8 days), with respect to previous applications of the method in the study area, probably played a major role in the improvement of our success rate (81% *vs.* 21.4%, Balestrieri et al. 2008; 30.3%, Balestrieri et al. 2010), although the effect of the increased experience of the surveyors cannot be ruled out.

As reported for several studies throughout Europe, in the study area small mammals and fruit were the main food resources for pine marten. Nonetheless, fruit consumption in winter and that of rodents in summer were much lower than those previously reported for other Mediterranean areas (on average, %RFO = 52.7 and 27.5, respectively; Zalewski 2004). The low consumption of small mammals in summer may depend on the availability of more profitable food resources, namely fruit (see Cavallini and Volpi 1996), which in the same season were exploited by pine marten more heavily than in most study areas (Zalewski 2004), and lagomorphs, which represented their main vertebrate prey in summer. The composition of the small mammal community assessed by the analysis of barn owl pellets agreed with that obtained by live-trapping in the central plain of the River Po (Giordano and Meriggi 2009). The dominant rodent, the wood mouse, is a widespread and opportunistic species, which can occur in a wide variety of habitats (Ouin et al. 2000), whilst the bank vole, although a typical forest-dwelling species, may also live in the small wood-patches scattered in agricultural landscapes (Fitzgibbon 1997, Butet et al. 2006).

The preference shown by pine marten for voles is in agreement with previous studies, which demonstrated that microtines are the most important food resource for the species throughout its range (De Marinis and Masseti 1995, Zalewski 2004). In accordance with the findings from continental Europe (Jędrzejewski et al. 1993, Pulliainen and Ollinmäki 1996), the bank vole seemed to be preferred to *Microtus* voles, although the last ones are considered to be more profitable prey (Buskirk and Macdonald 1984) and are selected by British pine marten (Gurnell et al. 1994, Putman 2000). Both wood mice and bank voles are

typical forest species, although they can be found in a wide variety of habitats (Canova and Fasola 1991, Caryl 2008), while *Microtus* voles are generally restricted to open grassland and hedgerows (Miklós and Žiak 2002, Caryl 2008). Pine marten preference for bank voles may thus depend on the behaviour of the predator rather than the profitability of the prey, pine marten being more inclined to search for prey under the tree canopy than in open fields (Jędrzejewski et al. 1993). The low interest in wood mice shown by pine marten has been considered as a consequence of their greater agility compared to voles (Jędrzejewski et al. 1993), while rats, although they can represent the main prey of insular pine marten (De Marinis and Masseti 1993b), are seldom preyed upon on the continent (De Marinis and Masseti 1995). In contrast, pine marten fed on coypu, probably carrions or young individuals, which are much more vulnerable to predation that adults (see also Rosell and Hovde 1998 about Eurasian beavers).

Medium-size mammals generally form less than 10% of the total diet, both in northern (e.g. Helldin 2000, Caryl 2008) and southern Europe (e.g. Marchesi and Mermod 1989, Clevenger 1993b), while, in our study area, hares and Eastern cottontails totalled about 18% of pine marten diet (%RFO ≈ 15). According to the generalized model of latitudinal variation in %RFO of the main food items in winter diet constructed by Zalewski (2004), such values are predicted for the northern range of the species (> 60° N).

In both study sites, lagomorph availability for pine marten is probably high (NRGV: 15-20 cottontails/km^2 and 1-2 hares/km^2, Balestrieri et al. 2005, NRSM: 80-100 cottontails/km^2 , 8-10 hares/km^2 and 8-10 rabbits/km^2; D. Massignani, pers. comm.), mainly as a consequence of the rapid spread of introduced cottontails (Vidus Rosin et al. 2008). As previously reported for the red fox (Balestrieri et al. 2005), cottontails, which select wood-field margins as resting sites (Swihart and Yahner 1982, Althoff et al. 1997), may represent a more profitable prey for pine marten than hares.

As in forested habitats (Jędrzejewski et al. 1993, Pulliainen and Ollinmäki 1996, Lynch and McCann 2007), the pine marten proved to be a generalist predator in the recently colonised plain of the River Po. The greater importance of fruit in summer and lagomorphs throughout the year with respect to previous studies may be explained as an adaptation to the reduced availability of small mammals. This feeding flexibility is likely to play a major role in enhancing the species success in the colonization of the heavy human-altered agricultural landscape of northern Italy.

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