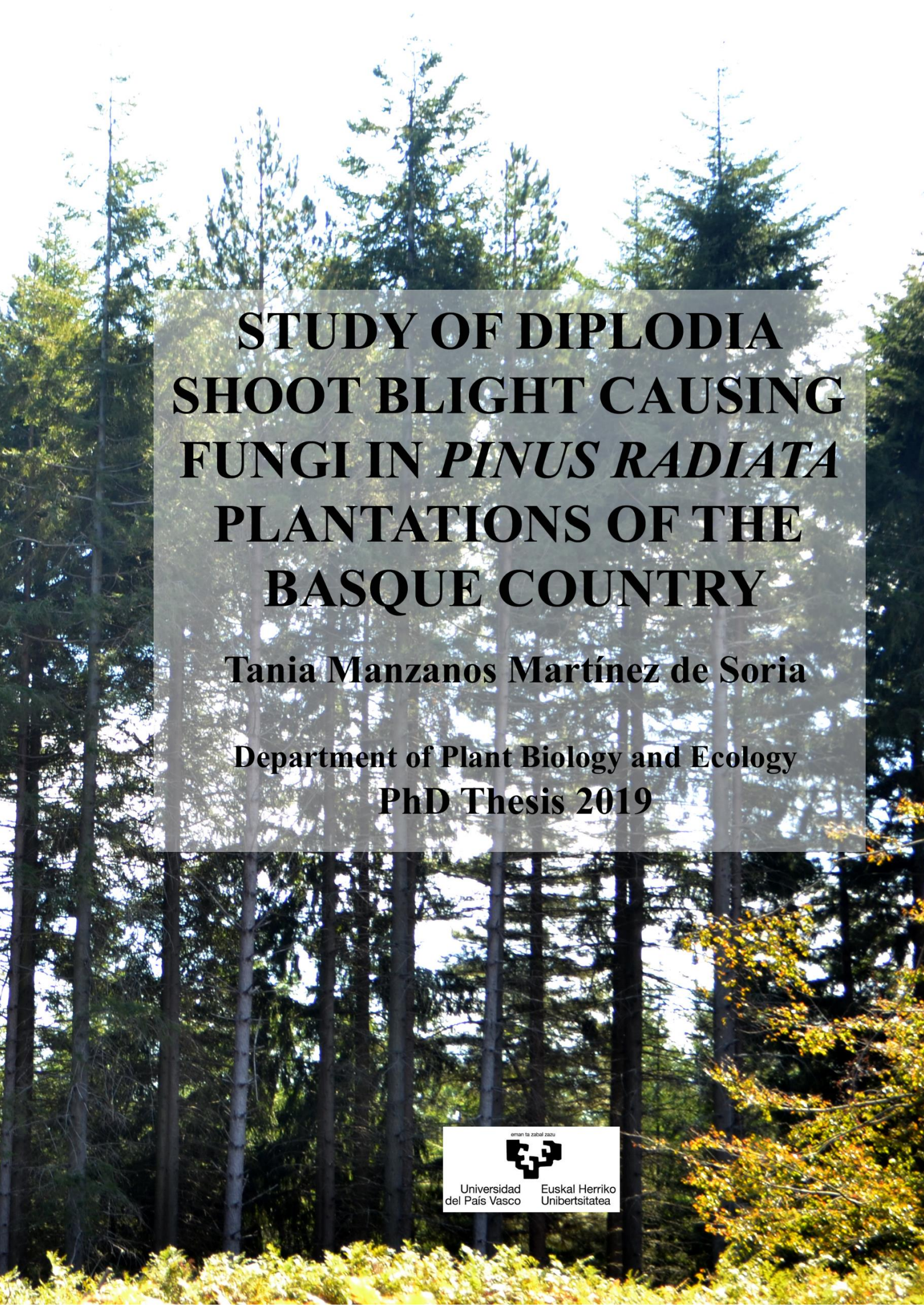

STUDY OF DIPLODIA SHOOT BLIGHT CAUSING FUNGI IN *PINUS RADIATA* PLANTATIONS OF THE BASQUE COUNTRY

Tania Manzanos Martínez de Soria

Department of Plant Biology and Ecology

PhD Thesis 2019

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Biodiversidad, Funcionamiento y Gestión de Ecosistemas

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Resumen

Durante las últimas décadas la entrada de animales, plantas, patógenos y microbios desde su área natural a otros países ha provocado daños severos. Las especies introducidas pueden volverse invasoras, esto es, pueden naturalizarse y reproducirse, propagándose y causando impactos negativos sobre las especies autóctonas.

En este sentido, los patógenos que afectan a las masas forestales han aumentado de manera notable en Europa. La introducción de estos patógenos forestales puede tener una serie de efectos perjudiciales, tanto para las funciones y servicios de los ecosistemas como para la extracción de materias primas de los árboles de las zonas afectadas.

En la mayoría de los casos la introducción y dispersión de los patógenos están causadas por el ser humano, debido al comercio de plantas vivas, el transporte de mercancías e incluso por actividades recreativas. Por lo general, estas introducciones se producen de forma involuntaria. Estos patógenos, en su área de distribución natural, se comportan de forma asintomática en sus relaciones con los hospedadores. Sin embargo, fuera de esa área de distribución, los hospedadores no son capaces de hacerles frente y son responsables de daños importantes.

En concreto, la enfermedad de la marchitez de los brotes está considerada como una de las más ampliamente distribuidas en el mundo, debido en gran medida a la facilidad que presentan sus propágulos (conidios y micelio) de dispersarse. Afecta principalmente a diferentes especies del género *Pinus*, aunque también se han descrito daños en otras especies de coníferas. Los estudios llevados a cabo en diferentes países han determinado que una de las especies de pino con menor tolerancia a los daños provocados por esta enfermedad es *Pinus radiata*. En su entorno natural (California y México), *P. radiata* y *Diplodia* coexisten, sin embargo, este patógeno no genera los mismos daños, aunque su hospedador sea el mismo.

Las mayores superficies ocupadas por *P. radiata* en España están localizadas en el extremo norte ya que es precisamente ahí donde se dan las condiciones climáticas idóneas para su crecimiento. En otros lugares también podemos encontrar esta especie, no obstante, las tasas de crecimiento para su aprovechamiento no son óptimas. En este contexto, las plantaciones del País Vasco toman especial relevancia ya que ocupan prácticamente un tercio de la superficie forestal de la comunidad y representan la mitad de las plantaciones de esta especie a nivel estatal. Las plantaciones de aprovechamiento en las

que se encuentra *P. radiata* son monocultivos, por lo que son áreas con escasa diversidad ecológica. Este factor hace que estos entornos sean menos resilientes ante posibles plagas.

El estudio desarrollado en esta Tesis Doctoral surge de la necesidad de conservar la sanidad de las plantaciones forestales de *P. radiata* en el País Vasco. Se trata de una de las especies forestales con una importancia económica y social más relevante, su mercado tiene un fuerte impacto en el Producto Interior Bruto de la comunidad y numerosos empleos están asociados a su producción. Desde hace algunas décadas, la especie se encuentra bajo la amenaza de diversos patógenos, tanto insectos como hongos, que están poniendo en jaque su supervivencia. Uno de los patógenos que históricamente más daños ha ocasionado pertenece al género *Diplodia*. Se trata de un hongo que se encuentra presente en la práctica totalidad de las plantaciones de *P. radiata* del País Vasco y que ocasionalmente, propiciado por condiciones ambientales adversas, como son el granizo o el viento, generan importantes daños. La situación se agrava cuando, a su vez, los árboles se enfrentan a algún tipo de estrés como puede ser un periodo de sequía, un hecho que se da cada vez con mayor asiduidad. Otro factor a resaltar es que, en ocasiones, hay más patógenos afectando a las plantaciones lo que supone el debilitamiento de estas especies forestales.

Es por todo ello por lo que esta Tesis Doctoral se ha centrado en determinar las especies responsables de causar la enfermedad de la marchitez de los brotes en la Comunidad Autónoma del País Vasco. Se va a proceder a explorar su ecología, biología, fisiología y diversidad. A su vez, se van a analizar aislados de distintos orígenes para determinar posibles intercambios genéticos.

Con el fin de identificar las especies responsables de causar la enfermedad del marchitamiento de los brotes, se ha llevado a cabo un muestreo generalizado en 100 plantaciones de *P. radiata* del País Vasco donde se tomaron muestras tanto de árboles asintomáticos como sintomáticos (Capítulo 2). Los resultados han mostrado que las dos especies responsables del desarrollo de esta enfermedad son *Diplodia sapinea* y *Diplodia scrobiculata*, siendo la primera la que aparece de forma mayoritaria en las plantaciones. Se trata de la primera detección de *D. scrobiculata* sobre *P. radiata* en España, y si bien se ha aislado de un árbol asintomático, en los estudios de patogenicidad llevados a cabo, ha mostrado ser capaz de generar daños severos sobre este hospedador. Esto supone una nueva realidad en la gestión forestal de la enfermedad.

Hasta este momento, tanto *D. sapinea* como *D. scrobiculata* han sido considerados hongos con una reproducción principalmente asexual, pero contrariamente a lo que cabría esperar por este tipo de reproducción, presentan elevadas tasas de diversidad. En esta situación, se ha querido analizar por medio de los MATs el tipo de apareamiento que está ocurriendo tanto en el País Vasco como en una población que incluye aislados de distintos orígenes (Capítulo 3). Los resultados obtenidos del estudio indican que ninguno de los aislados analizados presenta una reproducción diferente a la asexual, debido a que todos los aislados han resultados positivos para un único MAT. La presencia de los dos tipos de MAT en un ratio cercano al 1:1 puede ser indicativa de una reproducción de tipo críptica. El hecho de que un tipo de MAT pueda generar mayores lesiones sobre *P. radiata* parece no ser concluyente. No obstante, la media de las lesiones obtenida, señala que existe una mayor variación de la agresividad de la que se ha descrito previamente.

Tras estudiar el tipo de reproducción predominante, resulta necesario evaluar la diversidad genotípica que presentan estos hongos en el País Vasco (Capítulo 4). Para su determinación, se emplean dos metodologías distintas. Por un lado, un análisis de los grupos de compatibilidad vegetativa (VCGs), y por otro, un estudio de microsatélites. Los índices de diversidad genotípica muestran valores bajos para todas las poblaciones analizadas. Además, muchos de los marcadores SSR (Simple Sequence Repeat markers) analizados resultan ser monomórficos para los aislados estudiados. Los estudios de VCGs y microsatélites muestran valores similares de diversidad. A nivel de plantaciones la diversidad es menor, lo que demuestra la importancia que tiene la reproducción asexual en el ciclo de vida de estos hongos. Para el País Vasco, los valores de diversidad genotípica son significativamente menores que los descritos por otros autores en diferentes lugares del mundo donde el patógeno no es nativo.

Estos patógenos presentan una gran distribución por las plantaciones, especialmente del género *Pinus*, de todo el mundo. Para poder establecer posibles migraciones que hayan podido sufrir estos hongos, se han estudiado aislados del País Vasco, de Estados Unidos así como de diferentes lugares del mundo y se han comparado entre sí (Capítulo 5). Los resultados obtenidos muestran alelos y haplotipos compartidos entre aislados de *D. sapinea* y de alelos en el caso de *D. scrobiculata*, lo que indica que hayan podido tener un origen común. Sin embargo, los datos derivados del estudio de χ^2 han mostrado diferencias significativas entre las poblaciones, que unido a la identificación de alelos únicos, indican que se pueda producir un tipo de reproducción críptica. La asociación aleatoria de alelos

como respuesta a la adaptación en cada uno de los ecosistemas en los que se encuentran puede ser también una fuente de diversidad genética.

Por último, el estudio de degradación de carbohidratos ha aportado información sobre su comportamiento y ha aumentado el conocimiento sobre las características fisiológicas de *D. sapinea* y *D. scrobiculata* (Capítulo 6). Se han llevado a cabo dos analíticas. En una de ellas, se ha empleado el kit comercial API®, que incluye 31 tipos distintos de carbohidratos, y que va a servir de apoyo para la caracterización de los aislados. Otra de las metodologías llevadas a cabo ha sido el empleo de medio de celulosa tanto con tratamiento con fungicidas como sin él. Los resultados han mostrado que si bien los aislados de *D. scrobiculata* presentan lesiones menores sobre plántulas, se caracterizan por tener mayores tasas de degradación de celulosa, hecho que se aprecia tanto en los aislados del País Vasco como en los del resto de orígenes estudiados. De forma general, estos hongos son capaces de degradar en mayor o menor medida los compuestos incluidos en el sistema API®, lo que indica su gran capacidad patógena y constituye un apoyo para su diferenciación.

En conclusión, los estudios desarrollados durante esta Tesis Doctoral proporcionan nueva información sobre las poblaciones de *Diplodia* causantes de la enfermedad de la marchitez de los brotes en las plantaciones de *P. radiata* del País Vasco. Resalta la importancia de determinar cuál es la especie de hongo responsable de causar el brote de la enfermedad, ya que si bien durante mucho tiempo se ha considerado una única especie (*D. sapinea*), los resultados muestran que son dos (*D. sapinea* y *D. scrobiculata*) las responsables y que además presentan un comportamiento distinto. La información contenida puede ser de gran apoyo para la gestión y conservación de las plantaciones, pero también puede actuar como una herramienta a la hora de establecer planes de control de la enfermedad de cara a evitar posibles brotes.

Summary

During last decades, the entry of animals, plants, pathogens and microbes from their natural area to other countries has caused severe damage. Introduced species can become invasive, they can naturalize and reproduce, spreading and causing negative impacts on native areas.

In this context, pathogens that affect woods have increased significantly in Europe. The introduction of these forest pathogens can have important detrimental effects; for the functions and services of the ecosystems but also for the extraction of raw materials from the affected areas.

In most cases, the introduction and dispersion of pathogens is caused by humans, due to the trade of live plants, the transport of goods or even for recreational activities. In general, these introductions occur involuntarily. These pathogens in their natural distribution area behave asymptomatically in their relations with their hosts. However, outside that area of distribution, the hosts are not able to cope with them and are responsible for important damages.

Specifically, Diplodia shoot blight is considered as one of the most widely distributed forest diseases in the world, due to the ease of its propagules (conidia and mycelium) to disperse. Although damage has been reported in other conifer species, it mainly affects different *Pinus* species. The studies carried out in different countries have determined that one of the pine species with a lower tolerance to the damages caused by this disease is *Pinus radiata*. In its natural range (California and Mexico), *P. radiata* and *Diplodia* coexist, however, this pathogen does not report the same damage even if its host is the same.

The largest areas occupied by *P. radiata* in Spain are located in the north, and it is precisely in this area where the ideal climatic conditions for their growth occur. In other places, we can also find this species; however, the growth rates for it are not optimal. In this context, the Basque Country plantations take on special relevance. They occupy almost one third of the forest area of the community and represent half of the plantations of this species at state level. The plantations where *P. radiata* is found are monocultures, which mean that these areas will have little ecological diversity. This factor makes these environments less resilient to possible pests.

The study developed in this PhD thesis arises from the need to conserve the health of the forest plantations of *P. radiata* in the Basque Country. It is considered one of most important species based on its economic and social relevance. Its market has a strong impact on the Gross Domestic Product of the community and many jobs are associated with its production. For some decades, *P. radiata* has been under threat of various pathogens, both insects and fungi, which are impeding its survival. One of the pathogens that historically has caused more damage belongs to the *Diplodia* genus. It is a fungus that is present in almost all plantations of *P. radiata* in the Basque Country, and occasionally propitiated by adverse environmental conditions, such as hail or wind, generates significant losses. The situation could be aggravated if the trees are facing some kind of stress, such as a drought period, a fact that occurs more and more frequently. It has to be highlighted that, many times, there are more pathogens affecting plantations, which means the weakening of this forest species.

This PhD Thesis has been focused on determining the species responsible for causing Diplodia shoot blight disease in the Autonomous Community of the Basque Country. It will proceed to explore its ecology, biology, physiology and diversity. At the same time, isolates from different origins will be analysed to determine possible genetic exchanges.

In order to identify the species responsible for causing the Diplodia shoot blight, a generalized sampling was carried out in 100 plantations of *P. radiata* from the Basque Country. Samples were obtained from asymptomatic and symptomatic trees (Chapter 2). The results have shown that the two species responsible for the development of this disease are *Diplodia sapinea* and *Diplodia scrobiculata*. On the one hand, *D. sapinea* appears widely in the studied plantations. On the other hand, *D. scrobiculata* is the first time that is detected in this host in Spain. Although it has been detected in an asymptomatic tree, the pathogenicity trials found that *D. scrobiculata* cause severe damages on *P. radiata*. This represents a new reality in the forest management of the disease.

After long an intensive study, both *D. sapinea* and *D. scrobiculata* have been considered to reproduce mainly asexually, but contrary to what could be expected from this type of reproduction, they present high rates of diversity. In the light of above, the mating of these fungi was analysed in both, Basque Country's and worldwide populations (Chapter 3). The results obtained show that none of the analysed isolates present sexual reproduction, since all of them were only positive for a unique MAT. The presence of the two types of MAT in a ratio close to 1:1 can be an indicative of cryptic sexual stage. The hypothesis that a type

of MAT can cause bigger lesions on *P. radiata* does not seem to be conclusive. However, the mean lesions length indicates that there is a greater variation of the aggressiveness than it was previously described.

After determining the predominant type of reproduction, it becomes necessary to evaluate the genotypic diversity that these fungi present in the Basque Country (Chapter 4). Two different methodologies were used for its determination; an analysis of the vegetative compatibility groups (VCGs) and a microsatellites study. Genotypic diversity index show low values for all the analysed populations. In addition, many of the analysed SSR (Simple Sequence Repeat) markers seem to be monomorphic for the studied isolates. VCGs and microsatellites studies show similar values for diversity. These values at plantations are lower, which demonstrates the importance of asexual reproduction in the life cycle of these fungi. At Basque Country, values of genotypic diversity are significantly lower than those described by other authors in different parts of the world where the pathogen is non-native.

These pathogens are well distributed among, especially *Pinus* species, plantations worldwide. In order to establish possible migrations that these fungi may have suffered, isolates from the Basque Country, the United States as well as from different parts of the world have been studied and compared (Chapter 5). The results obtained show alleles and haplotypes shared between *D. sapinea* isolates and alleles in the case of *D. scrobiculata*, which indicates that they could have a common origin. However, the data derived from the study of χ^2 have shown significant differences between the populations and the identification of unique alleles indicate that a cryptic reproduction may be occurring. The random association of alleles as an adaptive response to the ecosystems in which they are found can also be a source of genetic diversity.

Finally, the study of carbohydrates degradation has provided information on their behaviour and increases knowledge about the physiological characteristics of *D. sapinea* and *D. scrobiculata* (Chapter 6). Two different analytical methods have been carried out. On the one hand, the API® commercial kit has been used. It includes 31 different types of carbohydrates and will serve as support for the characterization of the isolates. On the other hand, cellulose medium has been used with and without treatment with fungicides. The results revealed that although the isolates of *D. scrobiculata* present minor lesions lengths on *P. radiata* seedlings, they were characterized by having higher degradation rates. This fact was proved in both, the Basque Country and in worldwide populations. In

general, these fungi are capable of degrading to different extent the compounds included in the API® system, which indicates their great pathogenic capacity and constitutes a support for its differentiation.

Overall, the studies developed within the framework of this PhD Thesis have provided valuable insights about the species responsible of causing Diplodia shoot blight in *P. radiata* plantations in the Basque Country. It highlights the importance of determining which species of fungi are responsible for causing the outbreak of the disease. It has always been considered to be a single fungus the responsible (*D. sapinea*), now another species was described (*D. scrobiculata*). Furthermore, the results show a different behaviour for each of them. The information contained could support the management and conservation of plantations, but also, it can act as a tool when establishing disease control plans in order to avoid possible outbreaks.

Capítulo 1

Introducción general

1. Especies invasoras

En la actualidad, las enfermedades infecciosas emergentes de las plantas representan una de las mayores amenazas para la agricultura, la silvicultura y la conservación de la biodiversidad (Anderson *et al.*, 2004). Las enfermedades infecciosas que afectaban a las plantas fueron el resultado de la entrada accidental, durante el siglo pasado, de agentes patógenos invasores exóticos en nuevas áreas geográficas. Estas introducciones han sido favorecidas por el comercio y el transporte global (Bandyopadhyay & Frederiksen, 1999; Santini *et al.*, 2013). La dispersión epidémica de los patógenos, nativos o introducidos, ha sido debida a la asociación de dichos patógenos con artrópodos, actuando estos últimos como vectores (Wingfield *et al.*, 2016).

Estas especies, fuera de su entorno natural, no encuentran especies autóctonas que actúen como depredadoras o reguladoras. Sus hospedadores no han coevolucionado con ellas y por lo tanto no han desarrollado mecanismos de defensa ante los daños que estos les provocan (Stenlid & Oliva, 2016). El principal problema de las especies introducidas llega cuando éstas se naturalizan, esto es, cuando son capaces de reproducirse y establecerse como población (Eriksson, 2018).

En las especies forestales, las enfermedades emergentes están causadas principalmente por hongos (Anderson *et al.*, 2004). Estos tienen características como alta virulencia, alta tasa de persistencia ambiental en ausencia de hospedadores debido a su carácter saprófito, inóculos duraderos, amplio rango de hospedadores así como genomas altamente dinámicos. Todas ellas son características que les permiten resistir en un amplio rango de ambientes (Desprez-Lostau *et al.*, 2007).

Los daños generados por patógenos invasores tanto a nivel regional como global son complejos y difíciles de calcular, es por ello por lo que no existen muchos estudios que registren datos de las pérdidas económicas que estos suponen. Se ha estimado que las pérdidas causadas por especies patógenas invasoras a nivel mundial suponen 1.4 billones de dólares anuales, un 5% del Producto Interior Bruto (PIB) Mundial (año 1998) (Pimentel *et al.*, 2001). En Estados Unidos se han descrito pérdidas de aproximadamente 2.1 mil millones de dólares anuales (Pimentel *et al.*, 2005).

Además de las importantes pérdidas económicas debidas a la reducción de la producción, hay que añadir efectos secundarios negativos en el comercio de productos

forestales y plantas, los gastos indirectos producidos por las inspecciones, monitoreo, prevención y respuesta antes los patógenos, así como los importantes impactos ecológicos y ambientales producidos en los ecosistemas (Morse, 2005). Los efectos ecológicos negativos se producen a todos los niveles, desde los genes a todo el ecosistema (Loo, 2009). La pérdida de genes puede deberse a la hibridación de microorganismos no nativos con microorganismos nativos, lo que les va a generar una ventaja competitiva. Ante esta situación, los patógenos no nativos van a ser capaces de infectar nuevos huéspedes (Brasier *et al*., 1999). Los brotes epidémicos causados por estos patógenos pueden llegar incluso a modificar la diversidad, riqueza, composición y abundancia de las especies nativas alterando la estructura de los ecosistemas forestales, así como su productividad (Lovett *et al*., 2006).

2. Sector forestal en España

España es en la actualidad el segundo país con mayor superficie forestal de Europa tras Suecia (exceptuando Rusia) (Eurostat, 2012). La tendencia, al igual que en el resto de la Unión Europea, es al aumento de la superficie forestal total. La superficie forestal total incluye la superficie forestal arbolada, cuya fracción de cabida cubierta (FCC) es superior al 5% y la superficie forestal desarbolada, con una FCC menor al 5%. Se denomina FCC a la fracción de la parcela ocupada por la proyección vertical de las copas de arbolado. España contaba en el año 1990 con 25.9 millones de hectáreas de superficie forestal total aumentando hasta los 27.7 millones de hectáreas en 2010 y registrando así el mayor crecimiento de la Unión Europea. Además la cantidad de superficie forestal arbolada (FCC ≥ 5%) ha crecido considerablemente desde las 13.8 millones de hectáreas en 1991 hasta 18.2 millones de hectáreas en 2010 (Eurostat, 2012).

Atendiendo a los datos proporcionados por el Ministerio de Agricultura, Alimentación y Medio Ambiente (2012), Castilla y León es la comunidad con mayores tasas de superficie forestal arbolada (FCC≥ 5%) seguida por Andalucía y Castilla-La Mancha. Cataluña y el País Vasco cuentan con el mayor porcentaje de superficie forestal arbolada (FCC ≥ 5%) respecto al tamaño de la comunidad autónoma. Asturias y Canarias por su parte, presentan valores más altos de superficie forestal total (incluyendo la superficie arbolada y desarbolada).

La relación entre superficie forestal arbolada (que incluye monte arbolado con FCC ≥ 20% y monte arbolado ralo 10 ≤ FCC < 20%) y desarbolada (constituido por monte arbolado disperso 5 < FCC < 10 y desarbolado FCC < 5) se reparten prácticamente al 50%

(IFN3, 2007). España cuenta con 8.6 millones de hectáreas de frondosas (46.4% de la superficie forestal arbolada), 6.4 millones de coníferas (34.5%) y 3.5 millones de masas mixtas (19.1%). Las coníferas suponen el 57% (531.5 millones m^3) del total de las existencias de madera frente al 43% (396.2 millones m^3) de las frondosas (MAGRAMA, 2012).

Entre las especies que representan un mayor volumen maderable se encuentran: *Pinus sylvestris* L. (138329*10^3 m^3), *Pinus pinaster* Ait. (83408*10^3 m^3), *Fagus sylvatica* L. (69541*10^3 m^3), *Pinus halepensis* Mill. (68180*10^3 m^3), *Pinus nigra* J. F. Arnold (67156*10^3 m^3), *Quercus ilex* L. (62024*10^3 m^3), *Eucalyptus globulus* Labill. (53228*10^3 m^3), *Quercus pyrenaica* Willd. (46784*10^3 m^3) y *Pinus radiata* D. Don (41496*10^3 m^3) (IFN3, 2007).

Los principales destinos de las cortas han sido: aserraderos (36%), pasta de papel (27%), contrachapado (25%) y leña (22%). Los mayores aprovechamientos se han obtenido de las coníferas con un 52% del volumen de las cortas (año 2010) mientras que las frondosas han supuesto el 48%. Entre las coníferas destacan *Pinus pinaster* (3.2 millones de m^3) y *Pinus radiata* (1.6 millones de m^3) mientras que entre las frondosas destacan las especies de eucalipto (4.7 millones de m^3) (IFN3, 2007).

Aunque las masas forestales han aumentado durante la última década, la elaboración de productos maderables en España ha sido mantenida desde el año 1991 con 15 millones de m^3 anuales. Se considera por lo tanto que la productividad forestal española es baja (Montero & Serra, 2013). Un 66% de la superficie forestal es de propiedad privada (FAOSTAT, 2010), un valor similar al registrado en Europa.

Se estima que cerca de 312000 personas trabajan en el sector forestal a nivel nacional, lo que supone el 1.75% del total de la población activa (ASEMFO, 2012). Se calcula que el número de empresas que forman parte del sector son alrededor de 36700 (año 2011). Según datos de 2010, aporta el 0.9% al PIB nacional, unos 10 mil millones de euros (SECF, 2010). Sin embargo, la tasación del sector forestal no incluye valores indirectos que estos sistemas producen, como son los recreativos o los de biodiversidad.

La amenaza de los patógenos invasores, así como los brotes causados a lo largo de la historia, varían mucho dependiendo del país al que afecten. En Europa se han registrado importantes variaciones basadas principalmente en las condiciones climáticas y de las masas forestales (Santini *et al*., 2013). Otras variables que afectan a la introducción de patógenos exóticos son: la cantidad de territorio ocupado por áreas forestales, la contribución de la

silvicultura a la economía nacional, el uso recreativo de los bosques así como el volumen de comercio de plantas y productos derivados de la madera (Eriksson *et al*., 2018).

Los efectos que estos patógenos invasores tienen en el entorno generan una preocupación creciente en la sociedad. La conciencia de la población aumenta cuando son capaces de observar daños importantes en su entorno cercano. Estudios recientes muestran una preocupación mayor de la sociedad española y portuguesa por el nematodo de la madera o el chancro del pino (Eriksson *et al*., 2018). Es por ello por lo que se considera que los españoles muestran una mayor concienciación sobre los patógenos invasores capaces de generar daños sobre especies hospedadoras del género *Pinus*. Muchas de estas especies de pinos crecen en plantaciones, lo que supone una producción en monocultivo.

Las plantaciones forestales están compuestas por plantas pertenecientes, preferentemente, a una única especie creciendo en altas densidades y en grandes escalas espaciales (Mundt, 2002). Estos entornos son altamente propicios para la aparición y diseminación de patógenos. Las poblaciones de hospedadores uniformes facilitan la especialización de los patógenos que actúan sobre ellos, lo que concluye en un rápido crecimiento poblacional (van Baalen & Sabelis, 1995). Destaca sobre todo la rápida evolución que sufren los patógenos para superar los genes de resistencia de los hospedadores y pesticidas (McDonald & Stukenbrock, 2016). Los brotes epidémicos causados en cultivos monoespecíficos son a menudo devastadores y llamativos dado que puede conllevar la muerte de toda una plantación (Shipton, 1977).

La superficie destinada a la silvicultura intensiva en España supone el 6.8% de la superficie arbolada total del territorio nacional. La mayor superficie de plantaciones forestales se encuentra en el País Vasco (48%) y Galicia (34%) (MAGRAMA, 2012). Las plantaciones incluyen especies de crecimiento rápido como son *Eucalyptus* sp., *Pinus radiata* y *Populus* sp. Durante los últimos años, una de las especies que ha presentado un mayor volumen maderable y un porcentaje mayor de cortas anuales ha sido *P. radiata*.

Esta especie fue introducida en el siglo XIX, y sus plantaciones se localizaron especialmente en la zona norte donde cumplen los requisitos ambientales y climáticos necesarios para el crecimiento de la especie (Michel, 2003). El objetivo de estas plantaciones era obtener material para labores de entibamiento de minas de carbón así como para la fabricación de pasta de papel. En concreto, es en el País Vasco y Galicia donde toman especial relevancia las plantaciones de *P. radiata*. En el País Vasco se

encuentra el 47% del total nacional de las plantaciones de esta especie, Galicia tiene el 33% y Asturias el 8% (MAGRAMA, 2012).

3. Sector forestal en el País Vasco

Analizando la distribución por usos del suelo, el 68% del territorio está ocupado por superficie forestal (492233 hectáreas), siendo trece puntos superior a la media nacional. Del total de la superficie forestal, el 81% corresponde a superficie arbolada y el 19% restante corresponde a monte desarbolado que agrupa al arbolado disperso, matorral, pastizal y herbazal (Mapa Forestal, 2016).

Las frondosas representan el 50.2% del total de la superficie forestal de la comunidad, las coníferas el 46.2% y un 3.6% el bosque mixto (diversas especies de frondosas de cepa y semilla que nacen tras una tala final o un abandono agrario) (MAGRAMA, 2012).

En la actualidad el País Vasco cuenta con 123921 hectáreas de *Pinus radiata* lo que supone el 31.2% del total de la superficie forestal de la comunidad (397223 ha) y el 67.11% del total de las coníferas. Su importancia también queda reflejada en las cifras de existencias: estos pinares aportan el 23% de los pies mayores de la comunidad (diámetro normal $\geq$ 7,5 cm) y el 44% del volumen de madera muerta (fuste de pies mayores y menores muertos, ramas, tocones de brotes de cepa y acumulaciones) (Mapa Forestal, 2016).

En el País Vasco el 54% de la superficie forestal es de propiedad privada y, en concreto, el 85% de las plantaciones son de *P. radiata* (Figura 1.1.). La labor precursora de introducción de la especie corresponde a Carlos Adán de Yarza en el año 1850. Pero fue su hijo, Mario Adán de Yarza, quien tras observar el crecimiento que presentaba lo plantó en diversos montes de Bizkaia, siendo éste el comienzo de la reforestación en los montes vascos (Michel, 2003).

Figura 1.1. Situación de las plantaciones de *P. radiata* en el País Vasco.

Si bien es el tipo de plantación más extendida en la Comunidad Autónoma Vasca y representa entre el 85-90% de las cortas anuales, no obtiene altos valores de rentabilidad (1214 €/hectárea) en comparación con otras plantaciones forestales como pinares de *Pinus nigra* (2245 €/ha), eucaliptales (1911 €/ha) o bosques mixtos de frondosas mediterráneas (1730 €/ha) (IFN4, 2013).

El sector forestal en el País Vasco está muy arraigado en la sociedad. Las masas forestales proveen de gran cantidad de servicios: montes de uso comunal, aprovechamiento, ocio y recreo, así como reguladores de los regímenes de agua (Sáenz & Cantero, 2001). Si bien, todos los bienes que generan las masas forestales no se tienen en cuenta a la hora de calcular su aporte a la economía de la comunidad, se calcula que suponen el 1.53% del PIB, unos 1010 millones de euros anuales (Murua, 2016). El sector forestal genera alrededor de 18000 empleos. Cabría añadir que las existencias de madera son superiores a la demanda de este producto. Un sector forestal desarrollado y con elevadas existencias permitirían lograr los datos fijados para consumo de energía renovable propuesto para 2020, donde la biomasa puede tomar gran relevancia (Murua, 2016).

Encontrar rentabilidad a una masa forestal es, en general, una garantía de conservación del cultivo. Es por todo ello, por lo que resulta fundamental una buena sanidad forestal, que permita que las rentabilidades no se vean reducidas por la aparición de incendios, enfermedades forestales o plagas relacionadas con el abandono forestal (IFN4, 2013).

La presencia de especies patógenas en los ecosistemas forestales va a determinar la evolución y los efectos que estos vayan a sufrir en el futuro. Aquellas formaciones en las que existe un alto grado de manejo humano, como son las repoblaciones, es también donde se registran los valores mayores de especies invasoras. Las plantaciones de *P. radiata* presentan un 17% de especies invasoras (*Robinia pseudoacacia* L.), solo encontramos un valor superior en eucaliptales con un 22% y en las repoblaciones de *Quercus rubra* L. con un 20% (IFN4, 2013). Los daños, provocados por la meteorología y por el manejo forestal, son los principales factores de introducción y desarrollo de enfermedades y plagas, que afectan al estado fitosanitario de las masas forestales.

4. Localización de las áreas de estudio

Como se ha descrito, una de las especies más importantes en el sector forestal del País Vasco es *P. radiata*. Atendiendo a los requerimientos ambientales de la especie, en el País Vasco es posible encontrarla de forma mayoritaria en altitudes inferiores a los 600 metros, en Gipuzkoa, Bizkaia y en el extremo noroccidental de Araba, coincidiendo con los terrenos de cotas más bajas de la provincia (Figura 1.2.) (Mapa Forestal, 2016). Estas zonas presentan clima atlántico, caracterizadas por tener temperaturas moderadas y altas tasas de precipitación, entre 1200-2000 mm anuales (Euskalmet, 2011). Tolera rangos de precipitación bajos en torno a los 300 mm, pero no la sequía que se daría en los meses de verano en la zona sur de la comunidad (provincia de Araba). Se estima que necesitan precipitaciones de al menos 500 mm para obtener rentabilidad económica, sin embargo, esta aumenta considerablemente con precipitaciones hasta los 1000 mm (Lavery, 1986).

En Bizkaia y Gipuzkoa, las masas de aire frías se suavizan en contacto con las aguas oceánicas templadas. De modo que las variaciones de las temperaturas durante el día y entre estaciones no son muy acusadas. Esto permite que no sea común registrar temperaturas inferiores a 5ºC que provocan una reducción importante del crecimiento meristemático (Forde, 1966).

La localización de las plantaciones de *P. radiata* para obtener mejores crecimientos ha de situarse en terrenos profundos y bien drenados, principalmente de tipo arenoso por su reducida tolerancia al encharcamiento (Burdon *et al.*, 1998). Sin embargo, atendiendo a la orografía del País Vasco es fácil encontrarlas en laderas con importantes desniveles y suelos poco profundos.

P. radiata, por sus elevadas tasas de crecimiento es uno de los más exigentes del género *Pinus*, en cuanto a los requisitos de suelo se refiere. (Lavery, 1986). Estudios llevados a cabo por Gartzia-Bengoetxea y Arias-González (2015) en los que analizaron los suelos sobre los que se encuentran plantaciones de *P. radiata* en el País Vasco, determinaron que las deficiencias de nitrógeno son escasas y tanto el fósforo como el magnesio parecen ser los compuestos más limitantes para el crecimiento de esta especie. Además, la cantidad de materia orgánica necesaria para mantener la biodiversidad del suelo es muy reducida. Otro de los factores determinantes para su crecimiento es la presencia elevada de arcillas que favorecen la compactación del terreno.

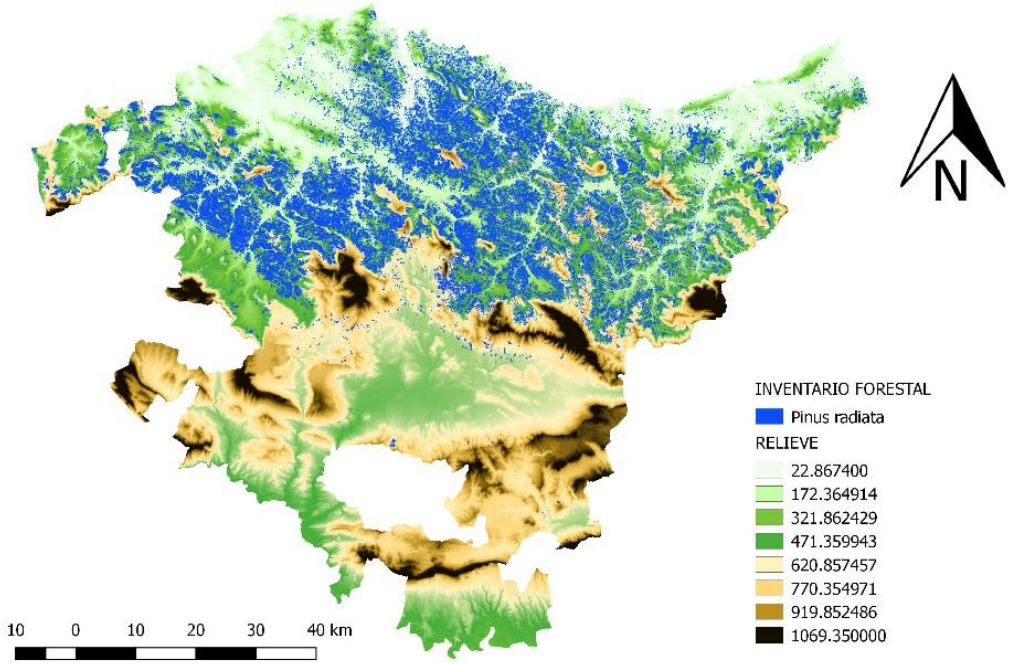

Figura 1.2. Localización de las plantaciones de *P. radiata* en el País Vasco (QGIS. 2.12.2., 2015).

5. La planta huésped: *Pinus radiata*

5.1. Situación de la especie

Es nativa de cinco áreas muy limitadas ubicadas en los condados de Santa Cruz, Península de Monterey y San Luis Obispo, a lo largo de la costa de California (Estados Unidos de América) así como en las islas de Guadalupe y Cedros (México) (Moran, 1996). Sin embargo, en la actualidad son muchos los países que han introducido esta especie para repoblaciones, entre otros destacan, Chile, Nueva Zelanda, Australia, España y Sudáfrica. Nueva Zelanda y Chile cuentan con aproximadamente 1,5 millones de hectáreas. En el sur de Australia llegan a las 700000 hectáreas y en España asciende a 263000, localizadas principalmente en el norte y centro-oeste de la península (MAGRAMA, 2012). Existen otras zonas donde las plantaciones de esta especie son importantes como en: Sudáfrica, Argentina, Italia, Ecuador, Kenia o Colombia (Lavery & Mead, 1998).

En muchos de los países donde la especie es introducida se han identificado invasiones en distintos hábitats. En Australia se ha visto afectado el monte arbolado disperso (caracterizado por la presencia de una especie vegetal dominante así como de matorral y pastizal natural) (Luken & Thieret, 1997), en Sudáfrica el fynbos (formación vegetal más extendida en el Cabo) y las zonas montanas húmedas (Henderson, 2001). En Nueva Zelanda los hábitats invadidos son matorrales, zonas abiertas y pastizales (Cronk & Fuller, 1995).

5.2. Descripción

Pinus radiata D. Don es también conocido como pino insigne o pino de Monterrey. Las tres poblaciones localizadas en California pertenecen a la variedad *radiata*, las de la isla de Guadalupe pertenecen a la variedad *binata* y, finalmente, la de la isla de Cedros pertenece a la var. *cedrosensis* (Libby *et al*., 1968). Tanto la variedad *binata* como la var. *cedrosensis* están caracterizadas por presentar acículas pares, a diferencia de la variedad *radiata* en la que se disponen en grupos de tres (Axelrod, 1980).

P. radiata es una especie que presenta un color verde intenso, tiene una densa copa y un denso follaje. La copa en los árboles jóvenes es puntiaguda aunque de forma irregular en el contorno. Los árboles maduros presentan copas redondeadas y en ocasiones aplanadas. Los árboles aislados suelen presentar forma cónica y un follaje más denso (Figura 1.3.). En las plantaciones presentan un crecimiento recto con una reducción de la densidad del

follaje en las zonas bajas del árbol, debido en gran medida a la falta de entrada de luz (Figura 1.3.).

Los árboles pueden alcanzar los 50 metros de altura, aunque lo normal es que no sobrepasen los 25 m (Burdon, 1994). Las hojas tienen forma de acícula, son suaves y flexibles, miden 7-15 cm de largo y 2-3 mm de ancho (Lindsay, 1932) (Figura 1.3.).

Los estróbilos masculinos del pino son cilíndricos y de menor tamaño que los femeninos, de 7-17 mm de largo, y a diferencia de éstos últimos, se agrupan en el conjunto de brotes de la rama final de una etapa de crecimiento (Burdon, 1992).

Las acículas persisten verdes de dos a cuatro años, dando a la copa un aspecto muy denso, de tonalidad más oscura que la del *P. pinaster* (McDonald & Laacke, 1990). Las piñas son una característica conspicua de la especie (Bannister, 1962) (Figura 1.3.). Tardan dos años en madurar desde la polinización. Una vez maduras, adquieren un color pálido o marrón claro, pudiendo llegar a medir de 7-17 cm de largo y de 4-7 cm de ancho con una forma ovoide-cónica y asimétrica. Las escamas son largas y gruesas (Burdon, 2000). Las piñas son serótinas, esto es, son capaces de permanecer sin abrir durante varios años hasta que las condiciones de temperatura y humedad sean las propicias. Después de la apertura, las piñas pueden permanecer unidas al árbol durante mucho tiempo. Pueden crecer aisladas, sin embargo, de manera habitual aparecen en grupos rodeando la zona de crecimiento de un nuevo brote (McDonald & Laacke, 1990).

Generalmente, el porte del tronco principal es recto y se divide en grandes ramas en las copas superiores de los árboles viejos (Bannister, 1962) (Figura 1.3.). El sistema radicular está dominado por una fuerte raíz en plántulas jóvenes, no obstante, el crecimiento en vivero puede modificar el desarrollo de este tipo de raíz principal. La profundidad de las raíces viene determinada por la profundidad y la compactación del suelo. En zonas de arena el sistema radicular es más profundo (Marris, 1969). La principal característica del sistema radicular de *P. radiata* reside en la alta regeneración que presentan las raíces y por lo tanto es muy fácil de trasplantar.

La madera es de color blanco amarillento, ligera, esponjosa y blanda (Figura 1.3.). Su fibra es larga y recta, y tiene una densidad aproximada de 500kg/m^3 con un 12% de humedad (Bamber, 1983).

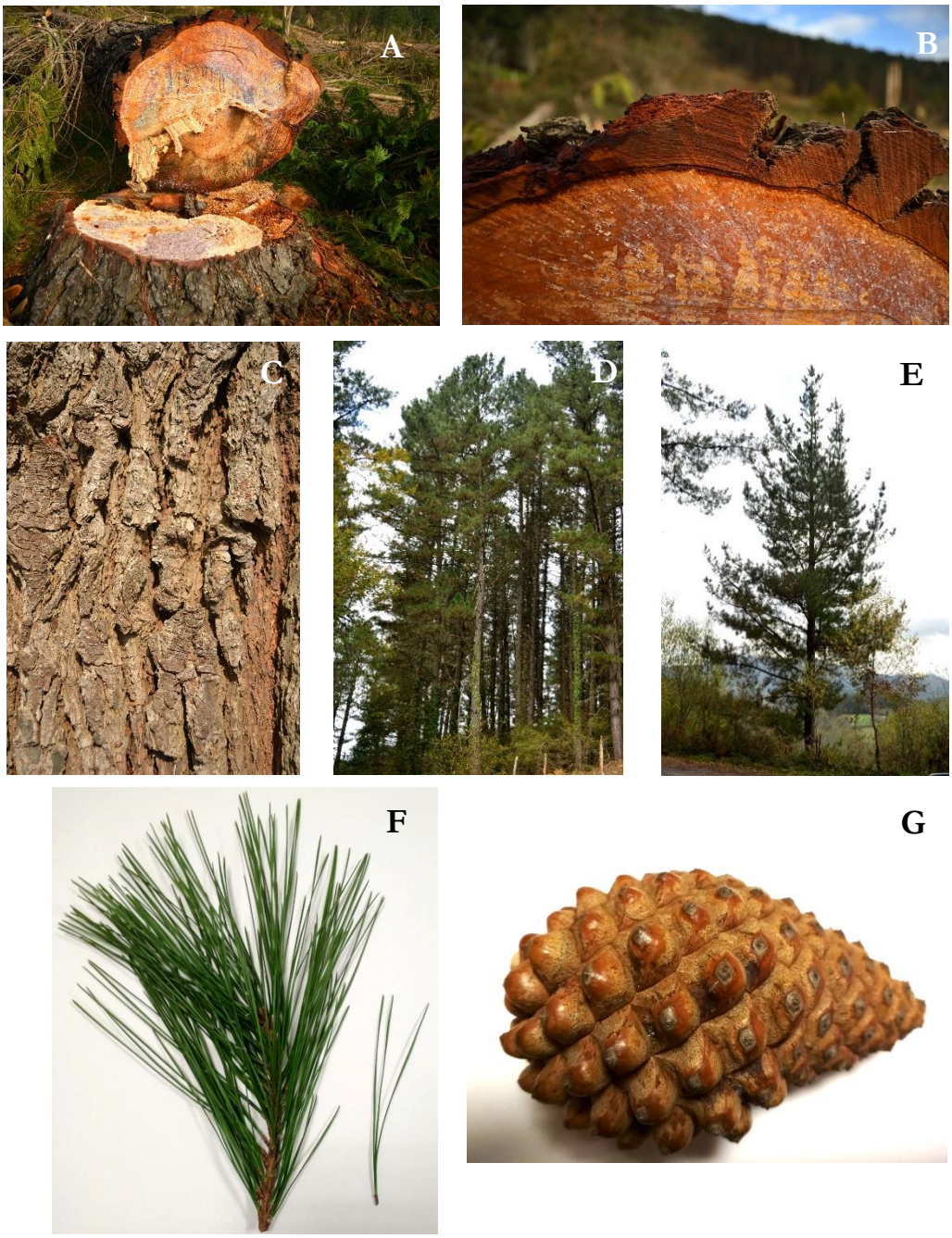

Figura 1.3. A) Árbol talado. B) Detalle de la madera. C) Corteza. D) Porte de *P. radiata* en una plantación. E) Porte de un árbol aislado. F) Acículas. G) Piña.

5.3. Reproducción y crecimiento

Los pinos son considerados especies diclino-monoicas ya que presentan conos masculinos y femeninos en el mismo individuo, pudiendo ser autofértiles (Griffin & Lindgren, 1985). La producción de polen comienza en una edad temprana, sobre los 5-6 años de edad. El inicio de la floración femenina comienza a edades similares, aunque puede variar en función de los individuos así como de su procedencia, lo que puede provocar un largo desfase en el inicio de la producción de semillas (Wilcox, 1983).

P. radiata presenta una temporada de crecimiento larga, comportándose de forma oportunista. Tras la etapa juvenil, el crecimiento se concentra en los meses de primavera, momento en el cual se desarrollan los brotes. En climas templados, por el contrario, el crecimiento puede desarrollarse hasta finales de invierno (Lavery, 1986).

5.4. Usos

Las grandes producciones que *P. radiata* tiene en el País Vasco se obtienen gracias al crecimiento en sistemas silviculturales. Es una especie de gran interés para la industria por la calidad de su madera y su rápido crecimiento, hace que su cultivo comience a dar beneficios en pocos años (± 32 años) (Bamber & Burley, 1983). Los usos derivados de esta especie son múltiples debido a la gran versatilidad que presenta su madera, hecho que permite un importante comercio internacional (Burdon, 2000).

Los destinos de estos productos son la construcción, la carpintería, los muebles o postes (p. ej. conducción eléctrica) (Scott, 1960). Pero la facilidad que presenta para el blanqueo la hacen idónea para la elaboración de pasta de papel así como para la fabricación de tableros de aglomerado, siendo estos últimos los destinos principales de los árboles del País Vasco (Murua, 2016).

Los residuos de la corta se emplean principalmente para fabricación de pasta. La corteza se quema como fuente de energía en grandes plantas de procesamiento pero también es usada en horticultura para mantillos (Kininmonth & Whitehouse, 1991). En cuanto a las piñas, su uso como combustible doméstico es muy popular. La asociación de esta especie con hongos micorrícicos permite la recolección y el consumo de los mismos (Burdon & Miller, 1992).

En algunos países la introducción de esta especie ha ido más allá de la obtención de beneficio económico. En la Cordillera de la Costa de Chile, el desarrollo de plantaciones de esta especie ha permitido rehabilitar grandes zonas gravemente erosionadas (Burdon, 2000). En Nueva Zelanda, han contribuido a mejorar la estabilización de dunas de arena así como evitar la erosión de tierras muy erosionables y fértiles (MacLaren, 1996). Además de evitar los problemas de erosión, se ha mejorado considerablemente la fertilidad del suelo (Burdon, 2000).

6. Problemas sanitarios

La reducción de las hectáreas de esta especie en su área nativa de distribución (de las 10000 hectáreas en el siglo XV a las 5300 ha en el año 2000), unido al desarrollo de numerosas plantaciones a lo largo del mundo, remarca la importancia no sólo de la perpetuación de la población nativa sino también de mantener la sanidad en las poblaciones alóctonas (Burdon, 2000). Además, según la Unión Internacional para la Conservación de la Naturaleza (Farjon, 2013), esta especie se considera en peligro de extinción en su área nativa, debido principalmente a la reducción de sus poblaciones, con solo cinco poblaciones en la actualidad, muy fragmentadas y con una ocupación de menos de 30 km^2.

El cese del desarrollo óptimo de las plantaciones viene determinado por el efecto de diferentes agentes bióticos y abióticos, siendo estos últimos más numerosos y difíciles de controlar (Madgwick, 1993). Hay que tener en cuenta que en el contexto de cambio climático en el que se encuentra nuestro planeta, los daños que provengan de factores bióticos van a ser cada vez más comunes y extremos (Eriksson *et al.*, 2018).

6.1. Patógenos que afectan a *P. radiata*

A continuación se describen algunas de las especies de hongos e insectos que históricamente han generado más daños en las plantaciones de *P. radiata* del País Vasco.

Thaumetopoea pityocampa Denis & Schiffermüller es uno de los insectos que afecta de manera especial a las especies del género *Pinus* y, más concretamente, a las plantaciones de *Pinus radiata*, que son una de las más susceptibles (Roques, 2015). Son fácilmente identificables especialmente por los bolsones sedosos que aparecen al final de las ramas en otoño e invierno así como por un aumento de la defoliación (Devkota & Schmidt, 1990). Dentro de los bolsones se encuentran las orugas pilosas, las cuales al final del invierno, se organizan en procesión, esto es, caminando en una sola línea desde la copa de los árboles hasta el suelo donde se entierran (EPPO, 2002). Los daños causados por estos insectos pueden provocar reducción del crecimiento y, si las defoliaciones son consecutivas, pueden favorecer la muerte de los árboles. Las defoliaciones generan estrés sobre los árboles lo que conlleva un aumento de la susceptibilidad frente a otras enfermedades secundarias (Tello *et al.*, 2005). Los daños causados por esta especie no se reducen únicamente a las masas forestales, son responsables de generar importantes reacciones alérgicas en los visitantes a las áreas afectadas (Fuentes *et al.*, 2006).

Tomicus piniperda L. es otro de los insectos que afecta a *P. radiata* en las plantaciones del País Vasco (Amezaga, 1996). Los signos que permiten su identificación son: la punta de la copa descolorida y los ramillos amarillentos y curvados. Se pueden distinguir grumos de resina y serrín alrededor de los orificios que realiza en el tronco (Browne, 1968). En el sistema de galerías (2-2.5 mm de ancho por 8-12 cm de largo) desarrollado por este insecto se pueden encontrar huevos, larvas, pupas y adultos inmaduros. Los adultos son de color negro o negro-rojizo con un tamaño de 3-5 mm. Los daños por la formación de galerías ocasiona frecuentemente la muerte en árboles debilitados e incluso en árboles sanos si se trata de ataques masivos (Day & Leather, 1997). Por otro lado, esta especie es vector de diferentes hongos.

Ips sexdentatus Böerner es una especie de insecto que afecta principalmente a especies del género *Pinus* pero también a otras coníferas como el abeto Douglas (*Pseudotsuga menziesii* (Mirb.) Franco). De forma general la copa adquiere un color rojizo, si bien en un primer momento se torna amarilla. El serrín provocado por la perforación del tronco es de color amarillento o anaranjado (Ciesla, 2001). Las galerías son muy reconocibles ya que presentan una forma de C, tiene de 2-5 ramas principales, llegando al metro de longitud. Los adultos presentan un color castaño claro que con el paso del tiempo se torna negro, miden de 5-8 mm de longitud con la parte final del cuerpo truncada y 6 espinas a cada lado (Cavey *et al*., 1994). Esta especie al igual que otros insectos es capaz de causar la muerte incluso a árboles sanos. Principalmente es responsable de la reducción del crecimiento y también es capaz de ser vector de otras enfermedades.

Fusarium circinatum Nirenberg & O'Donnell (teleomorfo *Gibberella circinatum*) es un hongo ascomiceto responsable de producir la enfermedad conocida como chancro resinoso del pino (Pine Pitch Canker = PPC) (Schoenherr, 1992). Es uno de los agentes patogénicos más virulentos de las coníferas, siendo capaz de infectarlas de forma directa a través de heridas o mediante insectos vectores y de forma indirecta a través del viento o el agua (Gordon, 2011). La enfermedad se hace evidente por la coloración amarillo-rojiza de las acículas y por la presencia de chancros exudantes de resina en el eje central del árbol (Martín-Rodrigues *et al*., 2013). La producción de resina conlleva un estrangulamiento de la zona afectada, que tiende a morir (Wikler *et al*., 2003). Tanto las piñas como las semillas pueden estar infectadas por el patógeno siendo una manera eficaz de dispersión del hongo en el entorno más cercano (Elvira-Recuenco, 2015), aunque también se considera una de las entradas más importantes del patógeno en otros países. Se trata de un hongo de

cuarentena cuyo control y erradicación viene determinado por el Real Decreto 637/2006 y el Decreto Foral 180/2006. Es una enfermedad con una gran incidencia descrita, en concreto, se ha estimado que un 16.8% de árboles pertenecientes a la especie *P. radiata* en el País Vasco se ven afectados. La severidad de la afección es media en un 43.1% y alta en un 53.1% de los árboles afectados (Iturritxa *et al.*, 2012). A su vez, se han detectado daños aislados en plantaciones de *Pinus pinaster* cercanas a plantaciones de *P. radiata*.

Heterobasidion annnosum (Fr.) Brefeld sensu lato es una de las principales enfermedades responsables de la pudrición de raíces de coníferas en el hemisferio norte. De las especies del género *Heterobasidion* es la única que se ha detectado en las masas de coníferas del País Vasco (Mesanza & Iturritxa, 2012). Es considerado uno de los patógenos forestales con mayor repercusión económica del mundo. Se estima que en Estados Unidos es responsable de unas pérdidas anuales que rondan los 12 millones de dólares (Hanson *et al.* 2010). Existen diversas vías de dispersión entre las que destacan: las basidioesporas, el contacto entre raíces sanas e infectadas así como insectos vectores (Manion, 1990; Adomas, *et al.*, 2005), siendo las dos primeras vías de infección las que se registran predominantemente en el País Vasco (Mesanza & Iturritxa, 2012).

La enfermedad de la banda roja *Mycosphaerella pini* E. Rostrup, anamorfo *Dothistroma pini* (Dorog.) Morelet así como la de la banda marrón *Mycosphaerella dearnessii* M. E. Bar, anamorfo *Lecanosticata acicola* (Thümen) H. Sydow son enfermedades que afectan especialmente a *Pinus* sp. Ambas enfermedades coexisten y están ampliamente distribuidas en el mundo, además producen una sintomatología similar entre ellas y con otras especies, por lo que resulta importante llevar a cabo análisis de laboratorio que permita su correcta identificación (Pehl & Wulf, 2001). De forma general, los síntomas aparecen sobre acículas con más de un año en forma de manchas amarillas y que más tarde se tornan marrones (Evans, 1984). Las acículas infectadas muestran tres zonas bien definidas: un ápice seco, la zona media con manchas marrones, y la base de las acículas verde (Gibson, 1972). Finalmente, la acícula se seca y cae de forma prematura. Los daños en plantas jóvenes pueden provocar su muerte. Los ejemplares adultos presentan signos importantes de clorosis, defoliación y una reducción importante en el crecimiento (Marks *et al.*, 1989).

Estas especies de hongos llevan muchos años presentes en las plantaciones de pino de la comunidad, sin embargo, ha sido este último año (2018) cuando se ha producido un brote importante de las enfermedades de la banda roja y marrón. Esto puede haberse debido a que las condiciones climáticas de este último año han sido óptimas para el

desarrollo de la enfermedad, con temperaturas templadas y muchos días con elevados valores de humedad. Se estima que alrededor de un 40% de las plantaciones de Gipuzkoa puedan estar afectadas con daños de diversa consideración. Los daños en Bizkaia son también numerosos y menos significativos en Araba.

6.2. Enfermedad del marchitamiento de los brotes y su agente causal

Las enfermedades foliares de las coníferas son importantes, ya que cuando son severas son capaces de reducir el crecimiento e incluso provocar la muerte del árbol. Es importante destacar que las coníferas no tienen tanta capacidad para generar nuevas acículas como las especies caducifolias y, por lo tanto, una enfermedad de este tipo provoca una reducción de la superficie fotosintética del árbol y en consecuencia una pérdida de vigor (Manion, 1990).

Esta Tesis Doctoral se va a centrar en el estudio los patógenos causantes de la enfermedad de la marchitez de los brotes (Diplodia shoot blight) que afectan de forma significativa a las plantaciones de *P. radiata* en el País Vasco.

6.2.1. *Nomenclatura*

En la actualidad, se considera que los principales hongos causantes de la enfermedad son *Diplodia sapinea* (Fr.) Fuckel (1980) y *Diplodia scrobiculata* de Wet *et al.* (2003). Sin embargo, hasta ahora se estimaba que era una única la responsable y le asignaron diversos nombres desde su primera identificación.

- *Botryodiplodia pinea* (Desm.) Petr. 1922
- *Diplodia conigena* Desm. 1846
- *Diplodia pinastri* Grove 1916
- *Diplodia pinea* (Desm.) J. Kickx F. 1867
- *Granulodiplodia sapinea* (Fr.) Zambett. 1955
- *Granulodiplodia sapinea* (Fr.) M. Morelet & Lanier 1973
- *Macrophoma pinea* (Desm.) Petr. & Syd. 1926
- *Macrophoma sapinea* (Fr.) Petr. 1962
- *Phoma pinastri* Lév.
- *Sphaeria pinea* Desm. 1842
- *Sphaeria sapinea* Fr. 1823
- *Sphaeropsis ellisii* Sacc. 1884
- *Sphaeropsis pinastri* (Lév.) Sacc. 1884
- *Sphaeropsis sapinea* (Fr.) Dyko & Sutton. 1980

Los estudios filogenéticos llevados a cabo han situado a *Diplodia sapinea* entre las especies de *Botryosphaeria* y hongos anamorfos que tienen conidios pigmentados (Jacobs & Rehner, 1998; Denman *et al*., 2000; Zhou & Stanosz, 2001). Los análisis moleculares permitieron la diferenciación de distintos grupos denominados por Palmer *et al*. (1987) como los morfotipos A y B. Otros autores diferenciaron un tercer grupo denominado morfotipo C (Stanosz *et al*., 1999; Zhou & Stanosz, 2001; Zhou *et al*., 2001; Burgess *et al*., 2001). Fueron de Wet *et al*. (2003) quienes posteriormente consideraron al grupo B como un taxón distinto y lo denominaron *Diplodia scrobiculata* de Wet *et al*. (2003).

6.2.2. *Distribución*

Son hongos con una amplia distribución tanto en el hemisferio norte como en el sur. Si bien es un hongo que es capaz de generar daños severos sobre especies de pino introducidas, rara vez causa daños importantes a los árboles que se encuentran dentro del rango natural de distribución. En concreto dentro de su área de distribución natural (California), *P. radiata* se encuentra asociado a *D. scrobiculata* y los daños generados por este hongo no son significativos (Burgess et al., 2004a).

Mientras *D. sapinea* presenta una distribución global y su número de hospedadores es muy elevado, *D. scrobiculata* presenta una distribución más limitada y un menor número de hospedadores (Bihon *et al*., 2010). A nivel general, se le considera menos agresiva que *D. sapinea* (Palmer *et al*. 1987; Blodgett & Stanosz, 1999), aunque en Sudáfrica se han detectado daños importantes causados por *D. scrobiculata* semejantes a los ocasionados por *D. sapinea* (Bihon *et al*. 2010).

Las especies que se ven más fuertemente afectadas por la enfermedad son aquellas que crecen en condiciones ambientales desfavorables. En Estados Unidos los daños más importantes se han registrado en *Pinus ponderosa* Douglas Ex. C. Lawson, *Pinus nigra* y *Pinus sylvestris* (Brookhouser & Peterson, 1971; Haddow & Newman, 1942). En Wisconsin afecta de forma especial a *Pinus resinosa* Aiton, donde se produce una mortalidad importante de la revegetación natural que se da en bosques dominados por esta especie (Smith *et al*., 2014). En el hemisferio sur *P. radiata* y *Pinus elliottii* Englem. son frecuentemente infectados (Waterman, 1943).

A nivel del País Vasco, *D. sapinea* se considera uno de los hongos más extendidos en las plantaciones de pinos y *P. radiata* es considerada la especie más susceptible (Iturritxa *et al*., 2013). Diversos estudios afirman que la zona con un mayor riesgo para desarrollar la

enfermedad es la zona noroccidental de la comunidad coincidiendo con la provincia de Gipuzkoa (Iturritxa *et al.*, 2015).

6.2.3. *Caracterización*

El tamaño de los cuerpos fructíferos denominados picnidios es de 200 μm de diámetro, son de color negro y aparecen sobre la superficie de las acículas muertas, las piñas y la corteza de las ramas, pudiéndose hallar desde finales de verano en el que se produce la infección hasta la primavera siguiente (Palmer & Nicholls, 1985). Los conidios o conidioesporas presentes en el interior de los picnidios son dispersados desde primavera hasta finales de otoño mediante la lluvia y el viento. Las esporas son marrón oscuras y son fácilmente observables mediante lupa. Las medidas rondan los 30-45 μm de largo y 10-16 μm de ancho (Wang *et al.*, 1985). Las esporas de *D. scrobiculata* son más alargadas, con una longitud de 37-41 μm y una anchura 13-15 μm (de Wet *et al.*, 2003), además en uno de los extremos la base es truncada.

El micelio de *D. sapinea* adquiere un color gris oscuro, es algodonoso y crece de forma rápida y simétrica en medio con PDA (Potato Dextrose Agar) (Wang *et al.*, 1985). Por su parte, *D. scrobiculata* se caracteriza por presentar un micelio de color gris claro, por crecer de forma irregular en los extremos y por mostrar un crecimiento más lento que el de *D. sapinea* bajo las mismas condiciones. Además, el micelio crece de forma lisa pegado al medio (de Wet *et al.*, 2003).

6.2.4. *Ciclo de la infección*

El hongo es capaz de penetrar de dos maneras distintas en los árboles: a través de heridas en la epidermis afectando a los brotes y a través de los estomas afectando a las acículas. Las heridas actúan como una de las principales vías de entrada de la enfermedad en los árboles. Son fácilmente visibles por exudaciones de resina que pueden conllevar la generación de un chancro (Wingfield & Knox-Davies, 1980). Estas heridas pueden estar producidas por insectos, viento o granizo, pero también por daños generados tras las labores de poda o clareo.

Las infecciones que conducen al marchitamiento de la punta comienzan en los brotes, y en ocasiones, en acículas inmaduras durante un período de 2 a 3 semanas en primavera, cuando los brotes comienzan a crecer (Wingfield & Knox Davies, 1980). Los conidios son capaces de germinar en pocas horas a 12-36ºC. Un período húmedo de 12 horas es

suficiente para la germinación e infección. En clima cálido, los síntomas aparecen 3-4 días después de la germinación (Peterson, 1981).

De forma general, la enfermedad solo afecta a los brotes del primer año y las piñas del segundo año en árboles que no presentan estrés. Los árboles que se enfrentan a distintos tipos de estrés tienen una mayor predisposición a la entrada de este patógeno (Bachi & Peterson, 1985). La sequía, elevados valores de humedad durante los meses de primavera, daño en las raíces, suelo compactado, presencia de otras enfermedades o un exceso de sombra son considerados los principales factores que pueden propiciar un desarrollo epidemiológico de la enfermedad (Slagg & Wright, 1943). En el caso en el que los factores de estrés se prolonguen en el tiempo, los daños en la copa y las ramas se hacen más evidentes. La invasión a las partes de más edad está favorecida por su presencia y crecimiento en la médula del árbol (Sutton, 1980).

Las piñas resultan infectadas de forma inmediata durante el crecimiento rápido que desarrollan durante su segundo año (Peterson, 1977). Si bien la infección de las piñas no supone un perjuicio importante para el estado sanitario general del árbol, supone una contribución importante para la dispersión de la enfermedad (Commonwealth Mycological Institute, 1964). Los conidios se acumulan en picnidios localizados en las piñas, y gracias al viento y la lluvia se dispersan a las zonas cercanas siendo responsables de causar una extensa infección en los nuevos brotes (Waterman, 1943). Los insectos pueden actuar también como vector de la enfermedad pudiendo transportar conidios o micelio desde zonas afectadas a otras que no lo estén. En otras ocasiones, se han descrito infecciones provenientes de material vegetal producido en viveros e incluso a través de las semillas de adultos enfermos (Palmer & Nicholls, 1985). Aunque también se han identificado importantes daños en viveros que se han visto afectados por infecciones provocadas por árboles adultos localizados cerca de las instalaciones (Palmer & Nicholls, 1985).

6.2.5. *Sintomatología*

Una reducción del crecimiento de los árboles viene determinada por el debilitamiento regresivo que causa esta enfermedad. Uno los signos más llamativos de la infección es el exudado de una gota de resina que brota desde una pequeña lesión (Peterson, 1981). Los exudados de la resina vuelven los brotes duros y frágiles. Las lesiones se agrandan rápidamente y las yemas o los brotes infectados dejan de crecer antes o durante la elongación de las acículas. Los brotes muertos se tornan de color amarillento en un primer momento y posteriormente rojizos (Marks & Minko, 1969). Estos síntomas (Figura 1.4.) se

pueden diferenciar fácilmente de los daños generados por insectos ya que presentan infiltraciones de resina, acículas persistentes, ausencia de túneles y se pueden observar los picnidios. En estados más avanzados de la enfermedad se puede observar la muerte de las ramas inferiores provocada por los chancros y por el marchitamiento continuado de los brotes (Swart *et al.*, 1958).

Figura 1.4. Síntomas de marchitamiento de los brotes (A, B, C, D, E) y picnidos observados sobre piñas (F) en las plantaciones de *P. radiata* del País Vasco.

6.2.6. *Daños*

Son numerosos los daños que se han descrito a lo largo de los años y que incluyen: inhibición de la germinación de las semillas, muerte de plántulas en vivero, desecación de los brotes, deformación de la copa, chancros en los tallos y el tronco (Flowers *et al.*, 2006). Generalmente, los daños en esta comunidad están asociados a factores climáticos adversos definidos con anterioridad, como son el viento o el granizo (Figura 1.5.), pero también a daños generados por el manejo forestal (Chou & Mackenzie, 1988).

Durante los últimos años se han registrado diferentes fenómenos de ciclogénesis explosiva caracterizados por fuertes vientos. Entre los años 2009 y 2010, se estima que se perdieron en torno a 200000 m^3 de madera en el País Vasco. Las medidas de aprovechamiento que se llevaron a cabo precisaron de ayudas del Gobierno Vasco así como de entidades forales para hacer frente a las pérdidas. Hay que destacar que los daños producidos por granizo son eventos comunes que se dan en las plantaciones. En concreto, en el año 2006 se registró una fuerte granizada que afectó a 1800 hectáreas de *P. radiata* con diferentes grados de afección (Omar, 2012).

Los daños que genera esta especie no se restringen a los causados cuando los árboles están en pie. Cuando se llevan a cabo podas o apeos también se observan daños importantes (Da Costa, 1955), es en estos momentos cuando los hongos encuentran zonas expuestas que actúan como vías de entrada y se les considera responsables de la coloración gris o azulada en la albura (parte joven de la madera). Además en la corteza exterior del tronco se pueden encontrar picnidios acompañados por micelio. Estos factores conllevan una pérdida económica importante ya que generan una devaluación de la madera (Sinclair & Lyon, 2005).

Figura 1.5. Daños generados por granizo observados en las ramas de árboles de *Pinus sylvestris* (arriba) y en plantaciones de *P. radiata* y *P. sylvestris* en Sarria (Araba) (abajo).

7. Justificación y objetivos

Pinus radiata es una de las principales especies de coníferas plantadas del País Vasco y se está viendo gravemente afectada por diversas enfermedades. Uno de los principales problemas sanitarios es la conocida como enfermedad de la marchitez de los brotes. Este estudio surgió de la necesidad de mantener la sanidad en las plantaciones de *P. radiata* por la importancia económica y social que presentan en el sector forestal del País Vasco. Para abordar esta realidad, el trabajo desarrollado se ha centrado principalmente en la descripción de las especies responsables de causar la enfermedad así como en su caracterización biológica.

Los objetivos específicos han sido los siguientes:

1. Caracterización morfológica y molecular de las especies responsables de causar la enfermedad de la marchitez de los brotes en la Comunidad Autónoma del País Vasco.
2. Determinar el tipo de reproducción, mediante análisis del tipo de MAT (mating types), de los aislados obtenidos de las plantaciones del País Vasco así como estudiar su distribución. Analizar si el tipo de MAT puede estar relacionado con la virulencia que puedan desarrollar sobre plántulas de *P. radiata*.
3. Explorar la diversidad genética presente en las plantaciones de *P. radiata* del País Vasco mediante dos técnicas distintas: microsatélites y grupos de compatibilidad vegetativa (VCGs).
4. Estimar la diversidad genética de aislados procedentes de distintos países y compararla con la obtenida para el País Vasco. Determinar posibles vías de migración que hayan podido ocurrir y que permitan identificar el origen de las introducciones.
5. Analizar las características fisiológicas del hongo determinando su capacidad de degradar diferentes tipos de carbohidratos así como su posible relación con la virulencia del mismo.

Chapter 2

Diplodia scrobiculata: A latent pathogen of *Pinus radiata* reported in northern Spain

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1. Abstract

Pinus radiata is a tree species native to the Central Coast of California and Mexico, which has been widely introduced in Europe for wood production. In Spain, especially in the northern region, it was introduced in the nineteenth century. Plantations located in the Basque Country (northern Spain) showing symptoms of Diplodia shoot blight were studied to confirm the causative pathogen species. Symptomatic and asymptomatic trees were sampled, and more than 150 fungal isolates obtained were morphologically characterized, with identities confirmed by sequencing the internal transcribed spacer (ITS) and the translation elongation factor 1-α (EF1-α) regions. Species-specific primers for *Diplodia sapinea* and *D. scrobiculata* were used to differentiate these fungi. *Diplodia scrobiculata* was detected on samples from asymptomatic trees, and BLASTN comparison was performed using the NCBI database. Lesions on *P. radiata* seedlings under controlled conditions were proved to be more substantial from *D. scrobiculata* than from *D. sapinea*. This is the first report of virulent *D. scrobiculata* in asymptomatic *P. radiata* trees in Spain.

Keywords: *Pinus radiata*, Diplodia shoot blight, asymptomatic, aggressiveness.

2. Introduction

Diplodia scrobiculata has been reported in Europe and Spain (Stanosz *et al.*, 1999; Moret & Muñoz, 2007). This fungus is known to coexist and interact with *D. sapinea*, one of the most common fungi found on pine trees (Burgess *et al.*, 2004), but *D. scrobiculata* has a much more limited distribution and host range (Bihon *et al.*, 2010). In its native range, *Pinus radiata* D. Don (Monterey pine) has been suggested to be exclusively associated with *D. scrobiculata* (Burgess *et al.*, 2004). Previous pathogenicity studies have shown *D. scrobiculata* to be less virulent than *D. sapinea* (Palmer *et al.*, 1987; Blodgett & Stanosz, 1999; Blodgett & Bonello, 2003), and biocontrol experiments have proved its ability to reduce Diplodia shoot blight (Muñoz *et al.*, 2008). Nevertheless, *D. scrobiculata* has been reported to be as

virulent as *D. sapinea* in South Africa (Bihon *et al.*, 2010). This paper presents results which identified the fungal species associated with Diplodia blight in northern Spain.

3. Material and methods

A survey for incidence of wood fungal pathogens was conducted in *Pinus radiata* plantations showing Diplodia shoot blight symptoms, located in the Basque Country (northern Spain, 42.989625°N, -2.618927°E). Samples were obtained from diseased and symptomless trees ranging from 9 to 52 years old. Wood cores were collected with a Pressler's increment borer (diameter = 5 mm) at 130 cm height (Grissino-Mayer, 2003). The cores were placed into sterilized tubes, labelled, transported to the laboratory, and stored at 4ºC.

The wood cores were surface-sterilized for 2 min with sodium hypochlorite (1% active chlorine) and rinsed with sterile deionized water. Thin disks cut from whole cross sections of the cores were placed on potato dextrose agar (PDA, Oxoid) and incubated in darkness for 7 d, and the cultures were placed into clear plastic boxes in an incubator at 20ºC. Developing fungal colonies were transferred to PDA and incubated in darkness for 7 d.

Botryosphaeriaceae isolates were incubated with sterilized pine needles according to the modified method of Smith *et al.* (1996) and were examined weekly for formation of pycnidia and conidia. The growth rates and colour of the isolates growing on PDA at 23°C in darkness were measured using colonies generated from 5 mm^2 diam. mycelial plugs obtained from the margins of 5-d-old PDA colonies. Fungal species were identified by colony and conidium morphology (de Wet *et al.*, 2003; Phillips *et al.*, 2013). Genomic DNA was extracted from mycelia cultured on PDA at 23ºC, using a commercial kit (Analytik Jena AG, Life Science). A total of two partial gene regions were used in this study: internal transcribed spacer (ITS) and the translation elongation factor 1-α (EF1-α). The ITS region was amplified with primer pairs ITS1 and ITS4 (White et al., 1990) as described by Alves *et al.* (2004). The primers EF1-728F and EF1-986R (Carbone & Kohn, 1999) were used to amplify part of the translation elongation factor 1-α (EF1-α), as described by Phillips *et al.* (2005), Alves *et al.* (2006) and Alves *et al.* (2008). PCR products were purified (Nucleospin®, Macherey-Napel) and sequenced (Eurofins. Genomics, Germany). Related sequences of *D. scrobiculata* used in the dendrograms were downloaded from the NCBI database.

BLASTN comparison of the sequences was performed using the NCBI database and E value, and identity percentages were determined. Sequences were aligned with multiple sequence comparison by log-expectation (MUSCLE), using Mega v 7.0.26 software (Tamura *et al.*, 2016). Phylogenetic analyses using Maximum Likelihood (ML) and Neighbour-Joining (NJ) (Saitou & Nei, 1987) were performed using MEGA v. 7.0.26 (Tamura *et al.*, 2016) with a Kimura 2-parameter model and statistical bootstrapping procedure involving 500 replicates.

To confirm the identity of fungal strains, the specific primers BotR, DpF, and DsF were used. These were developed for differentiation of the fungal pathogens *Diplodia sapinea* and *D. scrobiculata*, as described by Smith and Stanosz (2006).

Pathogenicity of fungal strains was tested by stem inoculation of each isolate on five *P. radiata* seedlings (18 months) maintained in a greenhouse at 22 ± 4ºC and 55-60% relative humidity (Figure 2.1.a). For each inoculation, a mycelial plug (3 to 4 mm^2 diam.) taken from the margin of an actively growing colony on PDA was placed in a shallow wound made by cutting the apical meristem of the seedling, and removing the first 5 cm.

4. Results

More than 150 isolates were obtained from *P. radiata* plantations suffering from Diplodia shoot blight throughout the Basque Country. *Diplodia sapinea* was the most common fungus isolated from symptomatic trees and is considered a widespread pathogen (99% of the cultures). *Diplodia scrobiculata* was isolated from an asymptomatic tree located in Bizkaia (43.352889ºN, -2.929818ºE).

Five weeks after incubation of fungi with sterilized pine needles, conidia appeared. They were brown, internally roughened without septa, clavate with truncate bases (Figure 2.1.g). Conidium dimensions were: length, 31 to 44 µm (mean = 38.1, standard error ± 3.06) and width 9 to 13 µm (mean = 11.1 ± 1.03), with a length/width ratio of 3.46 ± 0.41 (n=32).

When cultured on PDA at 20ºC, the colonies were white with sinuate edges and appressed mycelium that became light grey after 4 d (Figure 2.1.f). The strain was submitted to the Spanish Type Culture Collection (CECT-Universitat de Valencia, reference number CECT 20966).

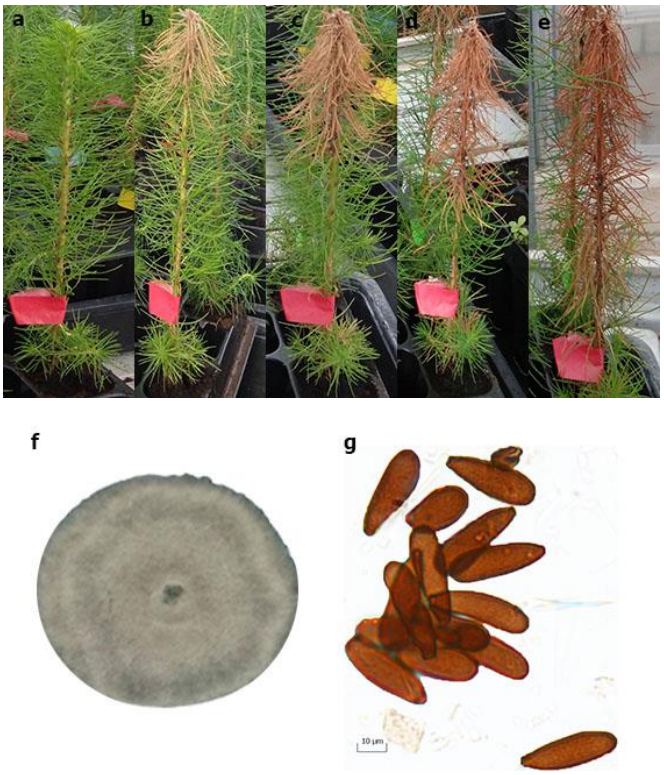


Figure 2.1. Disease caused by *Diplodia scrobiculata* on *Pinus radiata* seedlings. a: Uninoculated control. b: Symptoms produced 16 d after inoculation. c: at 30 d. d: at 55 d. e: at 75 d. f: Mycelium of the pathogen growing on PDA. g: Conidia of *Diplodia scrobiculata*.

Phylogenetic relationships among *Diplodia scrobiculata* isolates were assessed using the Neighbour-Joining method from the DNA sequences of the ITS region and EF1-α gene, and were constructed with MEGA v.7.0.26 software. Bootstrap values (500 replications) are provided to indicate support levels for tree nodes (Figures 2.2. and 2.3.). These dendrograms are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the dendrogram. The analysis for ITS phylogenetic tree involved 15 nucleotide sequences (Figure 2.2.) and eight for the EF1-α gene (Figure 2.3.). GenBank accession numbers of each reference sample are provided.

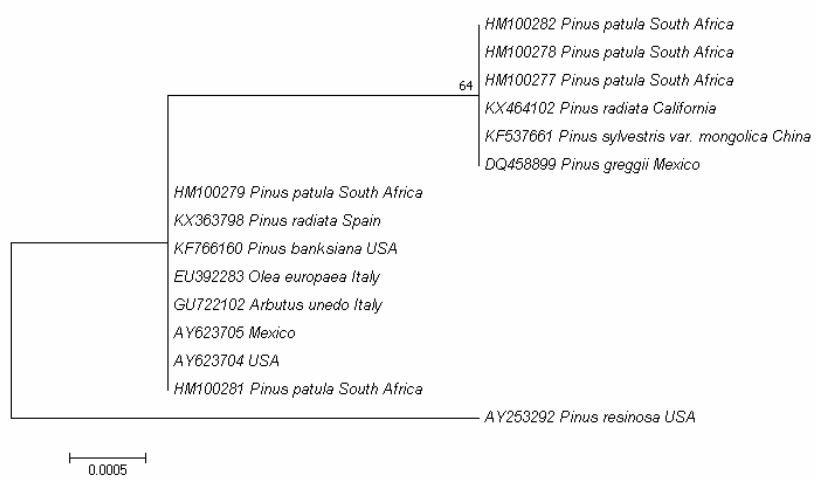


Figure 2.2. Dendrogram obtained from the dataset of 15 *Diplodia scrobiculata* isolates using the ITS region. Branches are labelled with the bootstrap values. Unlabelled branches have bootstrap values less than 50%.

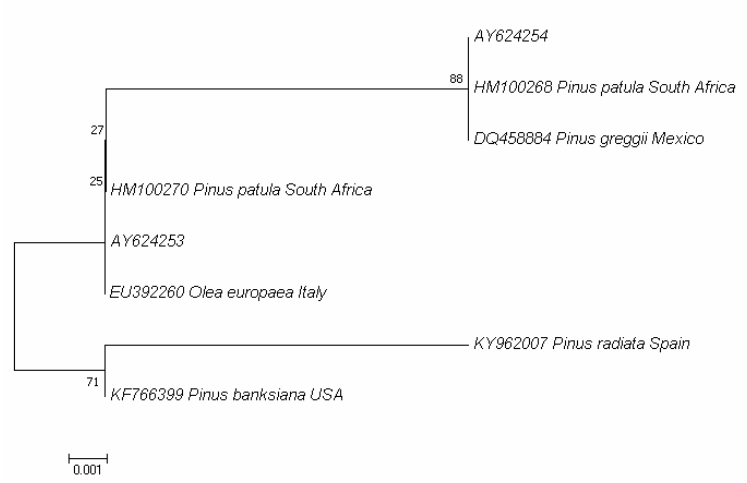


Figure 2.3. Dendrogram obtained from the dataset of eight *Diplodia scrobiculata* isolates using the EF1-α gene. Branches are labelled with bootstraps values. Unlabelled branches have bootstrap values less than 50%.

BLAST analysis of the ITS sequences revealed an E value 0, and 99% of sequence homology, with the external *D. scrobiculata* sequences from the GenBank database included. The representative ITS sequence of *D. scrobiculata* obtained in this study was deposited in GenBank (Accession No. KX363798). BLAST analysis of the EF1-α gene sequences have E values of KF766399, $1.67x10^{-6}$; EU392260 and DQ458884, $1.4\ x10^{-4}$; AY624253, $1.12x10^{-6}$; AY624254, $1.11x10^{-8}$; HM100270, $1.07x10^{-3}$; HM100268, $1.04x10^{-5}$, respectively, and 98% of sequence homology with the external *D. scrobiculata* sequences from the

GenBank database. The representative EF1-α gene of *D. scrobiculata* obtained in this study was deposited in GenBank (Accession No. KY962007).

In the pathogenicity test, infections first became visible 2 weeks after inoculation the needles in the top of the *P. radiata* seedlings became light brown. Although the inoculated plants produced resin, in almost all cases it was not enough to avoid the spread of the disease. The infections extended down through the main stems, the needles became dark brown, began to fall, and the stems turned tan. Twelve weeks after inoculation, the seedlings displayed dark brown discolouration lesions (Figure 2.1.e), located in both the bark and the wood tissues of the main stems ranging from 2.3 to 15 cm (mean = 6.3 ± 5.9 cm) for *D. scrobiculata* and from 0.6 to 11.5 cm (4.05 ± 0.99) for *D. sapinea*. The pathogens were successfully re-isolated onto PDA from symptomatic tissues and identified by the colony morphology and production of characteristic conidia, thus fulfilling Koch´s postulates. The control seedlings treated with sterile PDA plugs (non-inoculated) remained asymptomatic.

5. Discussion

This is the first report of *D. scrobiculata* affecting *P. radiata* in Spain. Although the fungus was isolated from an asymptomatic tree, in greenhouse conditions it showed a high aggressiveness. Further investigations should be undertaken to determine the distribution and impacts of this pathogen, and its interactions with *D. sapinea*. The roles of biotic and abiotic factors in the development of disease caused by *D. scrobiculata* on *P. radiata* should also be investigated.

6. References

Alves, A., Correia, A., Luque, J., & Phillips, A. J. L. (2004). *Botryosphaeria corticola*, sp. nov. on *Quercus species*, with notes and description of *Botryosphaeria stevensii* and its anamorph, *Diplodia mutila*. *Mycologia*, *96*, 598-613.

Alves, A., Correia, A., & Phillips, A. J. L. (2006). Multy-gene genealogies and morphological data support *Diplodia cupressi* sp. nov., previously recognized as *D. pinea f. sp. cupressi*, as a distinct species. *Fungal Diversity*, *23*, 1-15.

Alves, A., Crous, P.W., Correia A., & Phillips, A. J. L. (2008). Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. *Fungal Diversity*, *28*, 1-13.

Bihon, W., Slippers, B., Burgess, T., Wingfield, M. J., & Wingfield, B. D. (2010). *Diplodia scrobiculata* found in the southern hemisphere. *Forest Pathology*, *41*, 175-181.

Blodgett, J. T., & Bonello, P. (2003). The aggressiveness of *Sphaeropsis sapinea* on Austrian pine varies with isolate group and site of infection. *Forest Pathology*, *33*, 15-19.

Blodgett, J.T., & Stanosz, G. R. (1999). Differences in aggressiveness of *Sphaeropsis sapinea* RAPD marker group isolates on several conifers. *Plant Disease*, *83*, 853-856.

Burgess, T. I., Gordon, T. R., Wingfield, M. J., & Wingfield, B. D. (2004). Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. *Mycological Research*, *108*, 1399-1406.

Carbone, I., & Kohn, L. M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia*, *91*, 553-556.

de Wet J., Burgess, T. I., Slippers, B., Presig, O., Wingfield, B. D., & Wingfield, M. J. (2003). Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and identify a new species of *Diplodia*. *Mycological Research*, *107*, 557-566.

Felsenstein, J. (1985). Phylogenies and the comparative method. *The American Naturalist*, *125*, 1-15.

Grissino-Mayer, H. D. (2003). A manual tutorial for the proper use of an increment borer. *Tree-ring research*, *59*, 63-79.

Moret, A., & Muñoz, Z. (2007). Control of *Diplodia pinea* and *D. scrobiculata* in *Pinus halepensis* by 5-chloro-salicylic acid. *Phytopathologia Mediterranea*, *46*, 150-156.

Muñoz, Z., Moret, A., & Garcés, S. (2008). The use of *Verticillium dahliae* and *Diplodia scrobiculata* to induce resistance in *Pinus halepensis* against *Diplodia pinea* infection. *European Journal of Plant Pathology*, *120*, 331-337.

Palmer, M. A., Stewart, E. L., & Wingfield, M. J. (1987). Variation among isolates of *Sphaeropsis sapinea* in the north central United States. *Phytopathology*, *77*, 944-948.

Phillips, A. J. L, Alves, A., Correia, A., & Luque, J. (2005). Two new species of *Botryosphaeria* with brown, 1-septate ascospores and *Dothiorella* anamorphs. *Mycologia*, *97*, 513-529.

Phillips, A. J. L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M. J., Groenewald J. Z., & Crous, P. W. (2013). The *Botryosphaeriaceae*: genera and species known from culture. *Studies in Mycology*, *76*, 51-167.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406-425.

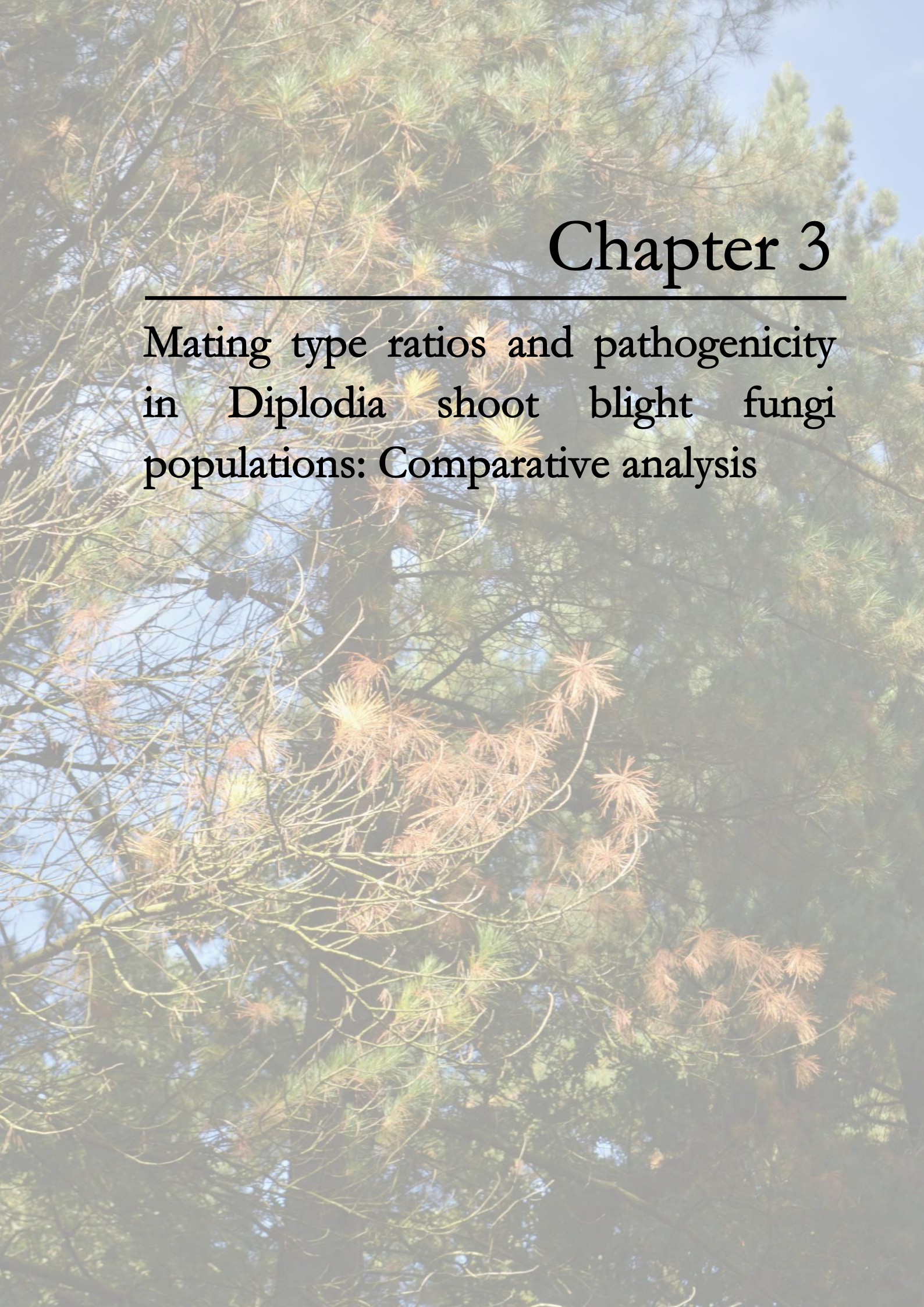
Smith, D. R., & Stanosz, G. R. (2006). A species-specific PCR assay for detection of *Diplodia pinea* and *D. scrobiculata* in dead red and jack pines with collar root symptoms. *Plant Disease*, *90*, 307-313.

Smith, H., Wingfield, M. J., Crous, P. W., & Coutinho, T. A. (1996). *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany*, *62*, 86-88.

Stanosz, G.R., Swart, W. J., & Smith, D. R. (1999). RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. *Mycological Research*, *103*, 1193-1202.

Tamura, K., Stecher, G., & Kumar, S. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0. *Molecular Biology and Evolution*, *33*, 1870-1874.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, *38*, 315-322.



Chapter 3

Mating type ratios and pathogenicity in Diplodia shoot blight fungi populations: Comparative analysis

In press as:

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1. Abstract

Diplodia sapinea and *Diplodia scrobiculata* are opportunistic pathogens of *Pinus* species. Several studies about taxonomy, impact and epidemiology of these fungi have been conducted in previous years, which have provided useful information and have raised new issues. These diseases produce a considerable impact on plantations resulting in significant economic losses. The main aims of this study are to increase the knowledge of the potential of genetic exchange and the relative aggressiveness of these organisms that can persist in healthy tissues of asymptomatic trees. A collection of 250 isolates among which are 149 strains collected from *Pinus radiata* plantations in Basque Country (Spain) and 101 strains from different countries was included in this work. Mating type ratios were analysed and compared using the structure of the *MAT* locus (*MAT1-1-1* and *MAT1-2-1*). Inoculations of *Pinus radiata* seedlings were performed in a biosafety greenhouse (P2) to confirm pathogenicity of isolates and compare their aggressiveness. The frequency of occurrence of both idiomorphs of *D. sapinea* in Basque Country isolates was close to 1:1, however, for collection of isolates of this fungus from around the world the ratio was 1:2. Furthermore, the spatial distribution of the two mating types in the Basque Country was random. Despite no detection of a sexual state, these results could suggest sexual reproduction behaviour. The pathogenicity of all strains in the collection was confirmed. Although aggressiveness (in terms of lesion lengths resulting from inoculation) varied greatly, no statistically significant effects of *MAT* type or pathogen species was detected.

Keywords: *Diplodia sapinea, Diplodia scrobiculata, Pinus radiata,* mating type, pathogenicity, *P. radiata*.

2. Introduction

Pinus radiata D. Don is a fast-growing forest tree typically managed in highly productive plantations. This species, native to the Monterey peninsula of California, was planted in more than 300000 ha in Spain during the second half of the 19th century, mainly in Atlantic areas. The Basque Country of northern Spain is the most productive forest region, in which *P. radiata* is the most abundant forest tree species. It occupies 31.2% of the forested area of that region, which is 47.1% of the total area of *P. radiata* forest in Spain (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2011; Inventario Forestal CAE, 2016).

Diplodia sapinea (Fr.) Fuckel causes a variety of diseases affecting all tree parts: seed rot; shoot blight and dieback on seedlings, saplings, and larger trees; branch and stem cankers; root diseases; and blue stain of sapwood. Infection of young seedlings can cause mortality; however, in more mature trees the death of terminal shoots can result in stem malformation and a reduction in the usable length of wood (Zwolinski *et al.*, 1990). Conidia from pycnidia produced on necrotic tissues are disseminated locally by rain splash and wind-driven rain, and longer distances to new areas by insects and human activities. Epidemics potentiated by hail damage lead to important economic losses in productive plantations (Zwolinski *et al.*, 1990). This pathogen also can long persist on or in asymptomatic pines in a latent phase, until unfavourable environmental or physiological factors predispose trees to disease development (Flowers *et al.*, 2001; Stanosz *et al.*, 2001).

Diplodia sapinea is the pathogen most frequently detected in plantations of *P. radiata* in Basque Country (Iturritxa *et al.*, 2013). An extensive survey in that region revealed presence of pycnidia of this fungus on dead tissue at >98% of survey points and shoot blight at 24% of survey points. This fungus is particularly injurious to *P. radiata*, compared to other pines that are found in the same region (Iturritxa *et al.*, 2013). The closely related species *Diplodia scrobiculata* J. de Wet, Slippers and Wingfield has also been recently detected in Basque Country (Manzanos *et al.*, 2017). The reported geographic distribution of *D. scrobiculata* is more limited and it may be less aggressive on some hosts than *D. sapinea* (Palmer *et al.*, 1987; Blodgett & Stanosz, 1997).

In spite of the long and intensive study of this pathogen on multiple continents and observation of spermatia (Wingfield & Knox-Davies, 1980; Palmer *et al.*, 1987), a sexual state of *D. sapinea* has never been observed and its reproduction is considered to be

predominantly asexual. However, genetic studies have revealed a high level of diversity that would be expected for sexually reproducing fungi as well as evidence for recombination (Burgess *et al.*, 2004; Bihon *et al.*, 2012a; Bihon *et al.*, 2012b). Subsequently, apparently functional copies of the *MAT* genes of ascomycete fungi were characterized in *D. sapinea*, and evidence was provided for existence of a heterothallic sexual cycle (Bihon *et al.*, 2014). Occurrence in populations of *D. sapinea* of the *MAT-1-1-1* and *MAT-1-2-1* mating types at frequencies not significantly different from 1:1 ratio also support the conclusion of a sexual cycle in this pathogen (Bihon *et al.* 2014). Sexual reproduction between individuals of the same species is governed by the genes found at a single mating type locus (*MAT-1*), which contains two core families (*MAT1-1* and *MAT1-2*) (Turgeon, 1998; Turgeon & Yoder, 2000; Lee *et al.*, 2010; Debuchy *et al.*, 2010; Ni *et al.*, 2011). In heterothallic species, individuals contain only one of the two gene families at the *MAT-1* locus (idiomorphs), and require an individual of the opposite *MAT-1* idiomorph for sexual reproduction (Kronstad & Staben, 1997; Groenewald *et al.*, 2008; Lee *et al.*, 2010; Martin *et al.*, 2011). The *MAT1-1* idiomorph contains at least a *MAT1-1-1* gene that encodes a protein with an α-1 box domain. The *MAT1-2* idiomorph contains at least a *MAT1-2-1* gene that encodes a regulatory protein with a DNA-binding domain known as high mobility group (HMG box) (Bihon *et al.*, 2014).

Evidence of a sexual cycle, although cryptic, in *D. sapinea* prompted the present study in a region where this pathogen is very common. The objectives were to evaluate the frequencies of the two mating types of *D. sapinea* across the broader Basque Country, in particular plantations, and also in a larger world-wide collection of both *D. sapinea* and *D. scrobiculata*. The spatial distribution of the mating types across the broader Basque Country was investigated. The relative severity of symptoms induced on *P. radiata* by isolates of the two mating types in these large collections of *D. sapinea* and *D. scrobiculata* following inoculation also was quantified.

3. Material and methods

3.1. Origin and identification of isolates

Three distinct sets of isolates were studied, including both *D. sapinea* and a lesser number of *D. scrobiculata* (Appendix A). The first, a Basque Country (Figure 3.1.a) wide set of isolates (BC, n=109) was obtained from a survey conducted in pine plantations distributed across the area occupied by *P. radiata* in that region, located on the Atlantic

coast of northern Spain (from sea level to 900 meters altitude, 43°27′N-42°28′N, 1°44′W-3°27′W) (Figure 3.1.b). Observations and sampling occurred in 2016 with most observations (>85%) in spring and summer, where symptomatic trees were observed. One symptomatic tree served as focal point from which samples were arbitrarily collected. If no symptomatic trees were immediately visible, an arbitrarily chosen tree served as the focal point. The foci were geospatially referenced (Garmin Oregon 400 GPS, accuracy level <10 m). The sample units were the plantations, which were composed of trees from the same species and age groups, from which each unique isolate per tree was obtained as described below. The second sample set (PS) consisted of isolates from three *P. radiata* plantations (PS): P1 Laukiniz (n=10), P2 Sollano (n=14), and P3 Hernani (n=16) (Figure 3.1.b). The third set (W) consisted of previously identified isolates of both *D. sapinea* and *D. scrobiculata* from a variety of *Pinus* species and some other host species in other parts of the world (n=101) (United States of America, Canada, Australia, New Zealand, France, United Kingdom, Spain, Tanzania, Madagascar, Mexico, Honduras and Turkey), obtained from the collection of Glen Stanosz, Department of Forest and Wildlife Ecology, University of Wisconsin-Madison, United States.

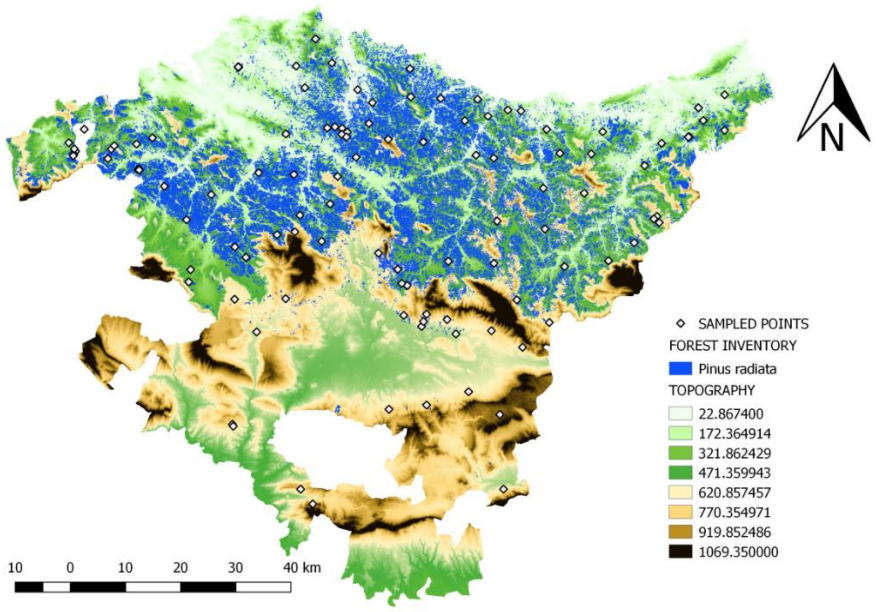


Figure 3.1. a. Location of Basque Country in Spain (QGIS. 2.12.2., 2015).

b. Spatial distribution of the samples from which isolates were obtained (BC+PS, n=149). (QGIS 2.12.2., 2015).

BC, PS (P1, P2, P3) and W isolate sets were obtained from fragments of branches, needles, cones, and pieces of wood. The samples were cut in small pieces, soaked in 30% commercial bleach (1.6% sodium hypochlorite) for 1 min, rinsed with sterile water. One single pycnidium was picked and spread on potato dextrose agar (PDA, Panreac), and then a single conidium was chosen to make monosporic cultures.

Monosporic isolates were grown on PDA in Petri plates in darkness at 20 ± 3 °C until they completely covered the medium surface. The mycelium was then scraped off and collected in a 2 ml tube with five sterile tungsten carbide beads (300 µm diam). The fungal material was disrupted by a Qiagen-Retsch MM300 Tissuelyser. In all cases, fungal DNA was extracted from 250-300 mg of mycelium using a DNA Plant Mini Kit (Analytik Jena AG, Life Science). Extractions were performed following the manufacturer's instructions. Specific primers, BotR, DpF, and DsF, developed by Smith and Stanosz (2006) were used for the identification of the species. The primers were synthetized by Integrated DNA Technologies (Belgium). The PCR mix consisted of the following ingredients: 15 µL H_2O, 2.5 µL PCR buffer, 1 µL (50 mmol L^{-1}) $MgCl_2$, 2.5 µL dNTP, 2 µL of each specific primer (10 µmol), 0.25 µL Platinum Taq-Polymerase, and 2 µL DNA template. The cycling profile

was: denaturation at 94°C for 60 s, followed by 35 cycles at 94°C for 30 s, 67°C for 30 s, and 72°C for 30 s, and a final extension at 70°C for 5 min.

3.2. *MAT1-1-1* and *MAT1-2-1* identification

Mating types of the BC, PS, and W isolate sets were identified using the two idiomorph primer sets (Bihon *et al.*, 2014). *MAT1-1-1* DipM1f: 5'-CAA GCC ATC GAC CGA AAC and DipM1r: 5'-GAA GAA GCG CAC CCT CAA T; and for *MAT1-2-1* DipHMGf: 5'-ACA AAG TTC AGC GGA GCG and DipHMGr: 5'-CCT CCG CAG GTC ACT CAT. PCR conditions consisted of PCR buffer (500 mmol L^{-1} KCl, 100 mmol L^{-1} Tris-HCL pH 8.8, 0.1% Tween-20, 15 mmol L^{-1} $MgCl_2$), 200 µmol L^{-1} dNTP, 6.4 pmol of each specific primer, 0.5 U *Taq* DNA Polymerase (BIORON GmbH), and 10-20 ng DNA template in a total volume of 20 µL. Cycling conditions consisted of 5 min denaturation at 94°C, 35 cycles of 30 s at 95°C, 30 s at 56°C, 45 s, at 72°C and a last extension at 72°C for 7 min.

3.3. Spatial distribution of *MAT*s

Indicator semivariogram analysis (Bivand *et al.*, 2013) was used to determine if mating types *MAT1-1-1* and *MAT1-2-1* tend to co-occur in the Basque Country at the landscape level (n=109). This excluded the 40 observations from the plantations P1 (Laukiniz), P2 (Sollano) and P3 (Hernani) which were sampled on a much finer spatial scale (plot level). This tool analyses the spatial behaviour of a variable over a defined area, obtaining as a result an experimental variogram that reflects the maximum distance and the way in which a point has influence on another point at different distances. Indicator semivariogram analysis applies ordinary semivariogram analysis to a binary (0/1) variable. In this case, the input variable was the mating type. To make sure to have at least 30 point pairs per distance lag, distance interval of 2.5 km was defined. The analysis was conducted in the open source statistical software R (R Core Team, 2018) using its package gstat (Pebesma, 2004).

3.4. Inoculation trial

Two-year-old *P. radiata* plug seedlings (Deba Seed Orchard, HS-Q-20/002, Basque Country, Spain) were obtained from the nursery "Explotaciones Forestales Jiménez" Araba (Vitoria-Gasteiz, Spain). Trees were then maintained in a biosafety level 2 greenhouse for cultivation at 22 ± 4°C, with a relative humidity of 55-60% and without supplemental light throughout the experimental period (May-August). After 4 weeks of acclimatization to greenhouse conditions, approximately 2 cm were removed from the tip of the leader to

expose the pith. An agar plug (approx. 3 to 4 mm^2) taken from the margin of an actively growing 7-day-old culture was placed on the wound (approx. 3 cm of the shoot apex was excised). Four trees were inoculated with each isolate. Control trees were treated in the same manner, but no inoculum plugs were placed on the wounds. Trees were maintained, for 5 weeks and irrigated to ensure they were not water stressed. After 5 weeks the inoculated seedlings displayed dark brown discolorations extending down from the point of inoculation and lengths of these lesions were recorded. At the end of the experiment 100 trees were chosen arbitrarily and re-isolations attempted from the symptomatic tissues on PDA to fulfil Koch's postulates. The pathogens were identified by colony and conidium morphology (de Wet *et al*., 2003; Phillips *et al*., 2013).

Measures of the lesion length were reduced to a unique value (mean ± SE) for each mating type and analysis of variance (ANOVA) was performed using SAS 9.1.3 service pack 3 (2008). Data were square root transformed prior to analysis to correct for normality. Each factor was tested using an error term of α=0.05. Means where compared by the Tukey multiple range test with a significance level of p<0.05. To distinguish potential differences in virulence among mating types contingency tables were constructed by mean lesion lengths and the mating type for each set (P1, P2, P3, PS, BC and W) and species (*D. sapinea* and *D. scrobiculata).* Mating type frequencies within each isolate set and species were calculated, and the χ^2 test of independence was performed with SAS software (2008).

4. Results

4.1. Isolates and *MAT* identification

Of the 149 isolates obtained from Basque Country plantations sampled in this study, all but one (identified as *D. scrobiculata*) were *D. sapinea*. Hosts were mostly from *P. radiata*, but occasional hosts included *Pinus attenuata* Lemmon, *Pinus contorta* Douglas, *Pinus halepensis* Mill., *Pinus nigra* J. F. Arnold, *Pinus pinaster* Ait. and *Pinus sylvestris* L. Isolates in the W set included 70 of *D. sapinea* and 31 of *D. scrobiculata*, with both of these pathogens having been obtained from a much greater variety of pine and nonpine hosts.

MAT1-1-1 and *MAT1-2-1* are widely distributed in the Basque Country region (BC) and in each of the three PS plantations (P1, P2 and P3) (Figure 3.2.). The ratio of the *MAT1-1-1* to *MAT1-2-1* mating types for *D. sapinea* ranged from P1=0.25, P2=6.00, P3=0.45, PS=0.9, BC=0.89 (Table 3.1.).

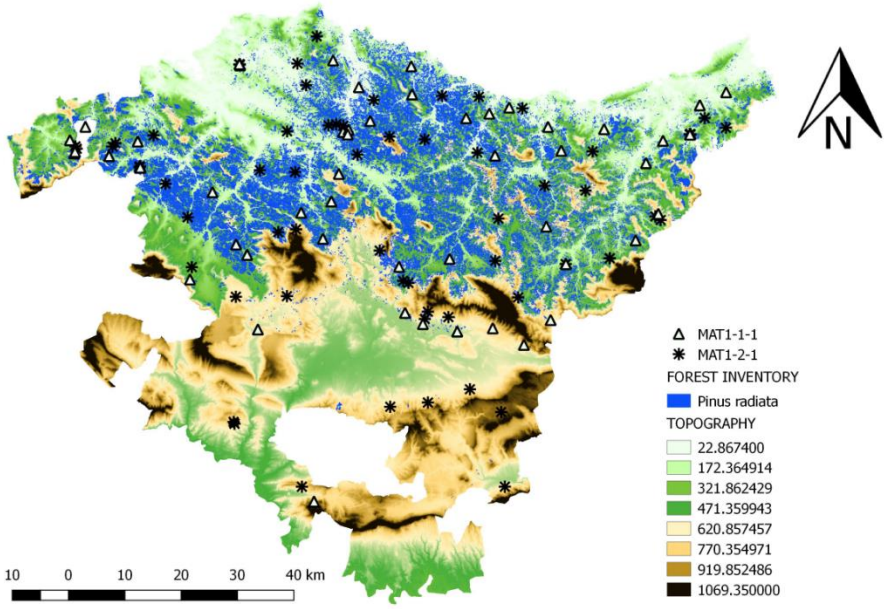


Figure 3.2. Spatial distribution of MAT types of isolates of *Diplodia sapinea* (n=148) and *Diplodia scrobiculata* (n=1) samples in Basque Country (Spain) (QGIS 2.12.2., 2015).

Both mating types were common and widely distributed in the W set of *D. sapinea* and *D. scrobiculata* isolates. However, among *D. sapinea* isolates in the W set, *MAT1-1-1* was only detected approximately half as often as *MAT1-2-1* (23 and 47 isolates, respectively) (Table 3.1.). In contrast, much more similar numbers of isolates of the two mating types (17 of *MAT1-1-1* and 14 of *MAT1-2-1*) were found for the *D. scrobiculata* isolates in the W set (Table 3.1.).

There was a statistically significant difference between occurrence of *MAT1-1-1* and *MAT1-2-1* in the study collections as a whole (BC, PS, and W), but this was due to differences of the frequencies of mating types in P2 (standardized residuals ≥ 2.58, significant at $p<.01$). The χ^2 analysis indicated that the ratios of *MAT1-1-1* to *MAT1-1-2* were not significantly different from 1:1 except for the collection P2 (Table 3.1.). The fairly low ratios (<0.50) of *MAT1-1-1* to *MAT1-2-1* in the P1, P3 and W were not significant at 5% level of probability.

Table 3.1. Relative frequencies of *MAT1-1-1* and *MAT1-2-1* in each three isolate sets.

D. sapinea	**No. of isolates**	***MAT1-1-1* No. (%)**	***MAT1-2-1* No. (%)**	**Ratio**
P1	10	2 (20.0)	8 (80.0)	0.25
P2	14	12 (85.7)	2 (14.3)	6.00
P3	16	5 (31.3)	11 (68.7)	0.45
PS (P1+P2+P3)	40	19 (47.5)	21 (52.5)	0.90
BC	108	51 (47.2)	57 (52.8)	0.89
W	70	23 (32.9)	47 (67.1)	0.49
TOGETHER (PS+BC+W)	218	93 (42.7)	125 (57.3)	0.74
D. scrobiculata				
BC	1	1 (100)	0 (0)	-
W	31	17 (54.8)	14 (45.2)	1.21
TOGETHER (BC+W)	32	18 (56.3)	14 (43.8)	1.29

4.2. Spatial analysis

Indicator semivariogram revealed no spatial autocorrelation at landscape level which can be interpreted as a lack of spatial clustering for either mating type. Both mating types frequently occur jointly at the landscape level, although different patterns may be present at finer spatial scales <2.5 km. The semivariogram value remained constant regardless of the number of isolates and the distance (Figure 3.3.). There was no clustering identified and mating type did not change with the distance between points.

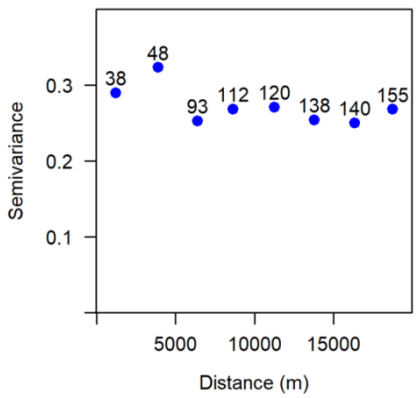


Figure 3.3. Indicator semivariogram from spatial analysis of *Diplodia sapinea* mating type distribution in Basque Country (BC) set of isolates (n=109). The numbers above the blue dots refer to the number of point pairs considered per distance lag.

4.3. Inoculation trial

All seedlings inoculated with all isolates of *D. sapinea* and *D. scrobiculata* developed lesions (Figure 3.4.). Symptoms first became visible 2 weeks after inoculation when the needles in the tips of inoculated shoots became light brown. The lesions became resinous and extended downward, the needles became dark brown, began to fall, and the stems turned tan. Five weeks after inoculation, all the inoculated seedlings displayed dark brown discoloured lesions, located in both the bark and the wood tissues of the main stems. The control seedlings treated with sterile PDA plugs (non-inoculated) remained asymptomatic till the end of the experiment, never produced lesions beyond what could be attributed to the wounding procedure (<1 mm) and were therefore excluded from analyses.

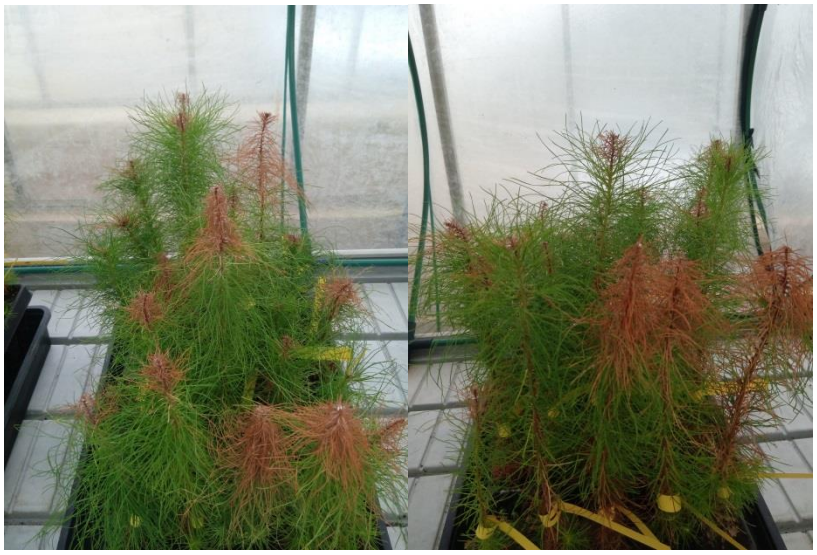


Figure 3.4. *P. radiata* seedlings inoculated with *D. sapinea* and *D. scrobiculata* isolates.

For all *D. sapinea* isolates collectively, the lesion length mean was 42.5 (Table 3.2.). In contrast, for all *D. scrobiculata* isolates collectively, lesion length mean was 25.1 (Table 3.2.). For individual *D. sapinea* isolates, mean lesion lengths ranged from 9.3 to 137.9 mm (*MAT1-1-1*) and from 4.4 to 141.5 mm (*MAT1-2-1*) (Table 3.2.). For individual *D. scrobiculata* isolates mean lesion lengths ranged from 20.6 to 38.4 mm (*MAT1-1-1*) and from 10.5 to 48.6 mm (*MAT1-2-1*) (Table 3.2.). Despite some differences in mean lesion lengths ($F=59.48$, $p=<0.0001$) among the various isolate sets and mating types ($F=1.26$, $p=>0.05$), these differences were not found to be statistically significant ($F=1.53$, $p=>0.05$). The differences in mean lesion lengths resulting from inoculation with *D. sapinea* or *D. scrobiculata* also were not found to be significant. In terms of the experiments, these results infer that mating type did not strongly influence the lengths of lesions induced by the

strains. However, it did represent a medium-sized effect d=0.68 according to χ^2 goodness-of-fit.

Table 3.2. Means, standard error and ranges of lesion length (mm) resulting from inoculation of *Pinus radiata* seedlings with 250 isolates of *Diplodia sapinea* and *Diplodia scrobiculata* from different origins and of two MAT types.

	No. of	Both MATs	*MAT1-1-1*		*MAT1-2-1*	
D. sapinea	**isolates**	**Mean + SE[a]**	**Mean + SE[b]**	**Range[c]**	**Mean + SE[d]**	**Range[e]**
P1	10	30.6 ± 2.1 a	27.9 ± 7.3	20.7-35.2	31.3 ± 2.1	25.5-43.6
P2	14	21.7 ± 2.0 a	20.6 ± 2.0	11.0-32.8	27.9 ± 6.3	21.6-34.2
P3	16	25.4 ± 2.8 a	22.6 ± 6.1	9.3-40.7	26.8 ± 3.0	20.1-54.8
PS (P1+P2+P3)	40	25.4 ± 1.5 a	21.9 ± 2.1	9.3-40.7	28.6 ± 1.9	20.1-54.8
BC	108	56.8 ± 2.5 b	54.2 ± 3.4	16.5-137.9	59.1 ± 3.6	11.9-141.5
W	70	29.9 ± 1.3 a	32.1 ± 1.9	17-56.5	28.8 ± 1.7	4.4-62.5
TOGETHER (PS+BC+W)	218	42.5 ± 1.6	42.1 ± 2.4	9.3-137.9	42.6 ± 2.2	4.4-141.5
D. scrobiculata						
BC	1	27.1	27.1	20.6-32.0	-	-
W	31	25.1 ± 2.1	20.8 ± 2.3	6.7-38.4	30.7 ± 2.9	10.5-48.6
TOGETHER (BC+W)	32	25.1 ± 2.0	21.1 ± 2.2	20.6-38.4	30.7 ± 2.9	10.5-48.6

N number of isolates per each population.

[a] Mean lesion lengths and standard error (SE) for each group of samples followed by different letters within a column are significantly different ($p \geq 0.005$) based on DHS of Tukey.
[b] Mean lesion lengths and standard error for *MAT1-1-1* samples.
[c] Range of lesions for *MAT1-1-1* samples.
[d] Mean lesion lengths and standard error for *MAT1-2-1* samples.
[e] Range of lesions for *MAT1-2-1* samples.

5. Discussion

The common occurrence of Diplodia shoot blight caused by *D. sapinea* in plantations throughout the Basque Country of northern Spain mirrors the prevalence of this disease and success of this pathogen species in natural forests and plantations worldwide (Stanosz *et al.*, 1999; Burgess *et al.*, 2004). The continued absence of detection of sexual stage of *D. sapinea*, despite the common occurrence of each mating type is puzzling. While the relative occurrence of the two *D. sapinea* mating types differed in local collections, the approximately 1:1 ratio of the two mating types was found in the larger number of isolates.

An even distribution of mating genes (Coppin *et al.*, 1997; Hull *et al.*, 2000; Lengeler *et al.*, 2002; Poggeler, 2002) supports potential for the presence of a cryptic sexual cycle in *D. sapinea* (Bihon *et al.*, 2014). A 1:1 mating type ratio is expected in randomly mating populations (Milgroom, 1996; Zhan *et al.*, 2002; Paoletti *et al.*, 2005; Groenewald *et al.*, 2006). Despite the possibility of a sexual cycle, it remains clear that prolific asexual reproduction is an important reproductive and dispersal strategy for *D. sapinea*, given the ubiquitous occurrence of pycnidia and abundant conidial production on killed organs of diseased trees.

Variation in mating type ratios, when seen in this study, suggests the importance of sample design in study of this trait and other characters of *D. sapinea* and similar species. Significant differences in mating type ratio were detected in P2 were a value of 6 was obtained. This difference could be due to different location and origin of the plant (Michel, 2003). Deviation from 1:1 ratio of mating types in P1, P2, and P3 populations were eliminated when these were pooled (PS) and not reflected in the large BC population from across the Basque Country. Similarly, localized subsets of isolates in the W set of isolates may reflect a skewing of mating type ratios only because of small numbers, or local sampling. Such a deviation from 1:1 also could be the result of an asexual reproduction, where the bottleneck that usually accompanies introduced populations is observable (Taylor *et al.*, 1999).

According to indicator semivariogram analysis, the distribution of the mating types appears to not have autocorrelation in the studied area. The results were unusually consistent given that all of the populations studied belong to different owners with different origins for the plant (Michel, 2003). In this region the seed suppliers are mainly from the Basque Country, New Zealand, Chile and France. The property of the exploitations is private; therefore, the management between different owners can vary enormously.

The many-fold differences in mean lesion lengths resulting from inoculation with the large number of isolates used in this study, although not related to mating type, suggest greater variation in aggressiveness of each pathogen than demonstrated previously. For example, Blodgett and Stanosz (1997) used nine *D. sapinea* isolates to inoculate wounded elongating terminal shoots of red pine (*Pinus resinosa*) seedlings. Mean distance from the inoculation site on red pine at which needles were necrotic 4 weeks later varied among isolates by less than 3 cm in each of two trials. Similarly, Bihon *et al.* (2011) found no

statistically significant differences among mean lesion lengths resulting 2 weeks after inoculation of *P. radiata* seedling stem wounds. It is possible that in addition to capturing much greater variation by testing a greater number of isolates, the longer (i.e., 5-week-long) incubation period utilized in the current study allowed differences in isolate aggressiveness to be detected. The implications of such variation among isolates will have practical significance in attempts to screen for resistance to Diplodia shoot blight, which was demonstrated to differ among *P. radiata* half-sib families (Swart *et al.*, 1996). Breeding and selection for resistance to a group of the most aggressive isolates within pathogen populations may offer the best opportunity to minimize losses to this disease.

Although only encountered once among samples collected for this study, results of inoculation trials contrast with the notion that *D. scrobiculata* is "probably best recognised as a relatively harmless endophyte" (de Wet, *et al.*, 2003). Despite, on average, lesions induced by *D. scrobiculata* isolates used in this study were not as long as those induced by *D. sapinea* isolates, the large range in lesion lengths produced by *D. scrobiculata* isolates were the product of individuals that were more aggressive than many *D. sapinea* isolates. Similarly, overlapping ranges in aggressiveness of *D. sapinea* and *D. scrobiculata* (using a variety of inoculation methods and measures of symptom severity) have been reported for *Pinus banksiana* (Blodgett & Stanosz, 1997), and both *P. radiata* and *Pinus elliotii* (Bihon *et al.*, 2011). These results should prompt further research on the factors that permit severe disease by *D. scrobiculata*, and are a reminder of the potential importance of both host and environment, in addition to pathogen, in disease development.

Even if occurrence of mating type genes and analysis of microsatellite markers suggest that recombination in *D. sapinea* (Bihon *et al.*, 2011), the sexual stage remains undetected. It is possible that some heretofore unexplained host substrate or environmental factors might be required to facilitate mating. However, rare occurrence of sexual reproduction may result from infrequent occurrence of anastomoses that would be prerequisite. The high diversity of distinct vegetative compatibility groups (VCGs) may already be present in local populations. Smith *et al.* (2000) found 62 VCGs present among 107 isolates from just three South African plantations. Particularly if other factors, including the success of particular asexually reproduced genotypes, reduce the potential for meeting of compatible strains, mating would be inhibited.

These findings have significance not only for understanding the biology and distribution of *D. sapinea* and *D. scrobiculata*, but they also have significant applied relevance

when considering development of disease by these fungi in the environment and when considering control strategies.

6. References

Bihon, W., Wingfield, M. J., Slippers, B., Duong, T. A., & Wingfield, B. D. (2014). MAT gene idiomorphs suggest a heterothallic sexual cycle in a predominantly asexual and important pine pathogen. *Fungal Genetics and Biology*, *62*, 55-61.

Bihon, W., Burgess, T., Slippers, B., Wingfield, M. J., & Wingfield, B. D. (2012a). High levels of genetic diversity and cryptic recombination is widespread in introduced *Diplodia sapinea* populations. *Australasian Plant Pathology*, *41*, 41-46.

Bihon, W., Slippers, B., Burgess, T., Wingfield, M. J., & Wingfield, B. D. (2012b). Diverse sources of infection and cryptic recombination revealed in South African *Diplodia sapinea* populations. *Fungal Biology*, *116*, 112-120.

Bihon, W., Slippers, B., Burgess, T., Wingfield, M. J., & Wingfield, B. D. (2011). *Diplodia scrobiculata* found in the southern hemisphere. *Forest Pathology*, *41*, 175-211.

Bivand, R. S., Pebesma, E., & Gómez-Rubio, V. (2013). *Applied spatial data analysis with R* (2th ed.). New York, NY: Springer.

Blodgett, J. T., & Stanosz, G. R. (1997). *Sphaeropsis sapinea* morphotypes differ in aggressiveness but both infect non wounded red or jack pines. *Plant Disease*, *81*, 143-147.

Burgess, T. I., Wingfield, M. J., & Wingfield, B. D. (2004). Global distribution of *Diplodia sapinea* genotypes revealed using simple sequence repeat (SSR) markers. *Australasian Plant Pathology*, *33*, 513-519.

Coppin, E., Debuchy, R., Srnaise, S., & Picard, M. (1997). Mating types and sexual development in filamentous ascomycetes. *Microbiology and Molecular Biology*, *16*, 411-428.

Debuchy, R., Berteaux-Lecellier, V., & Silar, P. (2010). Mating Systems and Sexual Morphogenesis in Ascomycetes. In: Ebbole, Borkovich, (Ed.), *Cellular and Molecular Biology of Filamentous Fungi*, 501-535. Washington, DC: ASM Press.

de Wet, J., Burgess, T., Slippers, B., Preisig, O., Wingfield, B. D., & Wingfield, M. J. (2003). Multiple gene genealogies and microsatellite markers reflect relationships between

morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. *Mycological Research*, *107*, 557-566.

Flowers, J., Nuckles, E., Hartman, J., & Vaillancourt, L. (2001). Latent infection of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. *Plant Disease*, *85*, 1107-1112.

Groenewald, M., Linde, C.C., Groenewald, J.Z., & Crous, P.W. (2008). Indirect evidence for sexual reproduction in *Cercospora beticola* populations from sugar beet. *Plant Pathology*, *57*, 25-32.

Groenewald, M., Groenewald, J. Z., Harrington, T.C., Abeln, E. C. A., & Crous, P. W. (2006). Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex. *Fungal Genetics and Biology*, *43*, 813-825.

Hull, C. M., Raisner, R. M., & Johnson, A. D. (2000). Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. *Science*, *289*, 307-310.

Inventario Forestal CAE. (2016). El bosque Vasco en cifras 2017. Retrieved from http://www.euskadi.eus/contenidos/informacion/inventario_forestal_2016/es_agripes/adjuntos/El%20bosque%20vasco%20en%20cifras%202017.pdf

Iturritxa, E., Ganley, R. J., Raposo, R., García-Serna, I., Mesanza, N., Kirkpatrick, S. C., & Gordon, T. R. (2013). Resistance levels of Spanish conifers against *Fusariun circinatum* and *Diplodia sapinea*. *Forest pathology*. *43*, 488-495.

Kronstad, J.W., & Staben, C. (1997). Mating type in filamentous fungi. *Annual Review of Genetics*, *31*, 245-276.

Lee, S.C., Ni, M., Li, W., Shertz, C., & Heitman, J. (2010). The evolution of sex, a perspective from the fungal kingdom. *Microbiology and Molecular Biology Reviews*, *74*, 298-340.

Lengeler, K. B., Fox, D. S., Fraser, J. A., Allen, A., Forrester, K., Dietrich, F. S., & Heitman, J. (2002). Mating-type locus of *Cryptococcus neoformans*: a step in the evolution of sex chromosomes. *Eukaryotic Cell*, *1*, 704-718.

Manzanos, T., Aragonés, A., & Iturritxa, E. (2017). *Diplodia scrobiculata*: a latent pathogen of *Pinus radiata* reported in northern Spain. *Phytopathologia Mediterranea*, *56*, 274-277.

Martin, S. H., Wingfield, B. D., Wingfield, M. J., & Steenkamp, E. T. (2011). Structure and evolution of the Fusarium mating type locus, new insights from the *Gibberella fujikuroi* complex. *Fungal Genetics and Biology* 48, 731-740.

Michel, M. (2003). *El pino radiata (Pinus radiata D. Don) en la historia forestal de la Comunidad Autónoma del País Vasco: análisis de un proceso de forestalismo intensivo.* Madrid, España: Universidad Politécnica de Madrid, PhD thesis.

Milgroom, M. G. (1996). Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology*, *34*, 457-477.

Ministerio de Agricultura, Alimentación y Medio Ambiente. (2011). Cuarto Inventario Forestal Nacional. Comunidad Autónoma del País Vasco/Euskadi. Retrieved from http://www.euskadi.eus/contenidos/informacion/inventario_forestal_2011/es_agripes/adjuntos/IFN4%20PAIS%20VASCO.pdf

Ni, M., Feretzaki, M., Sun, S., Wang, X., & Heitman, J. (2011). Sex in fungi. *Annual Review of Genetics*, *45*, 405-430.

Palmer, M. A., Stewart, E. L., & Wingfield, M. J. (1987). Variation among isolates of *Sphaeropsis sapinea* in the north central United States. *Phytopathology*, *77*, 944-948.

Paoletti, M., Rydholm, C., Schwier, E. U., Anderson, M. J., Szakacs, G., Lutzoni, F., Debeaupuis, J. P., Latgé, J. P., Denning, D. W., & Dyer, P. S. (2005). Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Current Biology*, *15*, 1242-1248.

Pebesma, E. J. (2004). Multivariable geostatistics in S: the gstat package. *Computer and Geoscience*, *30*, 683-691.

Phillips, A. J. L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M. J., Groenewald, J. Z., & Crous, P. W. (2013). The *Botryosphaeriaceae*: genera and species known from culture. *Studies in Mycology*, *76*, 51-167.

Poggeler, S. (2002). Genomic evidence for mating abilities in the asexual pathogen *Aspergillus fumigatus*. *Current Genetics*, *42*, 153-160.

QGIS. Release 2.12.2. (2015). QGIS Development Team.

R Core Team. (2018). R: A Language and Environment for Statistical Computing. Vienna, Austria.

SAS 9.1.3. Service Pack 3. (2008). SAS Institute INC. Cary, North Carolina, USA.

Smith, D. R., & Stanosz, G. R. (2006). A species-specific PCR assay for Detection of *Diplodia sapinea* and *D. scrobiculata* in dead red and jack pines with collar rot symptoms. *Plant Disease*, *90*, 307-313.

Smith, H., Wingfield, M. J., de Wet, J., & Coutinho, T. A. (2000). Genotypic diversity of *Sphaeropsis sapinea* from South Africa and northern Sumatra. *Plant Disease*, *84*, 139-142.

Stanosz, G. R., Blodgett, J. T., Smith, D. R., & Kruger, E. L. (2001). Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. *New Phytologist*, *149*, 531-538.

Stanosz, G. R., Swart, W. J., & Smith, D. R. (1999). RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. *Mycological Research*, *103*, 1193-1202.

Swart, W. J., Donald, D. G. M., & Theron, J. M. (1996). Screening of *Pinus radiata* progenies for resistance to *Sphaeropsis sapinea*. *South African Forestry Journal*, *175*, 15-18.

Taylor, J. W., Geiser, D. M., Burt, A., & Koufopanou, V. (1999). The evolutionary biology and population genetics underlying fungal strain typing. *Clinical Microbiology Reviews*, *12*, 126-146.

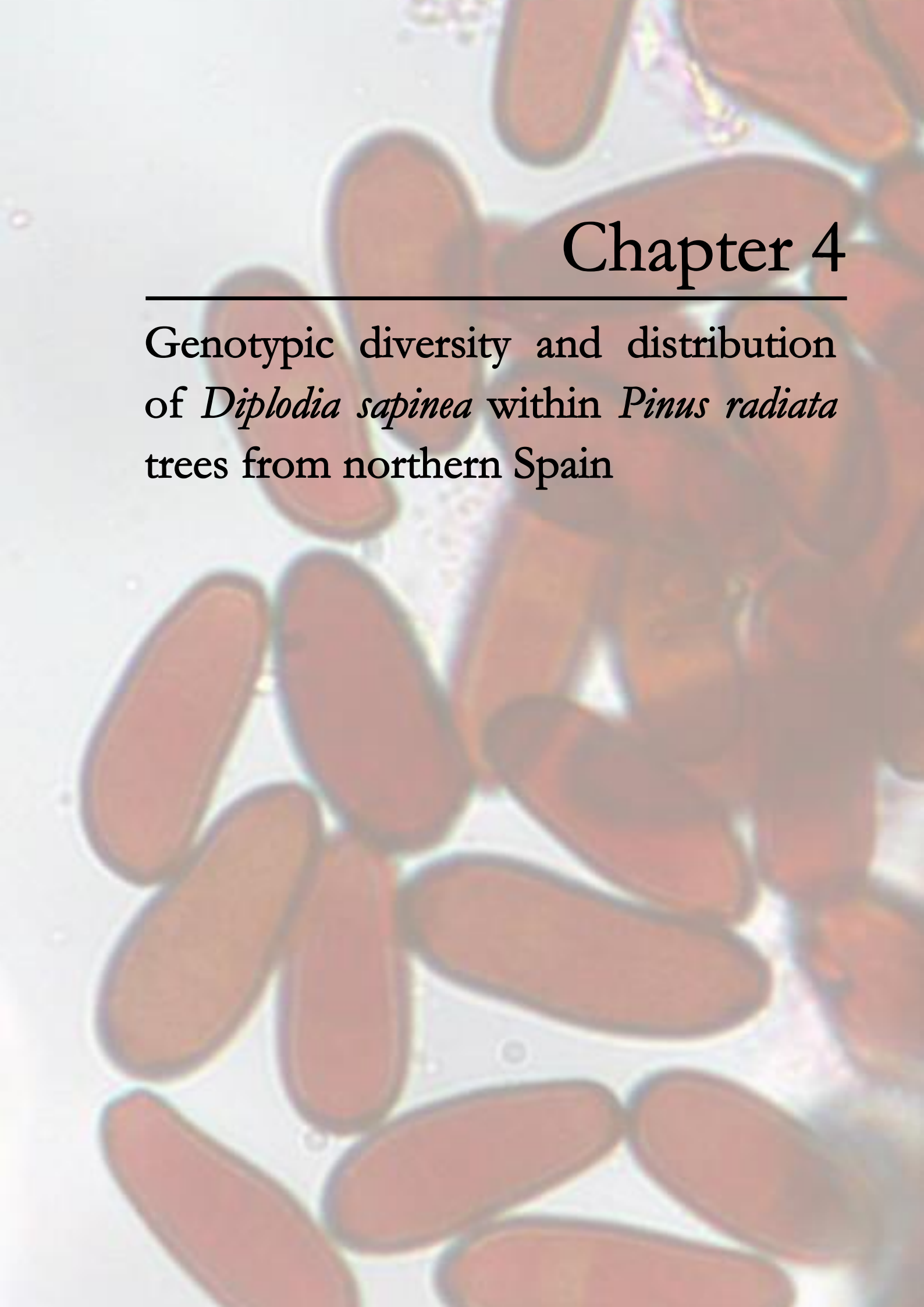
Turgeon, B.G. (1998). Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology* 36, 115-137.

Turgeon, B. G, & Yoder, O. C. (2000). Proposed nomenclature for mating type genes of filamentous ascomycetes. *Fungal Genetics and Biology*, *31*, 1-5.

Wingfield, M. J., & Knox-Davies, P. S. (1980). Association of *Diplodia sapinea* with a root disease of pines in South Africa. *Plant Disease*, *64*, 221-223.

Zhan, J., Kema, G. H. J., Waalwijk, C., & McDonald, B. A. (2002). Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. *Fungal Genetics and Biology*, *36*, 128-136.

Zwolinski, J. B., Swart, W. J., & Wingfield, M. J. (1990). Intensity of dieback induced by *Sphaeropsis sapinea* in relation to site conditions. *European Journal of Forest Pathology*, *20*, 167-174.



Chapter 4

Genotypic diversity and distribution of *Diplodia sapinea* within *Pinus radiata* trees from northern Spain

1. Abstract

Diplodia sapinea is an important latent pathogen of *Pinus* spp., whose outbreak causes considerable impact on plantations. This study considers the population diversity and distribution of *D. sapinea* in northern Spain at different spatial scales from single plantations to a wider area covered by *P. radiata* trees. Estimation of genotypic diversity is an important component of the analysis of the genetic structure of plant pathogen populations. Ten simple sequence repeat (SSR) markers were used, together with vegetative compatibility tests, to study the genetic diversity of *Diplodia sapinea* isolates. Polymorphism analysis at SSR loci is a simple and direct approach for estimating the genetic diversity. From a total of 86 isolates collected from 4 different areas, 14 microsatellite haplotypes and 13 vegetative compatibility groups (VCGs) were identified. The percentage of maximum genotypic diversity, based on Stoddart and Taylor's index, for the northern Spain population's microsatellites ranged from 14.6% to 38.1% and from 8.0% to 29.4% for VCGs. Analysis of these markers and vegetative compatibility groups confirmed that *Diplodia sapinea* reproduces mainly asexually due to its reduced genotypic diversity in spatially close populations. Isolates of *D. sapinea* from northern Spain populations were predominantly monomorphic at the tested SSR loci. VCGs also indicate a low level of genetic variability in these samples, which appear to be clonal.

Keywords: genotypic diversity, microsatellite marker, vegetative compatibility groups, *Pinus radiata*, *Diplodia sapinea*.

2. Introduction

Diplodia sapinea (Fr.) Fuckel is an opportunistic pathogen that causes disease when trees are subjected to biotic or abiotic stress (Zwolinski *et al.*, 1990; Blodgett & Stanosz, 1997). It is responsible of causing important damages in *Pinus radiata* D. Don, which was found out to be one of the most susceptible *Pinus* species in the area of study (Iturritxa *et al.*, 2013). *D. sapinea* causes Diplodia shoot blight and is responsible of several symptoms affecting all tree parts and all stages of development, from seeds to mature trees. The more significant symptoms include: death of new shoots, exudation of resin on needles or stem, decolored needles and necrotic bark (Swart *et al.*, 1987; Blodgett & Stanosz, 1997; Burgess & Wingfield, 2001; Stanosz *et al.*, 2001). Despite, the high genotypic diversity observed

(Burgess *et al.*, 2004b; Bihon *et al.*, 2012a; Bihon *et al.*, 2012b), this fungus has been considered to reproduce mainly asexually.

Conservation of *Pinus radiata* in this area becomes decisive based on its economic importance in northern Spain plantation forestry. The forest area covered up to 55.08% of the Basque Country's total area in 2016 (Hazi Fundazioa 2016). The area occupied by hardwoods exceeds that of the conifers; however, the extent of forest plantations continues to outstrip that of natural forests. Located in the Cantabria slope, *Pinus radiata* is the species that occupies the greatest extent, 123921 ha, 31.2% of the total forest area, and 67.1% of all coniferous forests (Hazi Fundazioa 2016). The Basque Country presents the 47% of the total *P. radiata* plantations in Spain. In this context, important losses have been reported during last decades associated or connected with biotic factors, specially strong winds and hailstorms led the loss of almost 2000 hectares of this tree species in 2006 (Omar, 2012).

An important part of successful disease management involves understanding the genetic diversity of a pathogen (McDonald & McDermott, 1993). In order to determine the diversity of *D. sapinea* population among *P. radiata* plantations in the Basque Country, different sample sets were established. Differences in diversity could be expected depending on the spatial distribution of the collected samples. Isolates from a single plantation could be genetically uniform characterized by asexual reproduction and dispersion into the nearest area. During last decades, *P. radiata* material was introduced from Chile and New Zealand, since material demand could not be covered with Basque Country's production (Michel, 2003). Consequently, isolates collected from a wider range of *P. radiata* plantations could present bigger diversity rates.

Vegetative compatibility groups (VCGs) and microsatellite markers are frequently used techniques to characterize and study genotypic diversity of fungal species. Vegetative compatibility is governed by a number of alleles at vegetative compatibility loci. Compatible isolates are identical at these loci and are represented by the same VCG (Leslie, 1993). Even if VCGs were useful for understanding genetic diversity, it is not possible to consider gene flow and the relative relationships among populations (Glass *et al.*, 2000). Most recently, microsatellite markers have been used to study population genetics (Bihon *et al.*, 2011) because they are highly polymorphic and relatively inexpensive to use once they have been developed (Zane *et al.*, 2002).

The health of a forest plantation depends not only on the resistance of the host population but also on the ability of the pathogen to overcome this resistance. The durability of host resistance, among other factors, is altered by the evolutionary potential of the pathogen (McDonald, 1997; McDonald & Linde, 2002). In this context, a pathogen with a high evolutionary potential is most likely to breakdown host resistance than a pathogen with low evolutionary potential. The structural analysis of fungal populations using microsatellite markers can be used to predict the risk of that pathogen causing epidemics. For asexually reproducing fungi such as *Diplodia sapinea*, high disease risk could be indicated by diverse pathogen populations resulting from high mutation rates, unrestricted gene flow or large population sizes (McDonald & Linde, 2002).

The main objective of this study was to determine the distribution and genetic diversity within the northern Spain *D. sapinea* population and to compare this with the diversity of three populations collected from different plantations. It was hypothesized that isolates from plantation sets would be relatively uniform, typical of an asexual reproduction. Whereas, isolates collected from a wider area among Basque Country, where diverse introductions have occurred, would be more diverse. Ten simple sequence repeat (SSR) markers and VCGs were used in order to assess diversity degree in the studied area.

3. Material and methods

3.1. Fungal collection and isolation

Sampling from symptomatic (die-back) *Pinus radiata* trees was conducted in the major pine-growing regions of the Basque Country (Appendix B). Isolates were obtained from cones where pycnidia in its surface were observable.

Four different sample sets were defined depending on sampling effort. First sample set enclose one sample randomly collected from each of the forty-five plantations of *P. radiata* surveyed in the Basque Country (BC=45). In addition, a more intensive survey was conducted in three plantations of *P. radiata* in Laukiniz (P1=10), Sollano (P2=15) and Hernani (P3=16), located all of them in the Atlantic slope of the Basque Country. For plantations sampling, a symptomatic tree was arbitrary chosen and served as focal point, a unique isolates was collected per tree.

Fragments of scales in which fruiting bodies were observed under a binocular loupe were soaked in 30% commercial bleach (1.6% sodium hypochlorite) for 1 min and rinsed

with sterile water. One single pycnidium from the cone's surface was transferred to water agar medium (agar, bacteriological European type, Panreac) and a single conidium was selected to initiate a monosporic culture. The single conidial isolates were grown on potato dextrose agar (PDA, Panreac) in Petri plates in darkness at 20 ± 3°C for 4 to 6 days. Fungal species were initially identified by colony and conidium morphology (de Wet *et al.*, 2003; Phillips *et al.*, 2013). All isolates are maintained in the Culture Collection of the Forestry Department, Neiker, Granja Modelo Arkaute, Vitoria-Gasteiz, Spain.

3.2. DNA extraction and species identification

The mycelia grown on Petri dishes were scraped off and collected in a 2 ml tube with five sterile tungsten carbide beads (300 µm diameter). The fungal material was disrupted using a Qiagen-Retsch MM300 Tissuelyser at a speed of 30 m/s for 3 minutes at room temperature. In all cases, fungal DNA was extracted from 250-300 mg pure monosporic cultures using a DNA Plant Mini Kit (Analytik Jena AG, Life Science). Extractions were performed following the manufacturer's instructions. The concentration and quality of DNA was estimated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Specific primers, BotR, DpF, and DsF, developed by Smith and Stanosz (2006) were used for the identification of species. The primers were synthetized by Integrated DNA Technologies (Belgium). The PCR mix consisted of the following ingredients: 15 µL H_2O, 2.5 µL PCR buffer, 1 µL (50 mmol L^{-1}) $MgCl_2$, 2.5 µL dNTPs, 2 µL of each specific primer (10 µmol), 0.25 µL Platinum Taq polymerase, and 2 µL DNA template. DNA was previously diluted to 20 µg/ml. The cycling profile was as follows: denaturation at 94°C for 60 s, followed by 35 cycles at 94°C for 30 s, 67°C for 30 s, and 72°C for 30 s, and a final extension at 70°C for 5 min. Fragment sizes were verified on 0.7% agarose gels in Tris-boric acid-EDTA buffer (TBE) with Bioline loading buffer (5x DNA).

3.3. PCR amplification of SSR loci

Ten microsatellite loci (Appendix C), SS1-5-9-10-11 previously described by Burgess *et al.* (2001b) and SS12-14-15-16 described by Bihon *et al.* (2011), were amplified for 86 *D. sapinea* isolates. The 13 µL reaction mixture consisted of 6.5 ml Master Mix (Quiagen), 0.2 µL forward and reverse primers for SS1, SS9, SS11, SS14 and SS15, 0.25 µL forward and reverse primers for SS16, 0.3 µL forward and reverse primers for SS5, SS10, SS12 and SS13, 2.5 µL distilled deionized water and 1.5 µL DNA. The reactions were carried out in an Eppendorf (Hamburg, Germany) thermocycler programmed for an initial denaturation

of 1 min at 95°C, followed by 2 min at 94°C, 15 cycles of 30 s at 58°C, 45 s at 60°C and 1 min at 72°C, and the last three interval 20 cycles of 55°C. Dilution of 1:100 for the thermocycler products was conducted before multiplex analysis in order to avoid detection error.

Ten fluorescently labelled SSR-PCR products were multiplexed and 1 μL of these multiplexed PCR products were separated on an ABI Prism 3500 Genetic Analyser (Applied Biosystems). The amplicon peaks were determined based on the four fluorescent dyes used and the sizes of the DNA fragments. The mobility of SSR products were compared to those of internal size standards (LIZ-500) and allele sizes were estimated by GeneMapper 4.0 computer software (Applied Biosystems). A reference sample was run on every gel to ensure reproducibility. The forward primers were labelled with a phosphoramidite fluorescent dye indicated as FAM, NED, PET and VIC.

3.4. Gene and genotypic diversity

For each isolate, a data matrix of multistate characters (one for each locus) was compiled by assigning a different letter to each allele at each of the five polymorphic loci (e.g., BAACA). The frequency of each allele at each locus for entire populations was calculated, and allele diversity was determined using the program POPGENE (Yeh *et al.*, 1999) and the equation $H = 1 - \sum x_k^2$, where x_k is the frequency of the kth allele (Nei, 1973). A χ^2 test was conducted to determine the differences in allele frequencies. Each genotype was assigned a number, and genotypic diversity (*G*) was estimated (Stoddart & Taylor, 1988) using the equation $G = 1/\sum p_i^2$, where p_i is the observed frequency of the ith phenotype in the population. To compare *G* between populations, the maximum percentage of genotypic diversity ($\hat{G}$ %) was obtained using the equation $\hat{G}=G/N \times 100$, where N is the population size (Chen *et al.*, 1994).

3.5. Molecular variance

The source of variation between and within populations was determined by AMOVA (analysis of molecular variance) tests, using 9999 permutations, with GeneAlEx version 6.2 (Peakall & Smouse, 2006). The null hypothesis was that variation within and between populations is equally responsible for the total genetic diversity in northern Spain isolates.

3.6. Vegetative compatibility groups (VCGs)

To support the population diversity study using SSR markers, VCGs were determined for all Basque Country isolates. Oatmeal (60 g/litre) was steamed in a water bath at 70°C

for 2 h with periodic stirring. The steamed oatmeal was filtered through a double layer of cheesecloth, resulting in approximately 600 ml of oatmeal suspension. Agar (39 g, Panreac) was melted in 400 ml of distilled water and then added to the oatmeal suspension and autoclaved (Smith et al. 2000). Isolates were placed on 6-well cell culture plates (SPL Life Science) containing OMA such that isolates could be paired, approximately 1 cm apart, in all possible combinations, as well as them with themselves as controls. The cultures were incubated at 22°C in the dark for 4-6 days until barrage lines were obvious, indicating incompatibility or different VCGs. After this time, mycelial growth became very dense and the reactions were no longer visible. Compatible isolates merged without the formation of any barrage lines. SAS 9.1.3 service pack 3 (2008) was used to construct dendrograms by simple agglomerative hierarchical clustering method UPGMA (unweighted pair group method with arithmetic mean).

4. Results

4.1. Fungal identification

Specific-identification of a total of 86 isolates was conducted and *D. sapinea* was identified to be causing Diplodia shoot blight fungi in the four studied populations of the Basque Country (Figure 4.1.).

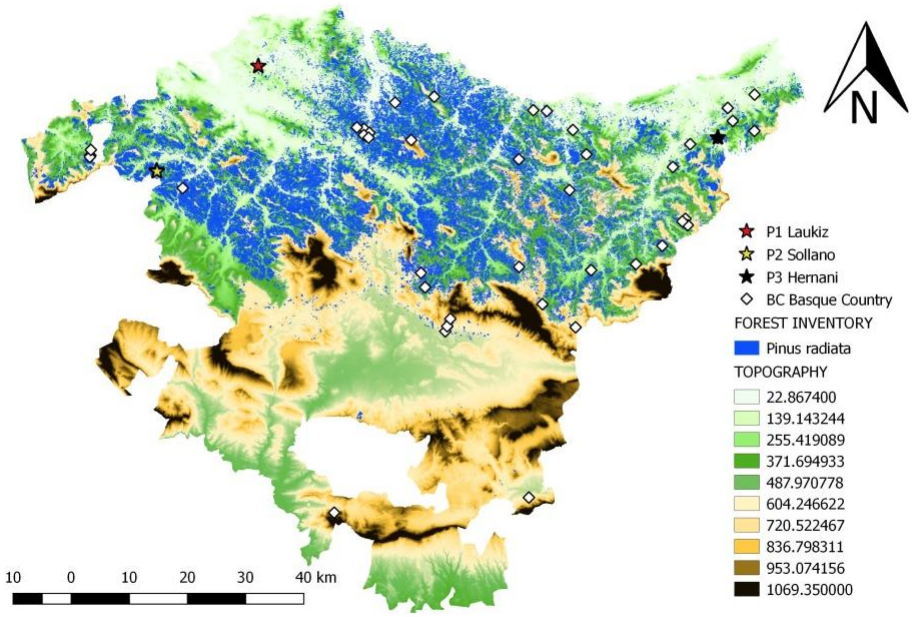


Figure 4.1. Spatial distribution of isolates of *Diplodia sapinea* (BC=45, P1=10, P2=15, P3=16) in Basque Country (Spain) (QGIS. 2.12.2., 2015).

4.2. Gene and genotypic diversity

The 10 SSR markers (Figure 4.2.) produced a total of 17 alleles among the 86 individuals. Of these, 12 where shared by all the populations. There were 16 alleles in the BC population, 13 in the P1, 14 in the P2, and 15 in the P3 (Table 4.1.). The analysed populations were monomorphic at five (BC and P3) and seven (P1 and P2) loci (Table 4.1.). Loci SS1, SS5, SS11, SS13 and SS16 were monomorphic in all isolates. Of the 17 alleles, 12 (70.6%) were present in all four populations. Some alleles were unique to specific populations of *Diplodia sapinea*: at locus SS9, allele 261 was unique to the P2 population and at locus SS14, allele 165 was unique to the BC population (Table 4.1.). The percentage of polymorphic loci within a single population ranged from 11 to 31.25%. The presence of unique alleles and monomorphic loci affects the measure of gene diversity in populations. The mean gene diversity for all 10 loci across all populations of *Diplodia sapinea* was 0.116. The diversity found in northern Spain plantations based on Nei's genetic diversity index ranged from 0.092 to 0.151 per plantation and from 0.000 to 0.495 per locus (Table 4.1.). The populations P1 and P2 had lower gene diversity in comparison to BC and P3 populations.

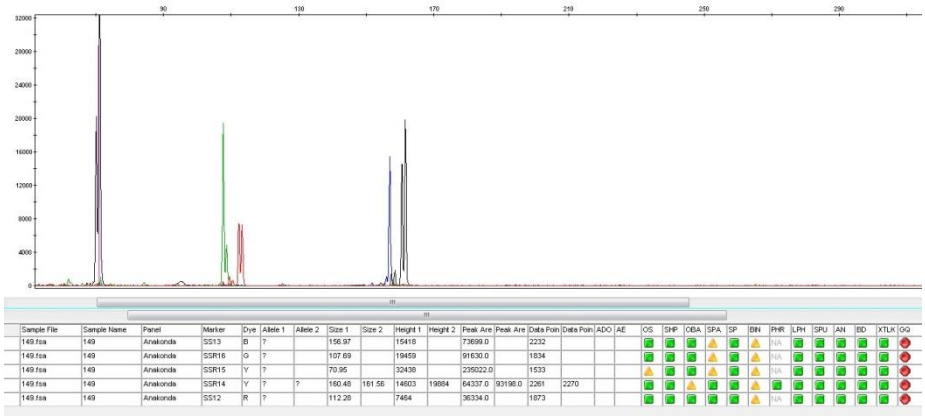


Figure 4.2. Multiplex genotyping of 5 microsatellites for *D. sapinea* in ABI Prism 3500 Genetic Analyser by capillary electrophoresis.

Table 4.1. Allele size (bp) and frequency at 10 loci (SS1, SS5, SS9-16) for *Diplodia sapinea* population collected from northern Spain (BC, P1, P2, P3).

Locus	Allele	BC	P1	P2	P3	H
SS1	407	1.000	1.000	1.000	1.000	0.000
SS5	500	1.000	1.000	1.000	1.000	0.000
SS9	261	…	…	0.067	…	
	263	0.756	0.200	0.600	0.750	
	267	0.244	0.800	0.333	0.250	0.442
SS10	312	0.822	1.000	1.000	0.938	
	314	0.178	…	…	0.063	0.171
SS11	173	1.000	1.000	1.000	1.000	0.000
SS12	112	0.956	0.800	1.000	0.938	
	118	0.044	0.200	…	0.063	0.111
SS13	157	1.000	1.000	1.000	1.000	0.000
SS14	162	0.822	1.000	0.933	0.750	
	165	0.022	…	…	…	
	171	0.156	…	0.067	0.250	0.243
SS15	69	0.511	0.200	0.200	0.625	
	71	0.489	0.800	0.800	0.375	0.495
SS16	108	1.000	1.000	1.000	1.000	0.000
No. Isolates	86	45	10	15	16	
No. Alleles	17	16	13	14	15	
No. Unique Alleles		1	0	1	0	
Polymorphic loci		5	3	3	5	
H		0.151	0.096	0.092	0.128	
SE		0.189	0.155	0.171	0.184	

H (Nei 1973).

In general, the Stoddart and Taylor genotypic diversity index of the *Diplodia sapinea* populations was low: 1.5 for P1, 2.9 for P2, 6.1 for P3 and 6.6 for BC (Table 4.2.). The values of maximum genotypic diversity confirm the low diversity found in the studied area: 14.6% for BC, 14.7% for P1, 19.5% for P2 and 38.1% for P3 (Table 4.2.). Many of the haplotypes of *Diplodia sapinea* were shared across the populations. In fact, the BCO haplotype was shared among the four populations from northern Spain (Table 4.2.). Some haplotypes were not found to be shared among populations: BCYL, BCG-2, BCPI, BCB-2 and BCR were found only at BC, BCW was only found at P2, and BCBR and BCGR were found only at P3 (Table 4.2.).

Table 4.2. *Diplodia sapinea* genotypes as estimated from multilocus profiles generated from the 10 SSR loci. Genotypes were distributed among populations collected from northern Spain (BC, P1, P2, P3).

Haplotype	BC	P1	P2	P3
BCBR				2
BCY	9		1	4
BCYL	7			
BCB	1	2		1
BCO	9	8	5	1
BCO-2	8		7	3
BCP	5		1	3
BCG	2			1
BCG-2	1			
BCPI	1			
BCB-2	1			
BCW			1	
BCGR				1
BCR	1			
N	45	10	15	16
N (g)	11	2	5	8
G	6.6	1.5	2.9	6.1
$\hat{G}$ **(%)**	14.6	14.7	19.5	38.1

N, number of isolates.
N(g), number of genotypes.
G, Genotypic diversity (Stoddart and Taylor 1988).
$\hat{G}$, % maximum genotypic diversity.

For the ten SSR loci, χ^2 tests indicated significant differences ($p<0.05$) in gene diversity between *D. sapinea* populations at three loci, SS9, SS10 and SS15 (Table 4.3.). Gene diversity did not differ significantly among any northern Spain populations, while P2 showed a lower value for the index (Table 4.3.).

Table 4.3. Gene diversity (*H*, Nei 1973) and contingency χ^2 test for differences in allele frequencies for the 10 SSR loci across populations of *Diplodia sapinea*, from all sample sets (BC, P1, P2, P3).

	Gene diversity (*H*)					
Locus	**BC**	**P1**	**P2**	**P3**	χ^2	**df**
SS1	0.000	0.000	0.000	0.000	-	-
SS5	0.000	0.000	0.000	0.000	-	-
SS9	0.375	0.420	0.524	0.375	13.0*	3
SS10	0.268	0.000	0.000	0.117	7.85*	3
SS11	0.000	0.000	0.000	0.000	-	-
SS12	0.087	0.320	0.000	0.117	4.72	3
SS13	0.000	0.000	0.000	0.000	-	-
SS14	0.305	0.000	0.124	0.375	4.36	3
SS15	0.500	0.420	0.320	0.469	9.84*	3
SS16	0.000	0.000	0.000	0.000	-	-
N	45	10	15	16		
Mean	0.154	0.116	0.097	0.145		
SE	0.060	0.060	0.058	0.059		

*Indicate significant ($p<0.05$) χ^2 values.

4.3. Molecular variance

The origin of genetic variation for the studied *Diplodia sapinea* populations was determined using AMOVA. The results indicated that only 6% was due to variation between populations and 94% was due to variation within each population (Table 4.4.).

Table 4.4. AMOVA of northern Spain *D. sapinea* populations hierarchically partitioned.

Source of variation	*df*	SS	MS	Est. Var.	%Variation
Among populations	3	102.673	34.224	0.968	6
Within populations	82	1.332.967	16.256	16.256	94
Total	85	1.435.640		17.224	100

df=Degree of freedom, SS=Sum of square, MS=Mean square, and Est. Var.=Estimated variance.

4.4. Vegetative compatibility groups

Genotypic differentiation was also estimated by VCGs (Figure 4.3.). Results for all isolates combination were determined and a letter was assigned for each pair reaction, I for isolates pair where no compatibility was detected and C for compatible pairs. A pairwise matrix was constructed with data obtained from VCG analysis. UPGMA method was performed and hierarchical clusters were formed for each studied population. The distance between clusters is taken to be the mean distance between elements of each cluster. The 86 *D. sapinea* isolates from the sampled trees were represented by 13 different VCGs. The 45 isolates obtained from the Basque Country sample set were represented by 13 different VCGs (Figure 4.4.). The 10 isolates obtained from P1 (Laukiniz), the 15 isolates obtained from P2 (Sollano) and the 16 isolates obtained from P3 (Hernani) were represented by two (Figure 4.5.), five (Figure 4.6.) and two (Figure 4.7.) VCGs, respectively. There was a clear difference between the number of VCGs identified in the BC sample set and the plantations (P1, P2, and P3). For P1, P2, and P3, the number of different VCGs was quite reduced, with the isolates showing higher compatibility levels. The 13 different VCGs identified in BC indicated that those isolates had lower compatibility levels and consequently are more diverse than the ones obtained from plantations.

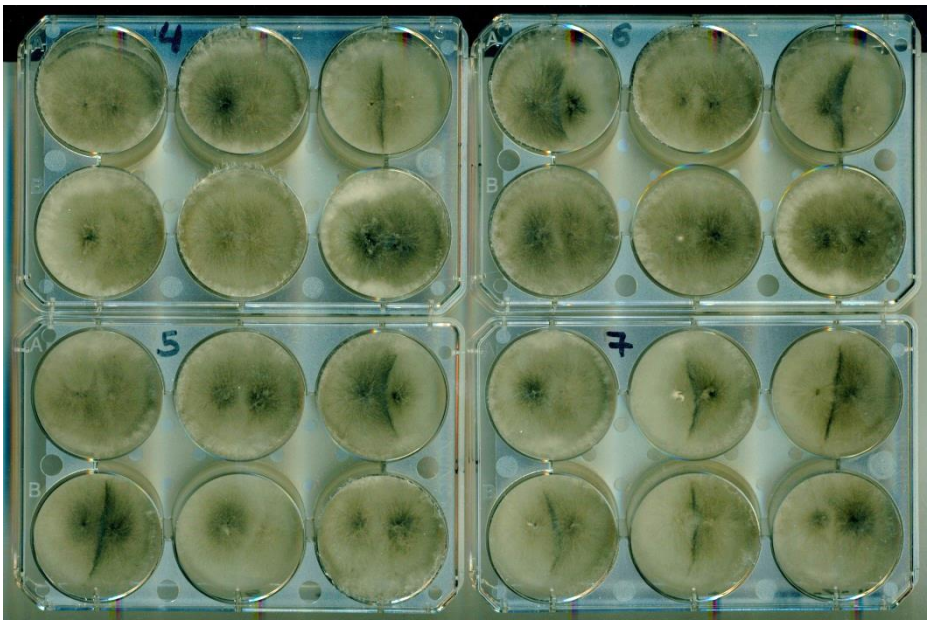


Figure 4.3. Different *D. sapinea* isolates placed together on oats media. Those in which a line is observed are considered incompatible isolates whereas, those where a barrier is not appreciated are compatible.

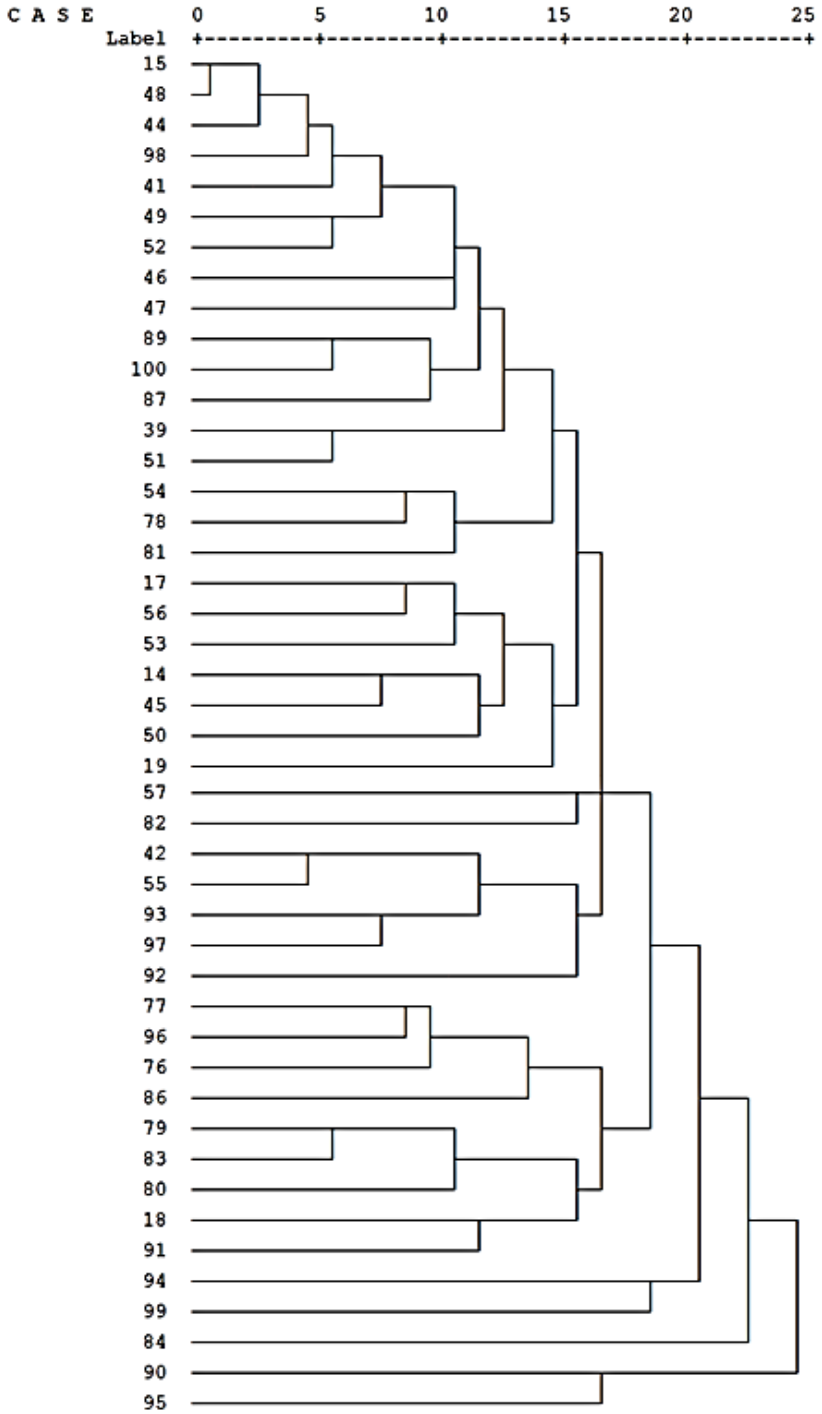


Figure 4.4. Phylogenetic tree showing the relatedness of BC isolates using UPGMA.

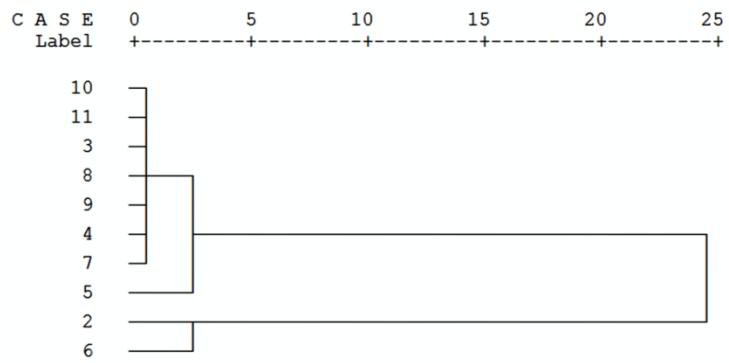


Figure 4.5. Phylogenetic tree showing the relatedness of P1 isolates using UPGMA.

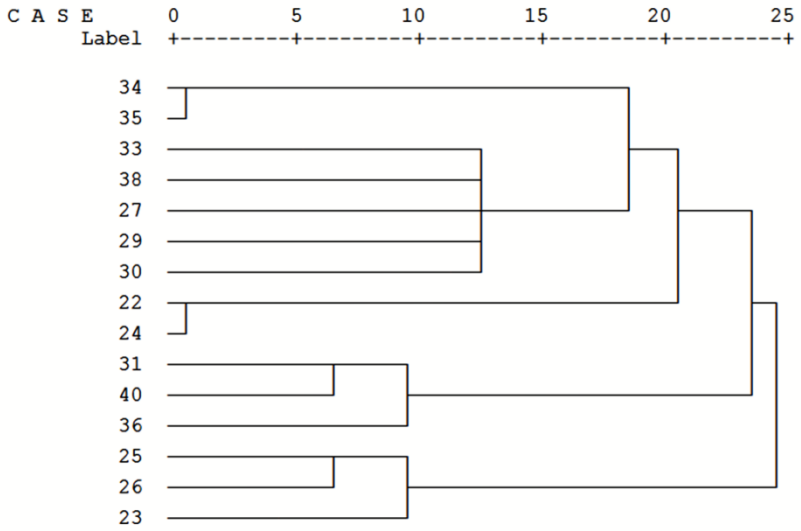


Figure 4.6. Phylogenetic tree showing the relatedness of P2 isolates using UPGMA.

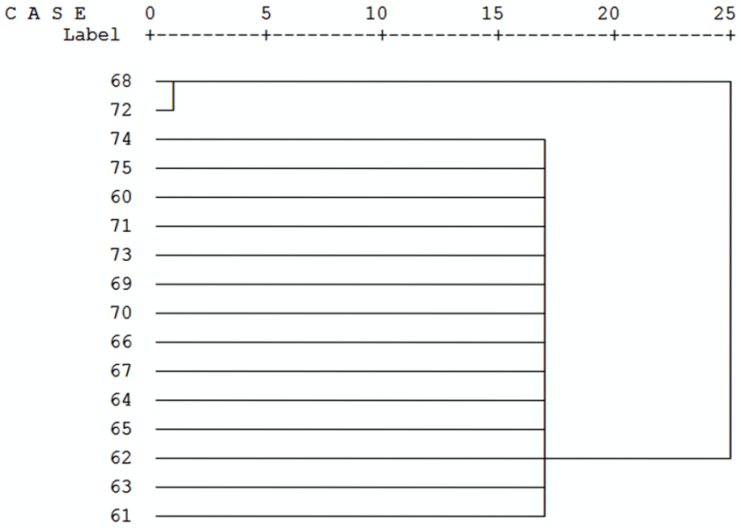


Figure 4.7. Phylogenetic tree showing the relatedness of P3 isolates using UPGMA.

The percentage of maximum genotypic diversity for the northern Spain sampled populations was 8% for P3, 14.7% for P1, 18.7% for BC and 29.4% for P2 (Table 4.5.). However, according to genotypic diversity, BC showed higher levels with a value of 8.4. P3 had a value of 1.3, P1 of 1.5 and P2 of 4.4 (Table 4.5.). All of the populations showed a high degree of clonality, with an observable dominant VCG.

Table 4.5. *Diplodia sapinea* genotypes as estimated from VCGs. Genotypes were distributed among populations collected from northern Spain (BC, P1, P2, P3).

VCG	BC	P1	P2	P3
1	7	8	2	2
2	1	2	5	14
3	1		2	
4	3		3	
5	2		3	
6	3			
7	7			
8	2			
9	5			
10	9			
11	2			
12	1			
13	2			
N	45	10	15	16
N (g)	13	2	5	1
G	8.4	1.5	4.4	1.3
$\hat{G}$ **(%)**	18.7	14.7	29.4	8.0

N, number of isolates.
N(g), number of VCGs.
G, Genotypic diversity (Stoddart and Taylor 1988).
$\hat{G}$, % maximum genotypic diversity.

5. Discussion

The analysis by SSR markers and VCGs allowed to determine gene and genotypic diversity among spatially separated populations of *D. sapinea* in northern Spain. The results revealed low levels of genetic and genotypic diversity and little population differentiation based on both microsatellite markers and VCGs. Nei's (1973) mean gene diversity (*H*) ranged from 0.097 to 0.154, and genotypic diversity ranged from 1.5 to 6.6 for both microsatellite markers and VCGs. Overall, there were almost same number of VCGs and microsatellite haplotypes for the 86 evaluated isolates, which show that both methods are equally valid for diversity studies. As expected, the more diverse population was BC where

11 and 13 different haplotypes were found respectively for microsatellite and VCG tests. BC samples were obtained from a wide range of *P. radiata* plantations, whereas P1, P2 and P3 samples belongs to three different plantations. The limited diversity of this pathogen in the examined collections and the lack of unique alleles suggest that asexual reproduction is more common in this area. Few genotypes at higher frequencies and a high degree of clonality are expected in populations that reproduce asexually (Chen & McDonald, 1996; Kohli & Kohn, 1998). Thus, there is little evidence of sexual stage reproduction in this region as a mechanism of genetic drift.

Study of genotypic diversity developed by Smith *et al.* (2000) where samples from Europe were analysed, show lower rates than those obtained in this study, 5.2%, whereas in Basque Country the maximum genotypic diversity values range from 8% to 38.1% for both microsatellite and VCG analysis. The higher diversity observed in northern Spain population could be explained by the fact that, the seed were mainly introduced from New Zealand and Chile due to the lack of available material for *P. radiata* silviculture system (Michel, 2003). Studies developed by Neiker's Diagnosis Laboratory, in which *D. sapinea* was successfully isolated from numerous introduced seeds, proved to act as vector of the disease (Iturritxa *et al.*, 2008). Founder effects could also affect the diversity of the populations since it is an introduced pathogen that is colonizing a new host. Furthermore, the absence of significant population differentiation and similar allelic frequencies are expected in spatially close populations (Slatkin, 1987; Linde *et al.*, 2002).

According to other studies, higher maximum genotypic diversity levels for microsatellite markers have been reported for this fungus in their native distribution area: 12-40% in California and 90% in Mexico (Burgess *et al.*, 2004a). It is generally accepted that the diversity of an introduced population will be lower than the diversity of an indigenous population (Burgess *et al.*, 2001a). However, high diversity was found in isolates obtained from asymptomatic trees out of the native area of distribution: 1.6-86.2% in South Africa (Burgess *et al.*, 2001b, Bihon *et al.*, 2011), and 5.2-36.8% in different southern hemisphere populations (Burgess *et al.*, 2004b). It has been observed that levels of diversity from asymptomatic trees are expected to be higher than those from diseased trees (Bihon *et al.*, 2011). Although the host does not show any lesions, endophytes are present and are able to cause disease. Once wounds occur, the population becomes more uniform, as the infections are mainly caused by asexual conidia.

According to Burgess *et al.* (2004b), the presence of the most ecologically fit genotypes in populations of *D. sapinea* could explain the predominance of certain genotypes and alleles over others. The low genetic diversity suggests that only a small number of genotypes represent genetically fit isolates from northern Spain. This could also be related to the similarity in climate, topography, environment and plantation productivity, which defines a similar selective pressure throughout the region. Thus, the low level of genetic diversity in *D. sapinea* populations could be explained by consistent selection pressures and prolonged asexual reproduction. Trees affected by pathogen populations with low genetic diversity are likely to become resistant over time (McDonald & Linde, 2002). Furthermore, the probability of having an outbreak with a more pathogenic strain is lower.

This study showed a low allele and genotypic diversity with little variation across populations, based on both microsatellite markers and VCG, in symptomatic populations of *D. sapinea* in northern Spain, but a higher level in comparison to previous studies where Europe's population was analysed. Isolates with the highest levels of genotypic diversity were obtained in the BC population, while the maximum genotypic diversity was found in P3 for microsatellite haplotypes and in P2 for VCGs.

6. References

Bihon, W., Slippers, B., Burgess, T., Wingfield, M. J., & Wingfield, B. D. (2012a). Diverse sources of infection and cryptic recombination revealed in South African *Diplodia sapinea* populations. *Fungal Biology*, *116*, 112–120.

Bihon, W., Burgess, T., Slippers, B., Wingfield, M. J., & Wingfield, B. D. (2012b). High levels of genetic diversity and cryptic recombination is widespread in introduced *Diplodia sapinea* populations. *Australasian Plant Pathology*, *41*, 41–46.

Bihon, W., Burgess, T., Slippers, B., Wingfield, M. J., & Wingfield, B. D. (2011). Distribution of *Diplodia pinea* and its genotypic diversity within asymtomatic *Pinus patula* trees. *Autralasian Plant Pathology*, *40*, 540-548.

Blodgett, J. T., & Stanosz, G. R. (1997). *Sphaeropsis sapinea* morphotypes differ in aggressiveness but both infect non wounded red or jack pines. *Plant Disease*, *81*, 143-147.

Burgess, T., & Wingfield, M. J. (2001). Exotic pine forestry in the southern hemisphere: A brief history of establishment and quarantine practices. *South African Forestry Journal*, *192*, 79-84.

Burgess, T. I., Gordon T. R., Wingfield, M. J., & Wingfield, B. D. (2004a). Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. *Mycological Research*, *108*, 1399-1406.

Burgess, T. I., Wingfield, M. J., & Wingfield, B. D. (2004b). Global distribution of *Diplodia sapinea* genotypes revealed using simple sequence repeat (SSR) markers. *Australasian Plant Pathology*, *33*, 513–519.

Burgess, T., Wingfield, B. W., & Wingfield, M. J. (2001a). Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*. *Mycological Research*, *105*, 1331-1339.

Burgess, T., Wingfield, M. J., & Wingfield, B. W. (2001b). Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and environmental microbiology*, *67*, 354-362.

Chen, R. S., Boeger, J. M., & McDonald, B. A. (1994). Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology*, *3*, 209-218.

Chen, R. S., & McDonald, B. A. (1996). Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. *Genetics*, *142*, 1119-1127.

de Wet, J., Burgess, T., Slippers, B., Preisig, O., Wingfield, B. D., & Wingfield, M. J. (2003). Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. *Mycological Research*, *107*, 557–566.

Glass, N. L., Jacobson, D. J., & Shiu, P. K. T. (2000). The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. *Annual Review of Genetics*, *34*, 165-168.

Hazi Fundazioa (2016). El bosque vasco en cifras 2017. Retrieved from http://www.euskadi.eus/contenidos/informacion/inventario_forestal_2016/es_agripes/adjuntos/El%20bosque%20vasco%20en%20cifras%202017.pdf. Accessed 9 August 2018.

Iturritxa, E., García-Serna, I., Mesanza, N. (2008). *Diplodia pinea* in forest plantations of *Pinus radiata* in northern Spain: Diversity and origin of the Spanish population. *Journal of Plant Pathology*, *90*, 366.

Iturritxa, E., Ganley, R. J., Raposo, R., García-Serna, I., Mesanza, N., Kirkpatrick, S. C., & Gordon, T.R. (2013). Resistance levels of Spanish conifers against *Fusariun circinatum* and *Diplodia sapinea*. *Forest pathology*. *43*, 488-495.

Kohli, Y., & Kohn, L. M. (1998). Random association among alleles in clonal populations of *Sclerotinia sclerotiorum*. *Fungal Genetics and Biology*, *23*, 139-149.

Leslie, J. F. (1993). Fungal vegetative compatibility. *Annual Review of Phytopathology*, *31*, 127-150.

Linde, C., Zhan, J., & McDonald, B. A. (2002). Population structure of *Mycosphaerella graminicola:* from lesions to continents. *Phytopathology*, *92*, 946-955.

McDonald, B. A. (1997). The population genetics of fungi: tools and techniques. *Phytopathology*, *87*, 448-453.

McDonald, B. A. & Linde, C. (2002). Pathogen population genetics, evolutionary potential and durable resistance. *Annual Review of Phytopathology*, *40*, 349-379.

McDonald, B. A. & McDermott, J. M. (1993). Population genetics of plant pathogenic fungi: electrophoretic markers give unprecedented precision to analyses of genetic structure of populations. *BioScience*, *43*, 311-319.

Michel, M. (2003). *El pino radiata (Pinus radiata D. Don) en la historia forestal de la Comunidad Autónoma del País Vasco: análisis de un proceso de forestalismo intensivo.* Madrid, España: Universidad Politécnica de Madrid, PhD thesis.

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA*, *70*, 3321-3323.

Omar, A. (2012). *Aspectos más relevantes de la sanidad forestal en Vizcaya/Bizkaia*. *Foresta*, *5*, 174-179. Retrieved from http://www.redforesta.com/wp-content/uploads/2012/11/GF22-Aspectos-mas-relevantes-de-la-sanidad-forestal-en-Vizcaya.pdf

Peakall, R. & Smouse, P.E. (2006). GENALEX 6: genetic analysis in excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, *6*, 288-295.

Phillips, A. J. L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M. J., Groenewald, J. Z., & Crous, P. W. (2013). The Botryosphaeriaceae: genera and species known from culture. *Studies in Mycology*, *76*, 51–167.

QGIS. Release 2.12.2. (2015). QGIS Development Team.

SAS Institute Inc. (2008). SAS 9.1.3. Service Pack 3. Cary, NC: SAS Institute Inc.

Slatkin, M. (1987). Gene flow and geographic structure of natural populations. *Science*, *236*, 787-792.

Smith, D. R., & Stanosz, G.R. (2006). A species- specific PCR assay for detetion of *Dipldia pinea* and *D. scrobiculata* in dead red and jack pines with collar rot symptoms. *Plant Disease*, *90*, 307-313.

Smith, H., Wingfield, M. J., de Wet, J., & Coutinho, T. A. (2000). Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. *Plant disease*, *84*, 139-142.

Stanosz, G. R., Blodgett, J. T., & Kruger, E. L. (2001). Water stress and *Shaeropsis sapinea* as a latent pathogen of red pine seedlings. *New Phytologist*, *149*, 531-538.

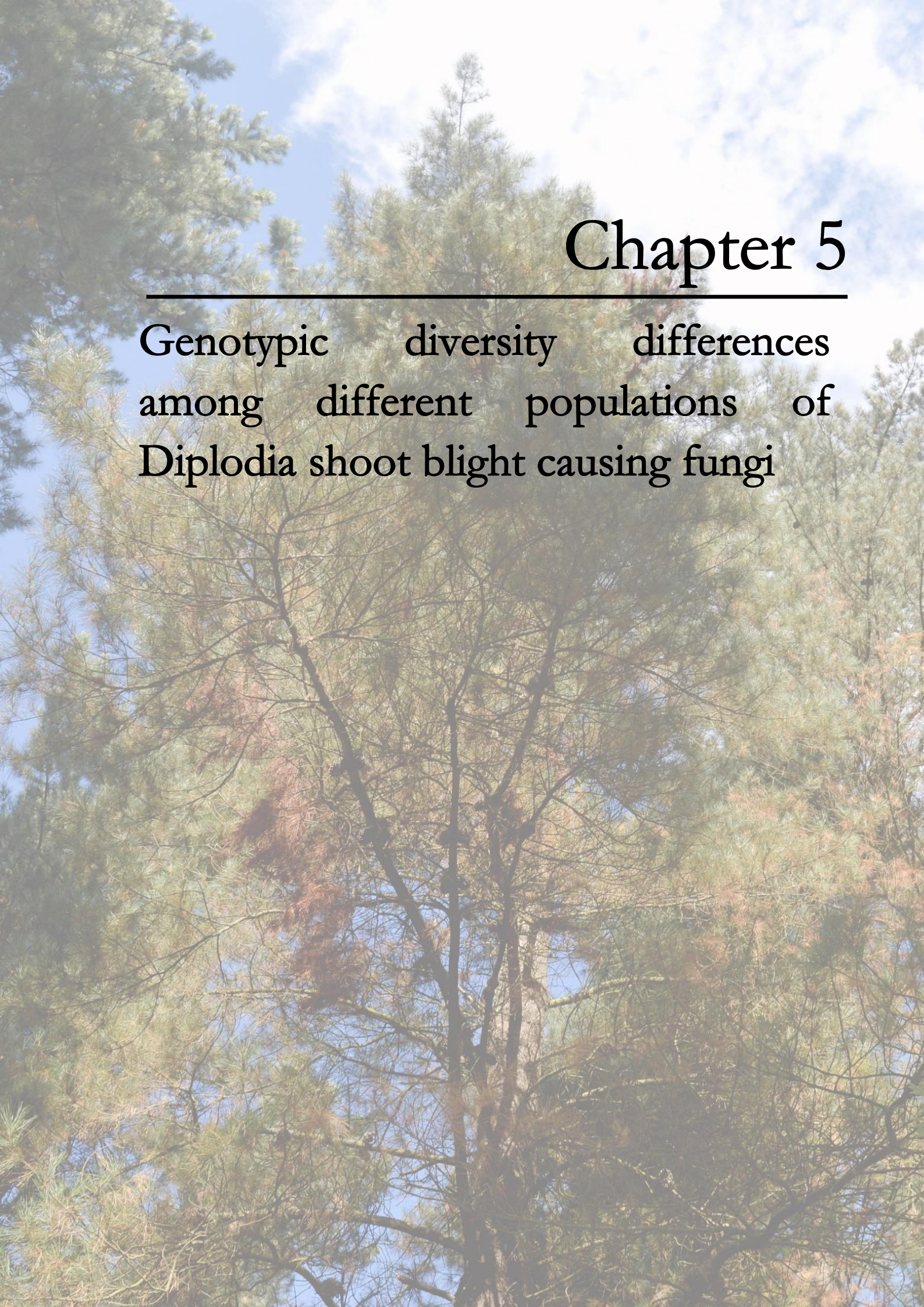
Stoddart, J. A. & Taylor, J. F. (1988). Genotypic diversity: estimation and prediction in samples. *Genetics*, *118*, 705-711.

Swart, W. J., Wingfield, M. J., & Knox-Davies, P. S. (1987). Factor associated with *Sphaeropsis sapinea* infection of pine trees in South Africa. *Phytophylactica*, *19*, 505-510.

Yeh, F. C., Yang, R. C., & Boyle, T. (1999). POPGENE Version 1.32. Microsoft Windows Based Freeware for Population Genetic Analysis, Alberta.

Zane, L., Bargelloni, L., & Patarnello, T. (2002). Strategies for microsatellite isolation: a review. *Molecular Ecology*, *11*, 1-16.

Zwolinski, J. B., Swart, W. J., & Wingfield, M. J. (1990). Intensity of dieback induced by *Sphaeropsis sapinea* in relation to site conditions. *European Journal of Forest Pathology*, *20*, 167–174.



Chapter 5

Genotypic diversity differences among different populations of Diplodia shoot blight causing fungi

1. Abstract

Diplodia sapinea and *Diplodia scrobiculata* endophytic pathogens are the responsible of causing Diplodia shoot blight fungal disease in worldwide plantations. Under biotic or abiotic stress conditions these fungi produce shoot-blight, cankers and seed mortality specially affecting *Pinus* species. In this context, the genotypic diversity of five populations was assessed and compared using ten Simple Sequence Repeat (SSR) markers. The five defined populations were: 2 from northern Spain (BC1 and BC2), 2 from United States of America (USA1 and USA2) and 1 that includes worldwide samples (W). From a total of 241 isolates collected, 79 microsatellite haplotypes were identified, 55 for *D. sapinea* and 24 for *D. scrobiculata*. This study reveals the presence of one haplotype shared in the five *D. sapinea* populations, other 3 haplotypes shared between BC and USA sample sets, and 6 haplotypes between BC and W sets, which may suggest a common source. Maximum genotypic diversity values for the studied populations based on the Sttodart and Taylor's index, ranged from 11.42% (USA1) to 48.15% (W) for *D. sapinea* and from 51.85% (USA2) to 100% (BC1 and W) for *D. scrobiculata*. Analysis of these markers show that *D. scrobiculata* isolates have higher levels of genotypic diversity in comparison to *D. sapinea*. Whereas *D. sapinea* isolates were predominately monomorphic at several SSR loci, *D. scrobiculata* was found to be more diverse. However, the absence of monomorphic SSR loci in USA sample sets, could reflect the randomly association of alleles in those populations. Even if these fungi have been considered to reproduce asexually, these results suggest that recombination is occurring with *D. sapinea* and *D. scrobiculata*.

Keywords: population diversity, SSR markers, microsatellite haplotypes, *Diplodia sapinea*, *Diplodia scrobiculata*.

2. Introduction

Diplodia shoot blight is an important fungal disease caused predominately by *Diplodia sapinea* (Fr.) Fuckel and *Diplodia scrobiculata* de Wet, B. Slippers & M. J. Wingfield pathogens. These endophytes are associated with symptomless infections, but a great variety of abiotic and biotic factors, such as drought, mechanical wounding or hail (Purnell, 1957; Chou, 1987) have been reported to predispose specially *Pinus* species to this disease. This endophytic behaviour could have led this pathogens to be widespread since it is occurring in conifers worldwide (Eldridge, 1961; Swart & Wingfield, 1991).

Pinus radiata D. Don is considered one of the most susceptible species to *D. sapinea* and *D. scrobiculata* pathogens. It is native to California and two islands in the Gulf of Mexico, where less than 5300 ha of natural forest remain (Burdon, 2000). In this area, *P. radiata* and *D. scrobiculata* coexist and it is not considered an important pathogen (Burgess *et al*., 2001a). Unlike in its natural distribution area, important losses caused by these pathogens have been reported in exotic plantations in many countries as Australia, New Zealand and South Africa (Iturritxa *et al*, 2013; Burgess *et al*., 2004a). In United States of America *Diplodia sapinea* has been also detected in both red (*Pinus resinosa* Ait.) and jack pine (*Pinus banksiana* L.), while *D. scrobiculata* has been detected from jack pine more frequently (Palmer, 1991; Smith & Stanosz, 2006).

Although in its natural environment, *P. radiata* does not stand out for its high growth rates, in exotic areas it is really successful and becomes an important species in worldwide forestry with harvest ages of 25-35 years. In Spain the mayor plantations are located in northern area, and especially in the Basque Country where the 31.2% or 123921 ha are occupied by this forest species (Inventario Forestal CAE, 2016).

In the north-central United States red pine is an economically important species and it is often grown and managed in plantations. These trees are thinned several times before final harvest at ages of approximately 55-80 years (Buckman, 2006). Red pine also can be maintained for 200 or more years, and plays many ecological roles in forest ecosystems throughout the region (Benzie, 1977). *P. resinosa* provides habitat for a diverse range of bird species and cover for other wildlife (Atwell *et al*., 2008).

Significant loses have been reported in these regions associated to hailstorm, strong winds and drought at the beginning of summer time, where wounds caused on branches and leaves serve as a gateway to the pathogen. The most notable damages are the death of guides and dieback of branches; however, it could also cause death of trees in plantations. All this entails the reduction of the total volume produced, a loss of wood quality and finally a decrease of economic return.

Damages in *P. radiata* plantations as consequence of the action of these fungi are quite common. In the Basque Country in 1999, 2003 and 2006, hailstorms and strong winds affected an extensive area, which forced to perform cuts and clears in 1800 ha in 2006 with different degree of affection (Omar, 2012). Other authors as Foster and Marks (1968) and Chou (2007) have reported losses up 40% due to the die of tips in Australia and New

Zealand. Zwolinski *et al.* (1990) also estimated losses of up to 55% in plantations following hail damage in South Africa.

In north-central United States, significant damage to red pine including shoot blight, collar rot, cankers, and mortality due to *Diplodia* species has been documented as consequence of hailstorm and drought (Palmer *et al.*, 1988, Nicholls & Ostry, 1990, Stanosz & Cummings Carlson, 1996, Blodgett *et al.*, 1997).

In this context, understanding the genetic diversity of a pathogen has always been considered as an important step for a successful disease management (McDonald & McDermott, 1993). Microsatellite markers are recognised to be a robust method to study fungi genetic diversity (Zane *et al.*, 2002). *D. sapinea* and *D. scrobiculata* have been regarded to reproduce exclusively asexually (Sutton, 1980), which results in clonal lineages within a population (McDonald, 1997). However, microsatellite analysis showed higher allelic diversity in population of *D. scrobiculata* than in populations of *D. sapinea*, where dominant and monomorphic alleles are quite common (Burgess *et al.*, 2004b). Nevertheless, previous studies of *D. sapinea* at different scale levels revealed high level of genotypic diversity and presence of unique alleles in populations where it has been introduced (Smith *et al.*, 2000; Burgess *et al.*, 2001a; Burgess *et al.*, 2004a,b; Bihon *et al.*, 2011).

Evidence of significant differences on diversity levels among introduced and native fungal populations as well as the need to characterize the introduction ways of this pathogen prompted the present study. The aims of this research were to determine the diversity of these pathogenic fungi and to identify common microsatellite haplotypes in five populations collected from different countries. This fact would let to assess if there has been an exchange of material among the studied populations and, therefore, how these pathogens could have been introduced. The diversity of 241 isolates was assessed using ten microsatellite markers developed by Burgess *et al.* (2001b) and Bihon *et al.* (2011).

3. Material and methods

3.1. Sample collection and isolation

241 samples were studied from five different sample sets (Appendix D). The first, a Basque Country set of isolates (BC1, n=27) was obtained from a survey conducted in three *P. radiata* plantations in Laukiniz, Oiartzun and Luiaondo, located all of them in the Cantabrian fringe in the northern Spain. Samples were taken from trunks, roots and pitch

cankers of symptomatic trees. The second set, includes samples obtained mainly from cones of *P. radiata* symptomatic trees in the Basque Country. However, samples from trunk and from different hosts as *Pinus contorta* Douglas ex Louden, *Pinus sylvestris* L. and *Pinus halepensis* Mill. were also included (BC2, n=63). Set 3 includes samples isolated from cones of symptomatic trees of *P. resinosa* from Washington, Vermont and Maine (United States of America) (USA1, n=50). Isolates included in the fourth set were obtained from cones, needle, bark, twigs, seeds and trunk from 14 different *Pinus* species, but mainly from *P. resinosa*, *P. bankisiana* and *P. radiata*, and collected from a wider region among United States (USA2, n=85). Finally, set 5 includes isolates from different countries; México, Madagascar, Tanzania, Israel, Canada, France, New Zealand, United Kingdom, Turkey and Honduras (W, n=16). Samples were collected from 12 different *Pinus*, *Cedrus* and *Picea* species. All 241 isolates are maintained in the Culture Collection of the Forestry Department, Neiker, Granja Modelo Arkaute, Vitoria-Gasteiz, Spain.

Fragments of collected samples where pycnidia were observable were soaked in 30% commercial bleach (1.6% sodium hypochlorite) for 1 min and rinsed with sterile water. Under binocular loupe one single pycnidia was transferred to water agar medium (bacteriological European type agar, Panreac) and a single conidium was selected. Monosporic cultures were grown on potato dextrose agar (PDA, Panreac) in Petri plates in darkness at 20 ± 3ºC for 4 to 6 days. Fungal isolates were initially identified by colony and conidium morphology based on de Wet *et al.* (2003) and Phillips *et al.* (2013) descriptions.

3.2. DNA extractions and species identification

Mycelia from monosporic cultures grown on PDA was scrapped from plate's surface and collected in a 2 ml Eppendorf tube with five sterile tungsten carbide beads (300 µm diameter). Qiagen-Retsch MM300 Tissuelyser at a speed of 30 m/s for 3 minutes at room temperature was used to disrupt the fungal material. DNA extractions were obtained from 250-300 mg pure monosporic cultures using a DNA Plant Mini Kit (Analytik Jena AG, Life Science) and following the manufacturer's instructions. The concentration and quality of DNA was estimated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Identification of species was developed using specific primers, BotR, DpF and DsF (Smith & Stanosz, 2006). The primers were synthetized by Integrated DNA Technologies (Belgium). The PCR mix consisted of the following ingredients: 15 µL H2O, 2.5 µL PCR buffer, 1 µL (50 mmol L^{-1}) $MgCl_2$, 2.5 µL dNTPs, 2 µL of each specific primer (10 µmol),

0.25 μL Platinum Taq polymerase, and 2 μL DNA template. DNA was previously diluted to 20 μg/ml. The cycling profile was as follows: denaturation at 94°C for 60 s, followed by 35 cycles at 94°C for 30 s, 67°C for 30 s, and 72°C for 30 s, and a final extension at 70°C for 5 min. Fragment sizes were verified on 0.7% agarose gels in Tris-boric acid-EDTA buffer (TBE) with Bioline loading buffer (5x DNA).

3.3. PCR amplification and separation of SSR loci

SSR analysis was performed on 241 isolates with five of the 12 fluorescent-labelled markers, SS1, SS5, SS9, SS10, SS11 described previously by Burgess *et al.* (2001b) and other five of the 16 fluorescent-labelled markers, SS12, SS14, SS15, SS16 developed by Bihon *et al.* (2011) (Appendix C). The 13 μL reaction mixture consisted of 6.5 ml Master Mix (Quiagen), 0.2 μL forward and reverse primers for SS1, SS9, SS11, SS14 and SS15, 0.25 μL forward and reverse primers for SS16, 0.3 μL forward and reverse primers for SS5, SS10, SS12 and SS13, 2.5 μL distilled deionized water and 1.5 μL DNA.

The reactions were carried out in an Eppendorf (Hamburg, Germany) thermocycler programmed for an initial denaturation of 1 min at 95°C, followed by 2 min at 94°C, 15 cycles of 30 s at 58°C, 45 s at 60°C and 1 min at 72°C, and the last three interval 20 cycles of 55°C. Dilution of 1:100 for the thermocycler products was conducted in ultra-pure DEPC treated water (Invitrogen). 1 μL of this dilution was combined with 10 μl of a suspension of Genescan 500 LIZ-labelled size standard (Applied Biosystems, Life Technologies) and formamide (Sigma-Aldrich). For formamide denaturalization a PCR was run and thermocycler was programed at 95ºC for 5 minutes.

After cooling the fluorescent-labelled SSR products were separated on an ABI Prism 3500 Genetic Analyser (Applied Biosystems). Allele size was estimated by GeneMapper 4.0 computer software (Applied Biosystems) and the mobility was compared to those of internal size standards (LIZ-500). A reference sample for *D. sapinea* and *D. scrobiculata* was run on every analysis to ensure reproducibility. The forward primers were labelled with a phosphoramidite fluorescent dye indicated as FAM, NED, PET and VIC.

3.4. Population genetic analyses

For each of the loci, individual alleles were assigned a different letter. Loci where no amplification was observable were considered as null alleles and were treated as missing data. For each of the 241 isolates a data matrix of ten multistate characters (one for each of the locus) was gathered by assigning each allele at each of the polymorphic loci, a different

letter (e. g., CCECACBEAB). POPGEN (Yeh, et al., 1999) program and the equation $H = 1 - \sum x_k^2$, where x_k is the frequency of the kth allele (Nei, 1973) were used to determine gene diversity of sample sets. This method analyses the frequency of each allele at each locus.

Genotypic diversity (*G*) was estimated by Stoddart and Taylor (1988) equation $G = 1/\sum p_i^2$, where p_i is the observed frequency of the ith phenotype in the population. The maximum percentage of genotypic diversity ($\hat{G}$%) obtained using the equation $\hat{G}$=*G*/N x 100, where N is the population size (Chen *et al.*, 1994), was used to compare genotypic diversity between populations.

Differences detected in allele frequencies were determined by χ^2 test. UPGMA dendrogram based on Nei (1978) was constructed to determine the genetic distance among the studied populations for both *Diplodia* species.

3.5. Analysis of molecular variance (AMOVA)

AMOVA test, using 99999 permutations was conducted to determine the difference sources of genetic variation between and within populations, using the software GeneAlEx version 6.2 (Peakall & Smouse, 2006). The null-hypothesis for studied sample sets was that variation within populations and between populations is equally responsible for the total genetic diversity of *D. sapinea* and *D. scrobiculata* strains.

4. Results

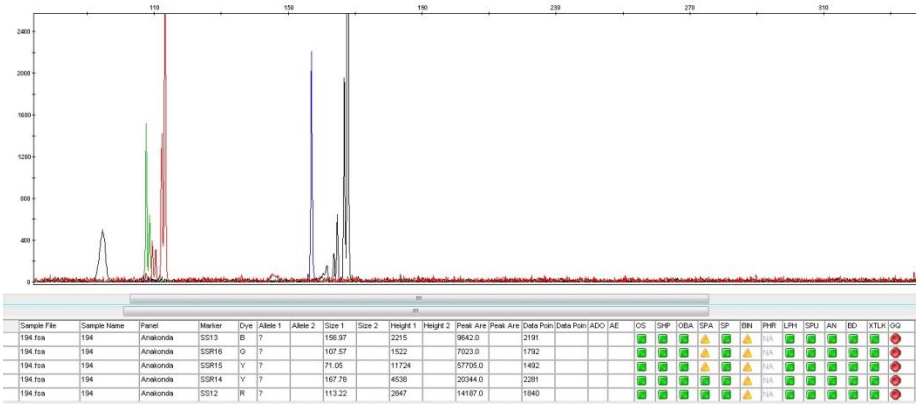
4.1. Fungal identification

From the 241 isolates obtained in this study, 209 were identified as *D. sapinea* and 32 as *D. scrobiculata*. All samples from BC1 and USA1 were only positive for *D. sapinea*. Whereas, 1 sample from BC2, 28 samples from USA2 and 3 from W populations were positive for *D. scrobiculata*.

4.2. Population genetic analyses

The SSR markers (Figure 5.1.) used in this study produced 51 alleles across the 10 loci examined for 209 *D. sapinea* isolates and 47 alleles for the 32 *D. scrobiculata* samples. Of these, 12 (23.53%) and 6 (12.77%) were respectively shared by all *D. sapinea* and *D. scrobiculata* populations. There were identified 17 alleles in BC1, 19 in BC2, 25 in USA1, 37 in USA2 and finally, 21 in W *D. sapinea* sample sets (Table 5.1.). For *D. scrobiculata*

populations 10 alleles were identified in BC2, 43 in USA2 and 20 in W (Table 5.2.). Two alleles from each locus were dominant within populations, while the rest were rarely found.



Sample File	Sample Name	Panel	Marker	Dye	Allele 1	Allele 2	Size 1	Size 2	Height 1	Height 2	Peak Are	Peak Are	Data Poin	Data Poin	ADO	AE	OS	SHP	OBA	SPA	SP	BIN	PHR	LPH	SPU	AN	BD	XTLK	GQ
194.fsa	194	Anakonda	SS13	B	?		156.97		2215		9642.0		2191										NA						
194.fsa	194	Anakonda	SSR16	G	?		107.57		1522		7023.0		1792										NA						
194.fsa	194	Anakonda	SSR15	Y	?		71.05		11724		57705.0		1492										NA						
194.fsa	194	Anakonda	SSR14	Y	?		167.78		4538		20344.0		2281										NA						
194.fsa	194	Anakonda	SS12	R	?		113.22		2847		14187.0		1840										NA						

Figure 5.1. Multiplex genotyping of 5 microsatellites for *D. scrobiculata* in ABI Prism 3500 Genetic Analyser by capillary electrophoresis.

In this context, the presence of unique alleles become relevant due to their influence on the genotypic diversity of the populations. For *D. sapinea* samples 2 unique alleles were detected in BC1, BC2 and W, 5 in USA1 and 11 in USA2 (Table 5.1.). As observed for *D. sapinea*, there were more unique alleles at USA2 population for *D. scrobiculata* samples, in BC2 1 allele was found and 2 in W sample set (Table 5.2.). The ten loci each contained three to ten alleles when considering all *D. sapinea* isolates and two to nine when considering all *D. scrobiculata* isolates.

The percentage of polymorphic loci within *D. sapinea* populations ranged from 50% in BC2 to 100% in USA2 (Table 5.1.) and from 0% in BC1 to 100% in USA2 for *D. scrobiculata* sample sets (Table 5.2.).

Nei's genetic diversity index for *D. sapinea* isolates ranged from H=0.1512 to 0.2631 per each population and from 0.0285 to 0.4547 per each locus. The mean total gene diversity across all *D. sapinea* populations was 0.1932, the values for individual populations were; 0.1512 for the BC1, 0.1625 for BC2, 0.1598 for USA1, 0.2631 for USA2 and 0.2296 for W (Table 5.1.). For *D. scrobiculata* Nei's genetic diversity index ranged from H=0.000 to 0.3835 per each population and from 0.2188 to 0.7107 per each locus. Mean gene diversity was 0.3806 and the values for individual populations were 0.3835 for USA2 and 0.3778 for W sample sets (Table 5.2.).

Table 5.1. Allele size (bp) and frequency at 10 loci (SS1, SS5, SS9-16) for five different *D. sapinea* populations collected (BC1, BC2, USA1, USA2, W).

Locus	Allele	BC1	BC2	USA1	USA2	W	H
SS1	335	…	…	…	0.018	…	0.0285
	337	…	…	0,020	0.018	…	
	407	1.000	1.000	0,980	0.964	1.000	
SS5	491	…	…	…	0.018	…	0.0654
	498	0.074	…	…	…	…	
	500	0.926	1.000	1,000	0.912	1.000	
	502	…	…	…	0.070	…	
SS9	246	…	…	…	0.018	…	0.4172
	250	…	0.016	0,020	0.035	…	
	251	…	…	0,020	…	…	
	253	…	…	…	…	0.077	
	263	0.481	0.694	0,920	0.807	0.538	
	265	…	…	…	0.053	…	
	267	0.519	0.290	0,040	0.087	0.385	
SS10	250	…	…	…	0.018	…	0.1689
	300	…	…	…	0.018	0.077	
	312	0.926	0.855	0,980	0.946	0.231	
	314	0.074	0.145	…	…	0.692	
	321	…	…	0,020	…	…	
	330	…	…	..	0.018	…	
SS11	173	1.000	1.000	0,640	0.474	1.000	0.3786
	176	…	…	0,360	…	…	
	177	…	…	…	0.526	…	
SS12	98	…	…	…	0.070	…	0.1891
	102	…	…	…	0.035	…	
	112	1.000	0.952	0,980	0.737	0.846	
	114	…	…	…	0.018	…	
	115	…	0.016	…	…	…	
	116	…	…	0,020	0.070	0.077	
	118	…	0.032	…	…	…	
	120	…	…	…	0.035	0.077	
	124	…	…	…	0.035	…	
SS13	141	…	…	…	0.018	0.077	0.0375
	157	0.963	1.000	1,000	0.982	0.923	
	167	0.037	…	…	…	…	
SS14	152	…	…	…	0.035	0.077	0.4547
	153	…	…	0,020	…	…	
	157	…	…	…	0.018	…	
	158	…	…	0,040	…	…	
	162	0.852	0.774	0,600	0.719	0.769	
	164	…	0.032	…	0.192	0.077	
	165	0.037	0.016	0,260	…	…	

	168	…	…	0,060	0.018	…	
	171	0.111	0.178	0,020	0.018	…	
	187	…	…	…	…	0.077	
SS15	69	0.222	0.597	0,980	0.824	0.385	0.4315
	71	0.778	0.403	0,020	0.158	0.615	
	78	…	…	…	0.018	…	
SS16	106	…	…	0,020	0.123	…	0.1358
	108	1.000	1.000	0,840	0.877	1.000	
	110	…	…	0,140	…	…	
No. Isolates	209	27	62	50	57	13	
No. Alleles	51	17	19	25	37	21	
No. Unique Alleles		2	2	5	11	2	
Polymorphyc loci		6	5	8	10	6	
H		0.1512	0.1625	0.1598	0.2631	0.2296	

H (Nei, 1973).

Table 5.2. Allele size (bp) and frequency at 10 loci (SS1, SS5, SS9-16) for three different *D. scrobiculata* populations collected (BC2, USA2, W).

Locus	Allele	BC2	USA2	W	H
SS1	335	…	0.273	1.000	0.4352
	407	1.000	0.727	…	
SS5	493	…	0.071	…	0.4160
	495	…	0.822	0.333	
	498	…	0.036	0.667	
	500	1.000	0.071	…	
SS9	243	…	0.036	…	0.3281
	246	…	0.893	0.333	
	248	…	0.071	0.333	
	250	…	…	0.333	
	267	1.000	…	…	
SS10	284	…	0.036	…	0.3145
	286	…	0.857	0.667	
	312	1.000	0.107	0.333	
SS11	170	…	0.143	…	0.2188
	173	1.000	0.857	1.000	
SS12	92	…	0.179	0.333	0.5918
	94	…	0.036	…	
	96	…	0.643	0.333	
	98	1.000	0.071	0.333	
	106	…	0.071	…	
SS13	154	…	0.056	…	0.7107
	157	1.000	0.388	1.000	
	162	…	0.056	…	
	163	…	0.111	…	
	167	…	0.111	…	

	170	…	0.111	…	
	172	…	0.056	…	
	179	…	0.111	…	
SS14	132	1.000	0.107	0.667	0.3535
	148	…	0.036	…	
	150	…	0.857	0.333	
SS15	57	…	0.036	0.333	0.4961
	74	…	…	0.333	
	75	…	0.785	…	
	77	…	0.036	…	
	78	1.000	0.107	0.333	
	99	…	0.036	…	
SS16	94	…	0.036	…	0.5508
	96	…	0.071	…	
	106	…	0.713	0.333	
	108	…	0.036	…	
	110	…	0.036	…	
	120	…	0.036	…	
	122	…	0.036	…	
	132	1.000	…	0.667	
	140	…	0.036	…	
No. Isolates	32	1	28	3	
No. Alleles	47	10	43	20	
No. Unique Alleles		1	24	2	
Polymorphyc loci		0	10	7	
H		0.0000	0.3835	0.3778	

H (Nei, 1973).

The Stoddart and Taylor genotypic diversity index (*G*) of *D. sapinea* populations was; 4.53 for BC1, 8.39 for BC2, 5.71 for USA1, 9.05 for USA2 and 6.26 for W (Table 5.3.). Whereas, the genotypic diversity for *D. scrobiculata* sample sets was; 1 for BC2, 14.52 for USA2 and 3 for W (Table 5.4.).

The values of maximum genotypic diversity index ($\hat{G}$ %) varies enormously among the five studied populations, from 11.42% in USA1 to 48.15% in W for *D. sapinea* isolates (Table 5.3.) and from 51.85 in USA2 to 100% in BC2 and W for *D. scrobiculata* samples (Table 5.4.).

In general, low values of diversity were observed for Nei's and Stoodart and Taylor diversity indexes in BC1, BC2 and USA1 populations. Low diversity values for *D. sapinea* were due to the predominance of certain haplotypes, for example in USA1 half of the isolates were represented by only two haplotypes (Table 5.3.).

Among the 55 haplotypes described for the 209 *D. sapinea* isolates, 6 haplotypes were shared by BC and W, 4 between BC and USA and 2 between USA and W populations (Table 5.3.). However, no shared haplotypes were observed among the 24 haplotypes described for *D. scrobiculata* populations (Table 5.4.). One single haplotype (BC1B) was shared among the five *D. sapinea* populations, but it becomes relevant since the greater number of isolates in almost all populations were represented by this haplotype (Table 5.3.). In the case of *D. scrobiculata*, the predominance of some haplotypes over others is not appreciated (Table 5.4.).

Table 5.3. *Diplodia sapinea* haplotypes as estimated from multilocus profiles generated from the 10 SSR loci. Haplotypes were distributed among the five studied populations (BC1, BC2, USA1, USA2, W).

Haplotype	BC1	BC2	USA1	USA2	W
BC1O	4	11		1	1
BC1G	11	8			1
BC1B	3	12	16	11	4
BC1P	2	7		1	
BC1PR	2				
BC1BR	2				
BC1Y	1	1			
BC1BL	1				
BC1W	1	1	6		
BC2G		4			
BC2GL		7			1
BC2GY		2			2
BC2B		1			1
BC2PL		1			
BC2P		1			
BC2Y		1			
BC2R		1			
BC2BL		1			
BC2T		1			
BC2GN		1			
BC2GN2		1			
U1OR			2		
U1Y			10		
U1YL			3		
U1YW			1		
U1O			4		
U1BR			1		
U1P			1		

U1GR			3		
U1G			2		
U1R			1		
WY1					1
WY2					1
WO1					1
U2Y5				1	
U2W1				1	
U2W3				2	
U2O1				1	
U2O2				1	
U2R1				5	
U2R2				13	
U2R3				2	
U2R4				1	
U2R5				1	
U2R6				1	
U2R7				2	
U2P1				3	
U2P2				2	
U2P3				1	
U2P4				1	
U2P5				1	
U2P6				1	
U2W4				1	
U2W5				1	
U2W6				2	
N	27	62	50	57	13
N (g)	9	18	12	24	9
G	4.53	8.39	5.71	9.05	6.26
***Ĝ* (%)**	16.77	13.54	11.42	15.88	48.15

N, number of isolates.
N(g), number of genotypes.
G, Genotypic diversity (Stoddart and Taylor 1988).
Ĝ, % maximum genotypic diversity.

Table 5.4. *Diplodia scrobiculata* haplotypes as estimated from multilocus profiles generated from the 10 SSR loci. Haplotypes were distributed among three populations collected (BC2, USA2, W).

Haplotype	BC2	USA2	W
BC2GR	1		
W1R			1
WO2			1
W1BL			1
U2Y1		1	
U2Y2		1	
U2Y3		1	
U2Y4		1	
U2Y6		1	
U2Y7		1	
U2G1		1	
U2G2		3	
U2G3		1	
U2G4		1	
U2W2		1	
U2B1		1	
U2B2		1	
U2B3		1	
U2B4		2	
U2B5		1	
U2B6		1	
U2B7		1	
U2B8		4	
U2B9		3	
N	1	28	3
N (g)	1	20	3
G	1	14.52	3
***Ĝ* (%)**	100	51.85	100

N, number of isolates.
N(g), number of genotypes.
G, Genotypic diversity (Stoddart and Taylor 1988).
Ĝ, % maximum genotypic diversity.

From the analysed populations, χ^2 values indicated that there were significant differences (p<0.05) for gene diversity at most SSR loci between populations, with the exception of SS1 and SS13 for *D. sapinea* (Table 5.5.). χ^2 test for *D. scrobiculata* isolates show significant differences at only four SSR loci; SS3, SS9, SS14 and SS15 (Table 5.6.).

Table 5.5. Gene diversity (*H*, Nei 1973) and contingency χ^2 test for differences in allele frequencies for the 10 polymorphic SSR loci across populations of *Diplodia sapinea*, from all sample sets (BC1, BC2, USA1, USA2, W).

	Gene diversity (*H*)						
Locus	**BC1**	**BC2**	**USA1**	**USA2**	**W**	χ^2	**df**
SS1	0.000	0.000	0.039	0.068	0.000	4.601	8
SS5	0.137	0.000	0.000	0.163	0.000	27.179*	12
SS9	0.560	0.434	0.150	0.337	0.556	77.737*	28
SS10	0.137	0.248	0.039	0.102	0.462	35.686*	20
SS11	0.000	0.000	0.461	0.499	0.000	151.838*	8
SS12	0.000	0.093	0.039	0.443	0.272	48.083*	32
SS13	0.071	0.000	0.000	0.068	0.142	5.731	4
SS14	0.261	0.368	0.556	0.442	0.391	113.904*	32
SS15	0.346	0.481	0.039	0.295	0.473	64.425*	8
SS16	0.000	0.000	0.274	0.216	0.000	38.386*	8
N	27	62	50	57	13		
Mean	0.151	0.163	0.160	0.263	0.230		
SE	0.187	0.201	0.203	0.164	0.228		

*Indicate significant (p<0.05) χ^2 values.

Table 5.6. Gene diversity (*H*, Nei 1973) and contingency χ^2 test for differences in allele frequencies for the 10 polymorphic SSR loci across populations of *Diplodia scrobiculata*, from three sample sets (BC2, USA2, W).

	Gene diversity (*H*)				
Locus	**BC2**	**USA2**	**W**	χ^2	**df**
SS1	0.000	0.397	0.000	4.947	2
SS5	0.000	0.314	0.444	22.714*	6
SS9	0.000	0.196	0.667	44.772*	8
SS10	0.000	0.253	0.444	6.693	4
SS11	0.000	0.245	0.000	0.653	2
SS12	0.000	0.543	0.667	9.828	8
SS13	0.000	0.790	0.000	4.889	14
SS14	0.000	0.253	0.444	10.060*	4
SS15	0.000	0.367	0.667	22.590*	10
SS16	0.000	0.367	0.444	24.308	16
N	1	28	3		
Mean	0.000	0.384	0.378		
SE	0.000	0.181	0.278		

*Indicate significant (p<0.05) χ^2 values.

Genetic relatedness between isolates within *D. sapinea* and *D. scrobiculata* populations was evaluated using UPGMA dendrogram. It shows that *D. sapinea* isolates from BC2 and W were more similar to each other (Figure 5.2.). Similarities were observed between USA2 and W in *D. scrobiculata* populations (Figure 5.3.).

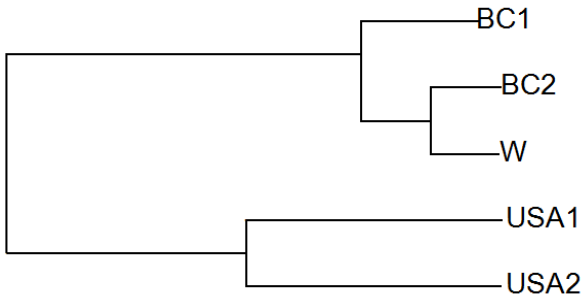


Figure 5.2. Phylogenetic tree showing the relatedness of *D. sapinea* isolates using UPGMA.

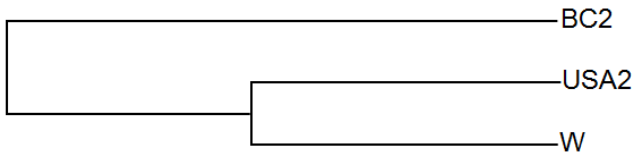


Figure 5.3. Phylogenetic tree showing the relatedness of *D. scrobiculata* isolates using UPGMA.

4.3. Analysis of molecular variance (AMOVA)

Genetic variation for all *D. sapinea* and *D. scrobiculata* isolates was partitioned within and among populations using AMOVA. The results indicated that 92% of the variation was mainly explained by variations within populations and the 8% was explained by variations among populations (Table 5.7.).

Table 5.7. AMOVA of the five populations (BC1, BC2, USA1, USA2, W) for both *D. sapinea* and *D. scrobiculata* samples hierarchically partitioned.

Source	df	SS	MS	Est. Var.	% Variation
Among populations	4	123484.19	30871.05	545.56	8%
Within populations	236	1488779.95	6308.39	6308.39	92%
Total	240	1612264.14		6853.95	100%

df=Degree of freedom, SS=Sum of square, MS=Mean square, and Est. Var.=Estimated variance.

5. Discusion

The origin of *D. sapinea* and *D. scrobiculata* in worldwide plantations has not been well determined (Burgess *et al.*, 2004b). In its native range, *P. radiata* appears to be exclusively associated with *D. scrobiculata* and the introduction of this species is by far less widespread than *D. sapinea*, even if both behave as endophytes. Molecular markers have been accepted as a valid method to assess the origin of both pathogens (Burgess *et al.*, 2004b).

It has been well documented that the first pines introduced by European colonialist were species from Europe, such as *Pinus pinea* L. and *Pinus sylvestris* L., which, for example, were well established in South Africa (Legat, 1930). These initial pine introductions were seedlings that may have been infected with *D. sapinea* when they were introduced. Importantly, these same European pine species seedlings were also planted by colonialist to North America, which could have served to introduce *D. sapinea* from what may have been its aboriginal home in Europe (Swart *et al.*, 1991; Stanosz *et al.*, 1999; de Wet *et al.*, 2000; Burgess & Wingfield, 2001).

The results of this study revealed interesting information among the 5 populations analysed from BC, USA and W. They are isolated populations and these pathogens have also been considered as asexually reproducing fungi. Therefore, a high degree of clonality is expected in those populations (Chen & McDonald, 1996; Kohli & Kohn, 1998; Burgess *et al.*, 2004a).

Significant similarities were observed at allele's level. In fact, the 23% of the alleles were found to be shared between *D. sapinea* populations and the 12% between *D. scrobiculata*. Moreover, 8% of haplotypes were shared among *D. sapinea* populations. UPGMA analysis supports the belief of certain similarities among these five populations. Indeed, BC and W *D. sapinea* populations were found to be more similar to each other than to USA ones. This could be due to the number of haplotypes shared, among the 18 and 9 haplotypes detected on BC2 and W respectively, 6 were shared. For *D. scrobiculata* important similarities were observed between USA and W sample sets.

However, only the 8% of diversity is explained by variation among all populations and species, which is not expected in asexually reproductive fungi. In fact, the χ^2 test detected significant differences among 8 of the 10 studied loci for *D. sapinea* populations and in 4 loci for *D. scrobiculata*. Furthermore, differences on polymorphic loci were relevant between

BC and USA isolates. In BC sample sets up to five monomorphic loci were detected, whereas, USA populations where highly polymorphic.

The maximum diversity value was obtained for W sample set for both species and is the only population to exceed the 17% of maximum diversity value. Although, some levels of genetic diversity among all studied populations based on both Nei's and Stoddart and Taylor indexes were detected. Lower diversity values in BC and USA populations could be explained by predominance of two haplotypes in each population and this could mean that the outbreak of the disease may be related to specific haplotypes (Burgess *et al.*, 2004b). Higher diversity values were appreciated in all *D. scrobiculata* populations. However, the values for BC2 and W populations were, at some point, not conclusive as few isolates were studied. These results are consistent with previous studies in which *D. scrobiculata* was found to be more diverse in comparison to *D. sapinea* (Burgess *et al.*, 2001a).

Evidence of genetic diversity and the presence of unique alleles in asexually reproductive fungi become puzzling. However, the absence of detection of previous sexual reproduction among the studied populations (Manzanos *et al.*, 2018), may indicate that cryptic sexual stage could be a mechanism of genetic drift. The diversity observed could also be explained by; randomly association of alleles in each population, new alleles arising through mutations, and the introduction of pathogens due to human activities.

The randomly association and mutation of alleles might occur to confer a fitness benefit to the fungus and therefore, play a significant role in the populations (McDonald & McDermont, 1993; FitzSimmons *et al.*, 1997; Milgroom & Cortesi, 1999), since it is expected that genetic drift and selection would have eliminate those unique alleles.

These alleles might also represent remnants of diversity introduced since the establishment of plantations into these regions. Furthermore, all the populations share both alleles and haplotypes, which could mean a common source. These exceptions most likely reflect occasional long distance dispersal due to human intervention by moving infected material.

Despite these pathogens may have been taken from Europe to other countries, it is considered that in the Basque Country during last decades it has been, to some extent, introduced with seeds from countries like Chile and New Zealand for the establishment of *P. radiata* plantations (Iturritxa *et al.*, 2008). Moreover, BC populations do not behave as an

indigenous population where higher values of diversity may be expected in comparison to the diversity of an introduced population (Burgess *et al*., 2001a).

In summary, these results indicate that these fungi populations have undergone adaptation in the environments in which they occur based on the detection of unique alleles. However, they also present common characteristics that may indicate the same origin. *D. sapinea* and *D. scrobiculata* were found associated with pine species across BC, USA and W and its dispersion was probably assisted by human's movement of exotic pines around the world.

6. References

Atwell, R. C., Schulte, L. A., & Palik, B. J. (2008). Songbird response to experimental retention harvesting in red pine (*Pinus resinosa*) forests. *Forest Ecology and Managent*, *255*, 3621-3631.

Benzie, J. W. (1977). Managers Handbook for red pine in the North Central States. *USDA Forest Service General Technical Reports*, NC-33.

Bihon, W., Burgess, T., Slippers, B., Wingfield, M. J., & Wingfield, B. D. (2011). Distribution of *Diplodia pinea* and its genotypic diversity within asymtomatic *Pinus patula* trees. *Autralasian Plant Pathology*, *40*, 540-548.

Blodgett, J. T., Kruger, E. L., & Stanosz, G. R. (1997). *Sphaeropsis sapinea* and water stress in a red pine plantation in central Wisconsin. *Phytopathology*, *87*, 429-434.

Buckman, R. E. (2006). Growth and yield of red pine in the Lake States. *USDA Forest Service General Technical Reports*, NC-271.

Burdon, R. D. (2000). *Pinus radiata*. In Last FT (Eds.), *Ecosystems of the World. Tree Crops Ecosystems, 19*, 99-161. Amsterdam, Netherlands: Elsevier.

Burgess, T. I., Gordon T. R., Wingfield, M. J., & Wingfield, B. D. (2004a). Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. *Mycological Research*, *108*, 1399-1406.

Burgess, T. I., Wingfield, M. J., & Wingfield, B. D. (2004b). Global distribution of *Diplodia sapinea* genotypes revealed using simple sequence repeat (SSR) markers. *Australasian Plant Pathology*, *33*, 513-519.

Burgess, T. & Wingfield, M. J. (2001). Exotic pine forestry in the southern hemisphere: a brief history of establishment and quarantine practices. *South African Forestry Journal*, *192*, 79-83.

Burgess, T., Wingfield, B. W., & Wingfield, M. J. (2001a). Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*. *Mycological Research*, *105*, 1331-1339.

Burgess, T., Wingfield, M. J., & Wingfield, B. W. (2001b). Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and environmental microbiology*, *67*, 354-362.

Chen, R. S., & McDonald, B. A. (1996). Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. *Genetics*, *142*, 1119-1127.

Chen, R. S., Boeger, J. M., & McDonald, B. A. (1994). Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology*, *3*, 209-218.

Chou, C. K. S. (2007). Perspectives of disease threat in large-scale *Pinus radiata* monoculture - the New Zealand experience. *European Journal of Forest Pathology*, *21*, 71-81.

Chou, C. K. S. (1987). Crown wilt of *Pinus radiata* associated with *Diplodia pinea* infection of woody stems. *European Journal of Forest Pathology*, *6*, 354-359.

de Wet, J., Burgess, T., Slippers, B., Preisig, O., Wingfield, B. D., & Wingfield, M. J. (2003). Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. *Mycological Research*, *107*, 557-566.

de Wet, J., Wingfield, M. J., Coutinho, T. A., & Wingfield, B. D. (2000). Characterization of *Sphaeropsis sapinea* isolates from South Africa, Mexico and Indonesia. *Plant Disease*, *84*, 151-156.

Eldridge, K. G. (1961). Significance of *Diplodia pinea* in plantations. *Review of Applied Mycology*, *41*, 339.

Fitzsimmons N. N., Moritz, C., Limpus, C. J., Pope, L., & Prince, R. (1997). Geographic structure of mitochondrial and nuclear gene polymorphisms in Austrian green turtle populations and male-biased gene flow. *Genetics*, *147*, 1843-1854.

Foster, R. C., & Marks, G. C. (1968). Fine structure of the host-parasite relationship of *Diplodia pinea* on *Pinus radiata*. *Australian Forestry*, *32*, 211-225.

Inventario Forestal CAE. (2016). El bosque Vasco en cifras 2017. Retrieved from http://www.euskadi.eus/contenidos/informacion/inventario_forestal_2016/es_agripes/adjuntos/El%20bosque%20vasco%20en%20cifras%202017.pdf

Iturritxa, E., Ganley, R. J., Raposo, R., García-Serna, I., Mesanza, N., Kirkpatrick, S. C., & Gordon, T.R. (2013). Resistance levels of Spanish conifers against *Fusariun circinatum* and *Diplodia sapinea*. *Forest pathology*. *43*, 488-495.

Iturritxa, E., García-Serna, I., & Mesanza, N. (2008). *Diplodia pinea* in forest plantations of *Pinus radiata* in northern Spain: Diversity and origin of the Spanish population. *Journal of Plant Pathology*, *90*, 366.

Kohli, Y., & Kohn, L. M. (1998). Random association among alleles in clonal populations of *Sclerotinia sclerotiorum*. *Fungal Genetics and Biology*, *23*, 139-149.

Legat, C. E. (1930). The cultivation of exotic conifers in South Africa. *Empire Forestry Journal*, *9*, 32-63.

Manzanos T., Stanosz, G. R., Smith, D. R., Muenchow, J., Schratz, P., Brenning, A., Aragonés, A., & Iturritxa, E. (2018). Mating type ratios and pathogenicity in Diplodia shoot blight fungi populations: Comparative analysis. *Forest Pathology*, e12475.

McDonald, B. A. (1997). The population genetics of fungi: tools and techniques. *Phytopathology*, *87*, 448-453.

McDonald, B. A., & McDermott, J. M. (1993). Population genetics of plant pathogenic fungi: electrophoretic markers give unprecedented precision to analyses of genetic structure of populations. *BioScience*, *43*, 311-319.

Milgroom, M. G., & Cortesi, P. (1999). Analysis of population structure of the chestnut blight fungus based on vegetative incompatibility genotypes. *Proceeding of the National Academy of Science, USA*, *96*, 10518-10523.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, *89*, 583-590.

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA*, *70*, 3321-3323.

Nicholls, T. H., & Ostry, M. E. (1990). *Sphaeropsis sapinea* cankers on stressed red and jack pines in Minnesota and Wisconsin. *Plant Disease*, *74*, 54-56.

Omar, A. (2012). Aspectos más relevantes de la sanidad forestal en Vizcaya/Bizkaia. *Foresta*, *5*, 174-179. Retrieved from http://www.redforesta.com/wp-

content/uploads/2012/11/GF22-Aspectos-mas-relevantes-de-la-sanidad-forestal-en-Vizcaya.pdf

Palmer, M. A. (1991). Isolate types of *Sphaeropsis sapinea* associated with main stem cankers and top-kill of *Pinus resinosa* in Minnesota and Wisconsin. *Plant Disease*, *75*, 507-510.

Palmer, M. A., McRoberts, R. E., & Nicholls, T. H. (1988). Sources of inoculum of *Sphaeropsis sapinea* in forest tree nurseries. *Phytopathology*, *78*, 831-835.

Peakall, R., & Smouse, P.E. (2006). GENALEX 6: genetic analysis in excel. Population genetic software for teaching and research. Molecular Ecology Notes, 6, 288-295.

Phillips, A. J. L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M. J., Groenewald, J. Z., & Crous, P. W. (2013). The *Botryosphaeriaceae*: genera and species known from culture. *Studies in Mycology*, *76*, 51–167.

Purnell, H. M. (1957). Shoot blight of *Pinus radiata* Don. caused by *Diplodia pinea (Desm.) Kickx. Forestry Commission Bulletin, 5*. Forestry Commission, Victoria.

Smith, D. R., & Stanosz, G. R. (2006). A species-specific PCR assay for detection of *Diplodia pinea* and *D. scrobiculata* in dead red and jack pines with collar rot symptoms. *Plant Disease*, *90*, 307-313.

Smith, H., Wingfield, M. J., de Wet, J., & Coutinho, T. A. (2000). Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. *Plant disease*, *84*, 139-142.

Stanosz, G. R., Swart, W. J., & Smith, D. R. (1999). RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous host and locations. *Mycological Research*, *103*, 1193-1043.

Stanosz, G., & Cummings Carlson, J. (1996). Association of mortality of recently planted seedlings and established saplings in red pine plantations with *Sphaeropsis* collar rot. *Plant Disease*, *80*, 750-753.

Stoddart, J. A. & Taylor, J. F. (1988). Genotypic diversity: estimation and prediction in samples. *Genetics*, *118*, 705-711.

Sutton, B. C. (1980). *The Coelomycetes*. Commonwealth Mycological Institute, Kew.

Swart, W. J., & Wingfield, M. J. (1991). Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. *Plant Disease*, *75*, 761-766.

Swart, W. J., Wingfield, M. J., Palmer, M. A., & Blanchette, R.A. (1991). Variation among South African isolates of *Sphaeropsis sapinea*. *Phytopathology*, *81*, 489-493.

Yeh, F. C., Yang, R. C., & Boyle, T. (1999). POPGENE Version 1.32. Microsoft Windows Based Freeware for Population Genetic Analysis, Alberta.

Zane, L., Bargelloni, L., & Patarnello, T. (2002). Strategies for microsatellite isolation: a review. *Molecular Ecology*, *11*, 1-16.

Zwolinski, J. B., Swart, W. J., & Wingfield, M. J. (1990). Intensity of dieback induced by *Sphaeropsis sapinea* in relation to site conditions. *European Journal of Forest Pathology*, *20*, 167–174.

Chapter 6

Physiological study of Diplodia shoot blight causing fungi

1. Abstract

Diplodia shoot blight is an important fungal disease caused mainly by *Diplodia sapinea* and *Diplodia scrobiculata*. It is responsible of tips and branches dieback especially affecting stressed *Pinus* species. In advanced stages of the infection it can also cause canker disease. The diversity of this fungus has been studied, but little is known regarding its physiology. In this context, an investigation of carbohydrates degradation and aggressiveness was conducted. A collection of 223 isolates was subjected to analysis, 145 from northern Spain and 78 from different origins. The commercially available API® 32C microbiological identification system was used to evaluate *D. sapinea* and *D. scrobiculata* fungi. Cellulose degradation was also determined and two different fungicides (benomyl and tebuconazole) were tested to observe growth reduction. The maximum cellulose degradation values were detected in isolates obtained from northern Spain populations. Fungicide susceptibility effect was also measured and attending to species, significant growth reduction rates were observable for *D. sapinea* isolates. Tebuconazole is the fungicide that shows greatest reduction in growth. Significant relations between carbohydrate degradation and pathogenicity were described for both species and populations.

Keywords: cellulose, carbohydrate, fungicide, aggressiveness, *D. sapinea*, *D. scrobiculata*.

2. Introduction

Diplodia shoot blight is a disease caused by different pathogens that belongs to *Diplodia* genus as *Diplodia sapinea* (Fr.) Fuckel and *Diplodia scrobiculata* J. de Wet, B. Slippers & M. J. Wingfield. These fungi are responsible for causing different diseases as seed rot, shoot blight, dieback, root disease and in older twigs that have been killed or in cut timbers, the bark turned dark brown and the sapwood stained grey or blue-back (Zwolinski *et al.*, 1990). *D. sapinea* is the most frequently detected species in plantations of *Pinus radiata* D. Don in the Basque Country (located in northern Spain) (Iturritxa *et al.*, 2013), but *Diplodia scrobiculata* has also been recently detected (Manzanos *et al.*, 2017). *D. sapinea* was found to be more aggressive in comparison to *D. scrobiculata* and has a widespread distribution (Palmer *et al.*, 1987; Blodgett & Stanosz, 1997). These organisms are considered opportunistic and saprophytic pathogens generally related to modest and severe damages as a consequence of episodic biotic or abiotic stress, thus it can also persist in asymptomatic trees (Flowers *et al.*, 2001; Stanosz *et al.*, 2001).

Pinus radiata is considered one of the most susceptible species to this disease in the area of study (Iturritxa *et al.*, 2013) and consequently, important losses have been reported associated or connected with biotic and abiotic factors. *P. radiata* is considered an important species in northern Spain plantation forestry as it occupies the greatest extent. Specifically, in the Basque Country, the 31.2% of the total forest area and the 67.1% of all coniferous forests is covered by *P. radiata* plantations (Hazi Fundazioa, 2016). Thus, conservation of *Pinus radiata* is important due to its economic relevance in northern Spain plantation forestry. In this context, it is important to study the enzymatic activity, through an analysis of the degradation of different carbohydrates, of the fungi belonging to the *Diplodia* genus that affect the health of these plantations. This could be connected with the pathogenic and saprophytic capacity of these isolates as well as with their development of resistance to the fungicides that have been systematically applied to the seeds and seedlings in nurseries of the study area.

Plant cell wall degradation is a typical feature of the metabolism of saprophytic fungi. Polysaccharides degrading enzymes that hydrolyse wall-bound carbohydrates such as peptic polymers and cellulose are involved in this process. These enzymes facilitate the wall penetration, tissue invasion and pathogenesis. Cellulose is a biopolymer and the most common component of the primary cell wall, 20-30% of the dry weight (Benhamou & Picard, 2001). The fungi that belong to Ascomycota division catalyse the degradation of polysaccharides, in particular of cellulose (Fogarty & Kelly, 1990). In order to obtain a high degradation of cellulose material, the synergic effect of the three cellulase enzymes is necessary. Cellulase is an enzyme complex formed by three mayor enzymes known as endo-β-(1-4)-D-glucanase, exo-β-(1-4)-D-glucanase and β-glucosidase (Duff *et al.*, 1987).

Benzimidazoles fungicides became available in the 1960s and benomyl (methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate) is one of the most important active maters of this group. In the plants it is converted to carbendazim and transported throughout the transpiration stream ending up in margins and tips of leaves and needles (Ypema, 2001). Benzimidazoles bind specifically to the β-tubulin of ascomycetes and basidiomycetes and inhibit the tubulin polymerization. It has been registered to control a range of fungal diseases affecting fruits, nuts, vegetables, turf, and field crops. It has been related to liver toxicity, reproductive effects, and chromosome abnormalities (Pearson & Miller, 2014; Zhou *et al.*, 2015). Recently, this fungicide has also been described as a substance implicated in neurotoxic pathways since its metabolites act as aldehyde dehydrogenase inhibitors

(Pearson & Miller, 2014). Therefore, the use of benomyl as a fungicide has been restricted recently in the European Community (EU Pesticides database, 2011). According to 1272/2008 regulation benomyl use is related to; skin and respiratory irritation, toxicity to aquatic life with long lasting effects, damage to fertility and unborn children, allergic skin reaction and genetic defects.

Since the use of benomyl has been prohibited, tebuconazole (1-(4-Chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)-3-pentanol) (EU Pesticides database, 2017) has been used as highly effective fungicide in controlling soil-borne and foliar fungal pathogens in a wide range of vegetables, fruits and crops. In the EU is primarily used on cereals and vines, but it has also been used in forest plantations seeds and nurseries (EFSA, 2014; FAO, 1994). This triazole group fungicide acts by inhibiting the biosynthesis of ergosterin in fungi (Strickland *et al.*, 2004). Although it has been related to acute toxicity to aquatic life with long lasting effects, acute toxicity if swallowed and it is suspect to be responsible of damaging unborn child, in agreement to 1272/2008 regulation (2008), its use has not been limited. Its effectiveness in the control of *Botryosphaeria* species has been recorded in several studies (Bester *et al.*, 2007; Amponsah *et al.*, 2012; Díaz & Latorre, 2013).

The aim of this study was to determine the carbohydrate degradation capacity of Diplodia shoot blight fungi isolates from northern Spain and different parts of the world. It was hypothesized that saprophyte pathogens were able to degrade different types of media and that could be related with pathogenicity of the isolates. Behaviour differences based on the origin of the isolates could be expected. Thus, single conidial cultures were generated for all the isolates. Inoculation trial, carbohydrate degradation and fungicide effect were used to characterize the diversity of 223 isolates. The effect of the most commonly used fungicides in *P. radiata* plantations from northern Spain was tested in order to determine the growth control over *D. sapinea* and *D. scrobiculata*.

3. Material and methods

3.1. Sampling and isolation

223 samples of *Diplodia sapinea* and *D. scrobiculata* were collected from fragment of branches, needles, cones, and pieces of wood (Appendix E). Five different sample sets were defined based on the origin of the isolates. P1 Laukiniz (n=10), P2 Sollano (n=13), and P3 Hernani (n=13) sample sets were obtained from different *P. radiata* plantations in Basque Country, located on the Atlantic coast of northern Spain. Fourth sample set (BC)

consist on 109 isolates obtained from a wider area occupied by *P. radiata* in the Basque Country. Last sample set (W) consisted on isolates from a variety of host species and different origins (n=78) (United States of America, Canada, Australia, New Zealand, France, United Kingdom, Spain, Tanzania, Madagascar, Mexico, Honduras and Turkey), obtained from the collection of Glen Stanosz, Department of Forest and Wildlife Ecology, University of Wisconsin-Madison, United States.

The samples were cut in small pieces, soaked in commercial bleach (30%) for 1 min and rinsed with sterile water. Monosporic cultures were obtained from single conidium and grown on potato dextrose agar (PDA, Panreac) in darkness at 20 ± 3°C for 7 days until they completely covered the medium surface. The mycelium was removed and collected in 2 ml tube and disrupted by Quiagen-Retsch MM300 Tissuelyser. DNA extractions were performed for all isolates using a DNA Plant Mini Kit (Analytik Jena AG, Life Science) and the quality of DNA was estimated using a NanoDrop ND-1000 spectophotometer (Thermo Fisher Scientific).

Species identification was performed using specific primers, BotR, DpF, and DsF developed by Smith and Stanosz (2006). The primers were synthetized by Integrated DNA Technologies (Belgium). The PCR mix consisted of the following ingredients: 15 µL H_2O, 2.5 µL PCR buffer, 1 µL (50 mmol L^{-1}) $MgCl_2$, 2.5 µL dNTP, 2 µL of each specific primer (10 µmol), 0.25 µL Platinum Taq-Polymerase, and 2 µL DNA template. The cycling profile was: denaturation at 94°C for 60 s, followed by 35 cycles at 94°C for 30 s, 67°C for 30 s, and 72°C for 30 s, and a final extension at 70°C for 5 min.

3.2. Cellulose enzyme and fungicide effect assays

All isolates were tested for cellulose hydrolysis by endoglucanase test using carboxymethyl cellulose. Screening of cellulose-producing fungi was done on CMC selective agar containing: 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethylcellulose (CMC) sodium salt, 0.02% peptone and 1.7% agar (Abu-Bakar *et al.*, 2010). Medium was autoclaved at 121ºC for 21 min (Autotester ST DRY PV II, Selecta). Mycelia obtained from isolates previously grown on PDA was transferred to CMC medium. Stock cultures were maintained on PDA agar at 4ºC for subsequent use as inoculum. Plates of CMC with inoculum were incubated at 21 ± 4ºC in darkness. Three plates were prepared for each isolate. Control plates were also prepared. After three days of incubation, plates were flooded with Gram's Iodine solution for 15 minutes. Gram's Iodine solution consists on KI 2 g, iodine 1 g diluted into 300 ml distilled water (Kasana *et al.*,

2008). After removing Gram's solution from plates, a clearance zone around the colony was observable where the cellulose was degraded and a photograph of each plate was taken. TpsDig2w32 (Rohlf, 2018) program was used to measure cellulose degradation. This program allows measuring the clearance zone where cellulose was degraded by fungi activity.

Same assay was performed under the use of two different fungicides, benomyl and tebuconazole. They are the most commonly used fungicides to treat plant pathogenic fungi in the studied areas. To prove their toxicity against fungi, 10 mg/l of benomyl and tebuconazole were added to previously prepared CMC media. Two replicates for each isolate and fungicide and control plates were prepared. Plates were conserved at 21 ± 4ºC in darkness for three days. Growth measure procedure was the same as the one previously defined for CMC test without fungicides.

3.3. API® identification system

API® is a standardized microbial identification system, it is commonly used to identify more than 600 different species of bacilli (Gram+ and Gram-), cocci (Gram + and Gram -) and yeast. For this study API® ID 32 C (bioMérieux, Marcy l'Etoile, France) was used. It consists of 32 cupules, each containing a dehydrated carbohydrate substrate, constituting in effect a miniaturized assimilation system. A semisolid, chemically defined minimal medium is supplied and a mycelia plug was added. The substrates analysed with ID 32 C system are: galactose, actidione, sucrose, N-acetylglucosamine, lactic acid, L-arabinose, cellobiose, raffinose, maltose, trehalose, potassium 2-ketogluconate, methyl-αD-glucopyranoside, mannitol, lactose, inositol, sorbitol, D-xylose, ribose, glycerol, rhamnose, palatinose, erythritol, melibiose, sodium glucuronate, melezitose, potassium gluconate, levulinate, glucose, sorbose, glucosamine and esculin. Sorbitol was used as a positive control, and a blank cupule is included as a negative control.

The strips were incubated for 7 days at 29 ± 4ºC in darkness. After a week the mycelia has grown in the cupules and media colour change was observable. Magnifying glasses were used to collect visual data; three values were stablished to differentiate growth process for the mycelia. 0 value was given when growth was not observable, 1 value when growth was not observable but media colour changes and 2 value when mycelia grow.

3.4. Inoculation trial

Pathogenicity test were performed using two-year-old *P. radiata* plug seedlings. Trees were maintained in a biosafety level 2 greenhouse at 22 ± 4ºC, with a relative humidity of 55-60% and without supplemental light. Plugs were maintained 4 weeks for acclimatization to greenhouse conditions. For inoculation approximately 3 cm were excised from the shoot in order to expose the pith. An agar plug (approx. 3 to 4 mm^2) taken from a growing culture was placed on the wound. Four trees were inoculated for each of the 223 isolates. Control trees were treated in the same manner; however, no inoculum plugs were placed on the wounds. Trees were maintained for 5 weeks, under described conditions, and irrigated to avoid water stress. The length of those lesions was recorded using a digital Vernier. At the end of the experiment, 100 trees where arbitrary chosen and pathogen was re-isolated from symptomatic tissues on PDA. Aggressiveness data was collected and three groups were defined attending to lesion lengths; isolates with lesion lengths between 0-20 mm were assigned with value 1, lesions between 20-40 mm were assigned with value 2 and for lesions bigger than 40 mm 3 value was assigned.

3.5. Statistical analysis

Measures of the cellulose degradation (decolourization zone) with and without fungicide effect were reduced to a unique value (mean ± SD) for each sample set. Waller-Duncan test was performed in order to analyse cellulose degradation according to the defined samples groups using SAS 9.1.3 service pack 3 (2008). An analysis of T-test was performed to establish relation between species and fungicide effect (SAS, 2008). ANOVA statistical test was performed with SAS 9.1.3 service pack 3 (2008) to identify any relation between cellulose degradation, fungicide effect and aggressiveness.

To distinguish potential differences among species (*D. sapinea* and *D. scrobiculata*) and populations (P1, P2, P3, BC and W) contingency tables were constructed by mean lesion lengths, and different media degradation (API®). Pearson's χ^2 test was run in order to detect significant differences (SAS, 2008). Each factor was tested using an error term of α=0.05.

4. Results

4.1. Samples identification

All the strains from plantations sets (P1=10, P2=13 and P3=13) were only positive for *D. sapinea*. Of the 109 isolates obtained from Basque Country sample set, all but one (identified as *D. scrobiculata*) were *D. sapinea*. Hosts were mostly from *P. radiata*, but occasional hosts included *Pinus attenuata* Lemmon, *Pinus contorta* Douglas, *Pinus halepensis* Mill., *Pinus nigra* J. F. Arnold, *Pinus pinaster* Ait. and *Pinus sylvestris* L. Isolates in the W set included 52 of *D. sapinea* and 26 of *D. scrobiculata*, with both of these pathogens having been obtained from a much greater variety of pine and nonpine hosts.

4.2. Cellulase enzyme and fungicide effect assays

All isolated fungi (223) were subjected to CMC agar for cellulose degradation analyses. Growth of the fungi was observed after 3 days of incubation and flooded with Gram's Iodine solution for decolourization zone observation and measurement. Decolourization zone made by fungi showed secretion of cellulase enzymes in order to degrade cellulose structure of CMC (Figure 6.1.). P1 *D. sapinea* (21.55 mm) and BC *D. scrobiculata* (27.35 mm diameter) isolates showed the largest decolourization zones compared to worldwide (W) samples (15.49 mm and 16.55 mm diameter respectively) (Table 6.1.). The growth reduction due to fungicides was also measured, for *D. sapinea* isolates, benomyl and tebuconazole fungicides were responsible of the reduction of 37.74% and 42.68% respectively for BC and of 14.11% and 19.25% for W population (Figure 6.2.) (Table 6.1.). The growth reduction for *D. scrobiculata* was 20.57% for benomyl and 33.65% for tebuconazole for BC and 7.73% and 17.81% for W (Table 6.1.).

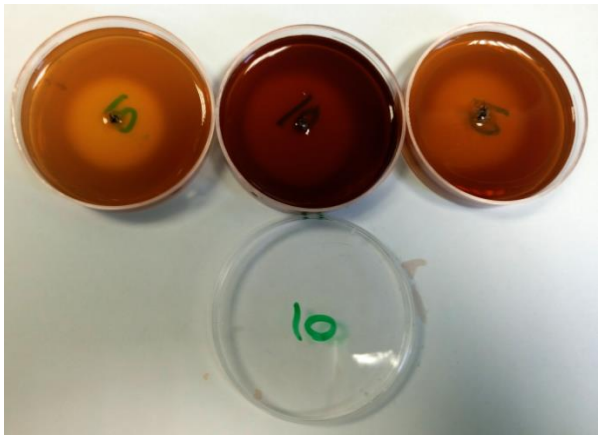


Figure 6.1. Fungi strain 10 showed degradation of CMC agar with appearance of decolourization zone after flooding with Gram's Iodine solution.

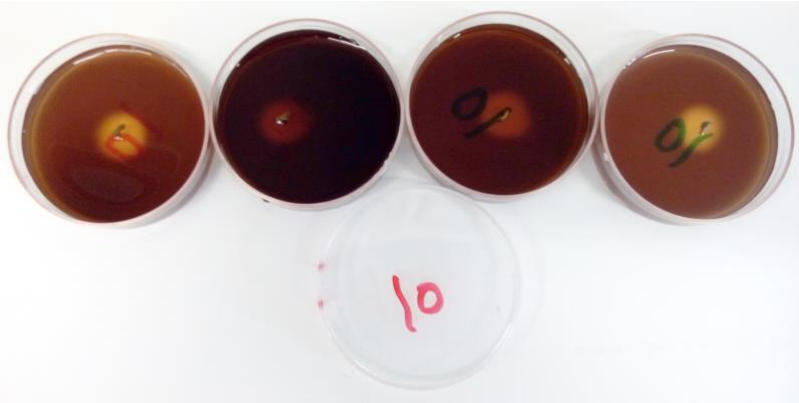


Figure 6.2. Fungi strain 10 showed degradation of CMC agar under benomyl (two plates in left side) and tebuconazole (two plates in right side) with appearance of decolourization zone after flooding with Gram's Iodine solution.

Table 6.1. Means, standard deviation (SD), ranges of lesion length (mm) and growth reduction (%) resulting from the study of 223 isolates of *Diplodia sapinea* and *Diplodia scrobiculata* from different origins.

D. sapinea	**N**	**Cellulose $\overline{X} \pm SD$[a]**	**Range cellulose[b]**	**Benomyl $\overline{X} \pm SD$[c]**	**Range benomyl[d]**	**Growth reduction Benomyl[e]**	**Tebuconazole $\overline{X} \pm SD$[f]**	**Range tebuconazole[g]**	**Growth reduction Tebuconazole[h]**
P1	10	21.55 ± 3.25	14.03-26.58	11.49 ± 2.9	4.65-16.60	46.07 ± 13.21	10.02 ± 3.12	4.82-16.88	52.65 ± 14.76
P2	13	18.54 ± 4.42	8.03-27.31	12.89 ± 2.95	6.90-17.83	27.28 ± 19.73	12.25 ±2.99	5.26-17.64	30.34 ± 24.46
P3	13	18.55 ± 4.08	11.09-27.09	12.74 ± 3.06	6.08-19.00	28.50 ± 23.21	11.06 ± 3.01	6.47-18.10	37.88 ± 20.55
BC	108	19.79 ± 4.56	2.91-35.79	12.01 ± 3.58	4.88-24.55	37.74 ± 18.57	11.04 ± 3.54	3.84-22.90	42.68 ± 17.69
W	52	15.49 ± 3.77	7.73-24.28	13.03 ± 3.94	3.05-20.38	14.11 ± 25.42	12.18 ± 3.60	2.87-19.59	19.25 ± 24.70
D. scrobiculata									
BC	1	27.35	26.53-28.42	21.72	18.62-24.82	20.57	18.81	17.30-18.99	33.65
W	26	16.55 ± 4.49	8.43-28.97	15.05 ± 3.58	8.40-21.92	7.73 ± 12.53	13.44 ± 3.74	6.76-21.46	17.81 ± 15.13

N number of isolates per each population.

[a] Mean growth lengths and standard deviation (SD) in cellulose media.
[b] Growth range in cellulose media.
[c] Mean growth lengths and standard deviation for benomyl.
[d] Growth range in cellulose media and benomyl fungicide.
[e] Growth reduction under benomyl effect and standard deviation expressed in percentage.
[f] Mean lesion lengths and standard deviation for tebuconazole.
[g] Growth range in cellulose media and tebuconazole fungicide.
[h] Growth reduction under tebuconazole effect and standard deviation expressed in percentage.

4.3. API® identification system

All isolates growth in API® system were visually analysed and categorized (Figure 6.3. and 6.4.). Simple agglomerative hierarchical clustering method UPGMA (unweighted pair group method with arithmetic mean) (SAS, 2008) was used to construct a dendrogram to observe how samples were distributed in relation to APIs data (Appendix F). Isolates from BC and W appear to form groups, but some samples were related despite their different origins and hosts. In general, isolates appear grouped together according to their origin so it can be established that they behave in a different way when growing in the tested media.

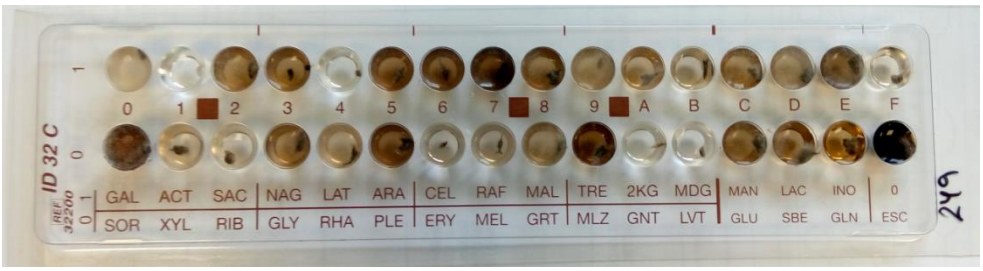


Figure 6.3. API® strip for fungi strain 249. This is an example of an isolate with good growth in almost all media.

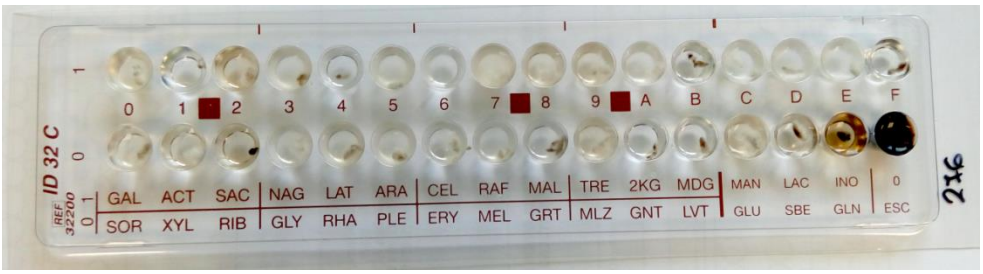


Figure 6.4. API® strip for fungi strain 276. In this case this sample show low growth rates in tested media.

4.4. Inoculation trial

Over all, *D. sapinea* isolates, show big mean lesion lengths in all sets except on P2 and P3 (Table 6.2.). For individual *D. sapinea* isolates, mean lesion lengths ranged from 4.4 to 141.5 mm, whereas *D.* scrobiculata ranged from 8.43 to 48.63 mm (Table 6.2.). Bigger lesion lengths were obtained in BC sample set with many isolates causing mean lesions over 40 mm (Table 6.2.).

Table 6.2. Means, standard deviation and ranges of lesions (mm) resulting from inoculation of *Pinus radiata* seedlings with 223 isolates of *Diplodia sapinea* and *Diplodia scrobiculata* from different origins.

D. sapinea	No. of isolates	Mean + SD[a]	Range[b]	Group		
				1[c]	2[d]	3[e]
P1	10	30.58 ± 6.49	20.65 – 43.6	-	9	1
P2	13	21.52 ± 7.70	10.98 – 34.16	6	7	-
P3	13	26.49 ± 12.18	9.28 – 54.85	3	8	2
BC	108	56.75 ± 25.81	11.89 – 141.5	6	26	76
W	52	29.75 ± 10.49	4.40 – 62.51	10	36	6
D. scrobiculata						
BC	1	27.12	20.6 – 32	-	1	-
W	26	26.41 ± 11.49	8.43 – 48.63	10	13	3

N number of isolates per each population.

[a] Mean lesion lengths and standard deviation (SD).
[b] Range of lesions.
[c] Number of isolates with lesion lengths between 0-20 mm.
[d] Number of isolates with lesion lengths between 20-40 mm.
[e] Number of isolates with lesion lengths from 40 mm and more.

4.5. Statistical analysis

T-test show significant differences in growth reduction attending to species under fungicide effect. Overall, *D. sapinea* isolates show greater reduction under fungicide effect for all sample sets, in particular, tebuconazole is the responsible of the 34% of the reduction growth whereas benomyl of the 27% (Table 6.3.). However, no significant reduction rates were appreciated in W sample set despite the species and fungicides.

Differences were detected in cellulose degradation attending to different sample sets according to Waller-Duncan test. Isolates from Basque Country (P1, P2, P3 and BC) show bigger degradation than those from W sample set (Table 6.3.). Three groups were established according to differences on cellulose degradation detected by the test. Results show higher degradation levels in P1 and BC, medium degradation levels were detected in P2 and P3 and lower levels in W (Table 6.3.).

Table 6.3. Means (mm), standard deviation (SD) and growth reduction (%) for each sample set.

Population	N	Cellulose $\overline{X} \pm SD^a$	Benomyl $\overline{X} \pm SD^b$	Growth reduction Benomyl[c](%)	Tebuconazole $\overline{X} \pm SD^d$	Growth reduction Tebuconazole[d] (%)
W	78	15.84 ± 3.46 (a)	13.70 ± 3.71	11.98	12.60 ± 3.44	18.77
P2	13	18.54 ± 4.11 (ab)	12.90 ± 2.07	27.28	12.25 ± 2.51	30.34
P3	13	18.55 ± 3.41 (ab)	12.74 ± 2.82	28.50	11.06 ± 2.74	37.88
P1	10	21.55 ± 2.53 (b)	11.49 ± 2.70	46.07	10.02 ± 2.83	52.65
BC	109	19.86 ± 4.05 (b)	12.10 ± 3.43	37.58	11.11 ± 3.25	42.60

N number of isolates per each population.

[a] Mean lesion lengths and standard deviation (SD) for each group of samples followed by different letters within a column are significantly different ($p \geq 0.005$) based on Waller-Duncan test.
[b] Mean lesion lengths and standard deviation for benomyl and group of samples.
[c] Growth reduction under benomyl effect expressed in percentage.
[d] Mean lesion lengths and standard deviation for tebuconazole and group of samples.
[e] Growth reduction under tebuconazole effect expressed in percentage.

Cellulose degradation, fungicide effect (benomyl and tebuconazole) and pathogenicity results were analysed by ANOVA statistical test. Results show significant relation between cellulose degradation and lesion lengths, those isolates with bigger cellulose degradation do not show the bigger lesions, as happens with *D. scrobiculata*.

Data obtained from API® system was related to species (*D. sapinea* and *D. scrobiculata*), population (P1, P2, P3, BC and W) and pathogenicity data through Pearson's χ^2 test. Significant differences were observable for species in raffinose, mannitol, glycerol and rhamnose media; for populations in actidione, L-arabinose and sodium glucuronate media; and for pathogenicity in raffinose, rhamnose, palatinose and sorbose media.

5. Discussion

Diplodia shoot blight caused by *D. sapinea* and *D. scrobiculata* has been proved to have a common occurrence in northern Spain *Pinus radiata* plantations, but also in worldwide forests (Burgess *et al.*, 2004, Phillips *et al.*, 2013; Luchi *et al.*, 2014). Damages in different parts of the tree ended in growth and vigour decrease, these facts joint with the high incidence of these two pathogens, entails important losses in plantations. Risk of an outbreak is highlighted by the predominance of *D. sapinea*, considered the more aggressive strain. Furthermore, it could behave as an endophyte and it presents many ways to

proliferate (Palmer *et al.*, 1987; Smith *et al.*, 1996; Blodgett & Stanosz, 1997; Flowers *et al.*, 2006).

The analysis of carbohydrates degradation in relation to lesion lengths allowed for greater resolution of population behaviour and for its physiology understanding. API® system and cellulose media were used to investigate the capacity of carbohydrate degradation among different species and populations.

Without regarding species, the results revealed bigger degradation of cellulose in Basque Country sample sets (P1>BC>P3>P2>W). In relation to fungicide degradation, tebuconazole presents bigger growth reduction rates for P1, P2, P3 and BC from 30.34% to 52.65%. For W isolates tebuconazole and benomyl were found to behave similarly and the growth reduction was insignificant. Based on these results, Basque Country's isolates could be considered to present resistances against benomyl fungicide. According to Iturritxa *et al.* (2011) benomyl has been widely used in northern Spain *Pinus radiata* nurseries and seed companies, it was applied in seedlings, cuttings and in seed treatments to control different diseases, among others, Diplodia shoot blight. Since it was considered toxic to wildlife and humans, its use has been forbidden by European Commission and tebuconazole has been used for same purposes. Worldwide samples seem to have undergone high levels of resistance for both of the fungicides tested and consequently they are no longer an option to control this disease in the studied areas.

According to previous studies, the diameter of clearance zone produced by fungi on CMC agar after 3 days of incubation ranged from 2.4-4.5 cm (Abu Bakar *et al.*, 2010). The results presented in this study show lower degradation rates with values between 1.5-2.7 cm. This means that both *D. sapinea* and *D. scrobiculata* pathogens without regarding the origin of the isolates have lower capacity to degrade cellulose in comparison to microbes obtained from oil palm empty fruit bunches.

D. scrobiculata show bigger cellulose degradation rates for BC and W isolates in comparison to *D. sapinea*. However, it was found to be less pathogenic in relation to inoculation trial results. Despite its reduced aggressiveness, it could lead to greater degradations of cellulose and consequently cause important damages and losses on wood quality (Gurnagul *et al.*, 1992). Furthermore, studied fungicides (benomyl and tebuconazole) have not shown to be as effective as for *D. sapinea* isolates. All this means that even if *D.*

scrobiculata has been considered less pathogenic (Palmer *et al.*, 1987; Blodgett & Stanosz, 1999; Blodgett & Bonello, 2003), it can cause important damages.

Overall, these pathogens were capable to degrade almost all the carbohydrates present in API® system. From all the 31 media analysed (1 control) degradation differences were found in; raffinose, mannitol, L-arabinose, palatinose and sorbose with important degradation rates. Whereas, actidione and glucoronate describe low degradation rates. These data showed the capacity that these fungi have to degrade different carbohydrates and therefore, it could be an indicative of their saprophytic and pathogenic condition.

It becomes important to highlight the fact that although *D. sapinea* isolates present greatest mean lesions lengths on *Pinus radiata* seedlings, *D. scrobiculata* isolates show the highest cellulase activity. In addition, the systematic application of the same fungicidal active ingredients, (originally benomyl and currently tebuconazole) during long periods of time could cause a reduction of the effectiveness of these fungicides, such reduction was evidenced in the performed tests results.

This study provides deeper understanding of *D. sapinea* and *D. scrobiculata* pathogens. The physiology analyses based on fungicidal active ingredients, carbohydrate degradation and pathogenicity have significant applied relevance when considering disease management and wood production. Identifying which species is the responsible of causing Diplodia shoot blight may be of more interest than previously thought.

6. References

Abu-Bakar, N. K., Abd-Aziz, S., Hassan, M. A., & Ghazali, F. M. (2010). Isolation and selection of appropriate cellulolytic mixed microbial cultures for cellulases production from oil palm empty fruit bunch. *Biotechnology*, *9*, 73-78.

Amponsah, N. T., Jones, E., Ridgway, H. J., Jaspers, M. V. (2012). Evaluation of fungicides for the management of *Botryosphaeria* dieback diseases of grapevines. *Pest Management Science*, *68*, 676-683.

Benhamou, N. & Picard, K. (2001). Encyclopedia of plant pathology. In O. C. Maloy & T. D. Murray (Ed.), *Cellulases*, 193-197. New York, NY: Willey.

Bester, W., Crous, P. W., Fourie, P. H. (2007). Evaluation of fungicides as potential grapevine pruning wound protectants against *Botryosphaeria* species. *Australasian Plant Pathology*, *36*, 73-77.

Blodgett, J. T., & Stanosz, G. R. (1997). *Sphaeropsis sapinea* morphotypes differ in aggressiveness but both infect non wounded red or jack pines. *Plant Disease*, *81*, 143-147.

Blodgett, J. T., & Bonello, P. (2003). The aggressiveness of *Sphaeropsis sapinea* on Austrian pine varies with isolate group and site of infection. Forest Pathology, 33, 15-19.

Burgess, T. I., Wingfield, M. J., & Wingfield, B. D. (2004). Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. *Australasian Plant Pathology*, *33*, 513-519.

Dehnad, D., Emam-Djomeh, Z., Mirzaei, H., Jafari, S.M., & Dadashi. S. (2014 a.). Optimization of physical and mechanical properties for chitosan–nanocellulose biocomposites. *Carbohydrate Polymers*, *105*, 222-228.

Dehnad, D., Mirzaei, H., Emam-Djomeh, Z., Jafari, S.M., & Dadashi, S. (2014 b.). Thermal and antimicrobial properties of chitosan–nanocellulose films for extending shelf life of ground meat. *Carbohydrate Polymers*, *109*, 148-154.

Díaz, G. A., Latorre, B. A. (2013). Efficacy of paste and liquid fungicide formulations to protect pruning wounds against pathogens associated with grapevine trunk diseases in Chile. *Crop Protection*, *46*, 106-112.

Duff, S. J. B., Cooper, D. G., & Fuller, O. M. (1987). Effect of media composition and growth conditions on production of cellulase and β-glucosidase by a mixed fungal fermentation. *Enzyme and Microbial Technology*, *9*, 47-52.

EU Pesticides database. (2011). *Benomyl*. Retrieved from European Commission website: http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.detail&language=ES&selectedID=1008

EU Pesticides database. (2017). *Tebuconazole*. Retrieved from European Commission website: http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.detail&language=EN&selectedID=1911

European Food Safety Authority (EFSA). (2014). *Conclusion on the peer review of the pesticide risk assessment of the active substance tebuconazole, 12*, 3485.

Flowers, J., Nuckles, E., Hartman, J., & Vaillancourt, L. (2001). Latent infection of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. *Plant Disease*, *85*, 1107–1112.

Fogarty, W. M., & Kelly, C. T. (1990). *Microbial enzymes and biotechnology*. Elsevier Science Publishers.

Food and Agriculture Organization of the United Nations (FAO). (1994). *Report of the Joint FAO/WHO Meeting on Pesticide Residues (JMPR), Rome*. Retrieved from FAO website: http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation94/tebucona.pdf

Gurnagul, N., Page, D. H., & Paice, M. G. (1992). The effect of cellulose degradation on the stranght of wood pulp fibres. *Nordic Pulp & Paper Research Journal*, *7*, 152-154.

Hazi Fundazioa. (2016). El bosque vasco en cifras 2017. Retrieved from www.euskadi.eus/contenidos/informacion/inventario_forestal_2016/es_agripes/adjuntos/El%20bosque%20vasco%20en%20cifras%202017.pdf

Iturritxa, E., Ganley, R. J., Raposo, R., García-Serna, I., Mesanza, N., Kirkpatrick, S. C., & Gordon, T.R. (2013). Resistance levels of Spanish conifers against *Fusariun circinatum* and *Diplodia sapinea*. *Forest pathology*. *43*, 488-495.

Iturritxa E., Desprez-Lostau, M. L., García-Serna, I., Quintana, E., Mesanza, N., & Aitken, J. (2011). Effect of alternative disinfection treatments against fungal canker in seeds of *Pinus radiata*. *Seed technology*, *33*, 88-110.

Kasana, R.C., Salwan, R., Dhar, H., Dutt, S., & Gulati, A. (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. *Current Microbiology*, *57*, 503-507.

Luchi, N., Oliveira Longa, C. M., Danti, R., Capretti, P., & Maresi, G. (2014). *Diplodia sapinea*: the main fungal species involved in the colonization of pine shoots in Italy. *Forest Pathology*, *44*, 372-381.

Manzanos, T., Aragonés, A., & Iturritxa, E. (2017). *Diplodia scrobiculata*: a latent pathogen of *Pinus radiata* reported in northerm Spain. *Phytopathologia Mediterranea*, *56*, 274-277.

Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December. (2008). *Official Journal of the European Union L353/1*. Retrieved from European Union Law website: https://eur-lex.europa.eu/legal-content/ES/TXT/?uri=CELEX%3A32008R1272

Palmer, M. A., Stewart, E. L., & Wingfield, M. J. (1987). Variation among isolates of *Sphaeropsis sapinea* in the north central United States. *Phytopathology*, *77*, 944–948.

Pearson, M. A., & Miller, G. W. (2014). Benomyl. *Encyclopedia of Toxicology*. 411-412.

Phillips, A. J. L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M.J., Groenewald, J.Z., & Crous, P. W. (2013). The *Botryosphaeriaceae*: genera and species known from culture. *Studies in Mycology*, *76*, 51-167.

Ramani, R., Gromadzki, S., Pincus, D. H., Salkin, I. F., & Chaturvedi, V. (1998). Efficacy of API 20C and ID 32C systems for identification of common and rare clinical yeast isolates. *Journal of Clinical Microbiology*, *36*, 3396–3398.

Righi, S., Morfino, A., Galletti, P., Samori, C., Tugnoli, A., & Stramigioli C. (2011). Comparative cradle-to-gate life cycle assessments of cellulose dissolution with 1-butyl-3-methylimidazolium chloride and N-methyl-morpholine-N-oxide. *Green Chemistry*, *13*, 367-375.

Rohlf, F. J. (2018). TpsDig2w32. Retrieved from: http://life.bio.sunysb.edu/morph/soft-dataacq.html.

SAS 9.1.3. Service Pack 3. (2008). SAS Institute INC. Cary, North Carolina, USA.

Smith, D. R., & Stanosz, G. R. (2006). A species-specific PCR assay for Detection of *Diplodia sapinea* and *D. scrobiculata* in dead red and jack pines with collar rot symptoms. *Plant Disease*, *90*, 307-313.

Stanosz, G. R., Blodgett, J. T., Smith, D. R., & Kruger, E. L. (2001). Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. *New Phytologist*, *149*, 531-538.

Strickland, T.C., Potter, T.L., & Joo, H. (2004). Tebuconazole dissipation and metabolism in Tifton loamy sand during laboratory incubation. *Pest Management Science*, *60*, 703-709.

Ypema, H. L. (2001). Encyclopedia of plant pathology. In O. C. Maloy & T. D. Murray (Ed.), *Systemic fungicides*, 999-1003. New York, NY: Willey.

Zhou J., Xiong K., Yang, Y., Ye, X., Liu, J., & Li, F. (2015). Deleterious effects of benomyl and carbendazim on human placental trophoblast cells. *Reproductive Toxicology*, *51*, 64-71.

Zwolinski, J. B., Swart, W. J., & Wingfield, M. J. (1990). Intensity of dieback induced by *Sphaeropsis sapinea* in relation to site conditions. *European Journal of Forest Pathology*, *20*, 167–174.

Discusión general

Pinus radiata es una de las especies más comunes en repoblaciones forestales en el País Vasco (Mapa Forestal, 2016). Esta especie se localiza principalmente en la vertiente atlántica de la Comunidad Autónoma Vasca y hasta los 600 m de altitud se encuentra en forma de monocultivo. Este tipo de explotación favorece el desarrollo y dispersión de diferentes tipos de patógenos (Bengtsson *et al*., 2000).

En este contexto, uno de los que aparece de forma más habitual en las plantaciones es *Diplodia sapinea* (Burgess *et al*., 2004a). Se trata de un hongo con comportamiento endofítico, (Eldridge, 1961; Swart & Wingfield, 1991; Hernández-Escribano *et al*., 2018) pero que tras la aparición de diferentes condiciones de estrés como son: el granizo, la sequía e incluso los fuertes vientos, es capaz de debilitar al hospedador ocasionando lesiones (Blodgett & Stanosz, 1997). Las heridas producidas por estos fenómenos favorecen la entrada y dispersión de forma patogénica de este hongo, causando la enfermedad de la marchitez de los brotes. Los principales daños observables son: la marchitez de los brotes, la podredumbre de la semilla, chancros en ramas y tallos, enfermedades en la raíz y el azulado de la madera (Zwolinski *et al*., 1990). En los casos más extremos se puede producir la mortalidad en plántulas jóvenes así como la muerte de los tallos terminales que provoca la malformación de los mismos y que conlleva una reducción de su crecimiento (Chou, 1976; Palmer & Nicholls, 1985). Los daños generados por *Diplodia sapinea* no se restringen únicamente al País Vasco, de hecho, se trata de un hongo con una amplia distribución en plantaciones forestales de todo el mundo y que afecta a un amplio rango de hospedadores (Waterman, 1943; Sutton, 1980; de Wet *et al*., 2000), aunque se considera que *P. radiata* es uno de los más susceptibles (Palmer, 1991; Burgess *et al*., 2004a; Smith & Stanosz, 2006).

Hasta la fecha se ha considerado que el desarrollo de la marchitez de los brotes en el País Vasco sobre *P. radiata* se asociaba únicamente a la especie *D. sapinea* (Iturritxa & Ganley, 2007). Sin embargo, en el desarrollo de esta Tesis Doctoral se ha detectado la presencia de *Diplodia scrobiculata* sobre este hospedador. La capacidad de generar menores daños relacionado con su carácter endófito, es un aspecto fundamental que dificulta su detección y por lo tanto su distribución se considera más limitada (Bihon *et al*., 2010). En el entorno natural de distribución de *P. radiata* se encuentra asociada a esta especie y no a *D. sapinea* (Burgess *et al*., 2001a). Diversos autores han considerado que *D. scrobiculata* no supone un problema sanitario en las plantaciones dado que los daños que provoca son poco significativos (Palmer *et al*., 1987; Blodgett & Bonello, 2003). Por lo que se ha

establecido que *D. sapinea* es la especie responsable del desarrollo de la enfermedad. No obstante, los resultados derivados de las pruebas de inoculación de 12 semanas han mostrado que el aislado de *D. scrobiculata* detectado en el País Vasco es capaz de generar lesiones mayores que *D. sapinea*.

Las dos especies del género *Diplodia* detectadas en las plantaciones del País Vasco fueron estudiadas y caracterizadas para determinar su tipo de reproducción. Diversos estudios llevados a cabo han concluido que el tipo de reproducción es asexual, aunque los elevados datos de diversidad observados hacen pensar que se esté produciendo otro tipo de reproducción (Bihon *et al*., 2012b; Burgess *et al*., 2004b). Por el contrario, no se han hallado elementos que defiendan la presencia de una reproducción sexual que pueda responder a la diversidad detectada (Bihon *et al*., 2011a). El estudio del tipo de apareamiento llevado a cabo en muestras del País Vasco, Estados Unidos y de diferentes países (Nueva Zelanda, México, Canadá, Israel…) no ha detectado una reproducción sexual, como se demuestra por la ausencia de los dos tipos de MAT (MAT1-1-1 y MAT1-2-1) en el mismo aislado. En cambio, el ratio de distribución de ambos MATs en valores cercanos al 1:1 en muestras del País Vasco sugiere la presencia de reproducción críptica.

Dado que los resultados de apareamiento han indicado la existencia de una posible reproducción críptica (Bihon *et al*., 2012a), se ha querido analizar la diversidad genotípica en las plantaciones del País Vasco. La diversidad hallada no ha sido elevada en ninguna de las poblaciones, aunque sí se han apreciado diferencias entre ellas. A nivel del País Vasco, debido al análisis de diferentes poblaciones, y dado que el material (incluyendo también a los hongos) podría provenir de diferentes orígenes, los valores de diversidad son algo mayores. A nivel de plantación, debido a que la entrada del hongo podría tener un mismo origen y unido al tipo de reproducción asexual característica, se detectaron valores menores de diversidad (Wingfield & Knox-Davies, 1980). Además, los picnidios producidos en los tejidos necróticos se diseminan de forma local por medio de la lluvia y el viento (Bihon *et al*., 2011b). Por lo que, aunque aparezca el tipo de reproducción críptica, la reproducción asexual también es importante en el desarrollo de la enfermedad.

Como se ha citado previamente, estos hongos se encuentran ampliamente distribuidos por todo el mundo y además son responsables de importantes pérdidas económicas (Currie & Toes, 1978; Nicholls & Ostry, 1990). Su presencia en un número importante de países se ha visto propiciada por el transporte de materiales, introduciendo de manera fortuita estos y otros muchos patógenos, que por sus características endófitas son difíciles de detectar y

prevenir (Sinclair & Lyon, 2005). Por todo ello, se ha llevado a cabo otro estudio de diversidad cuyo objetivo no fue únicamente determinar los valores de diversidad, sino identificar posibles similitudes entre aislados de distintos orígenes. Gracias a este estudio, se ha conseguido detectar en todas las poblaciones estudiadas alelos y haplotipos iguales, lo que puede indicar que estas muestras hayan podido tener un origen común. La prevalencia de estos alelos y haplotipos compartidos pueden resultar necesarios para el desarrollo de estas comunidades de hongos. El resultado más significativo derivado de este estudio ha sido la identificación de una importante cantidad de material genético compartido entre las poblaciones del País Vasco y del mundo. Legat (1930) considera que, en concreto, *D. sapinea* ha podido ser distribuido desde Europa al resto del mundo. Sin embargo, en el País Vasco, su presencia en *P. radiata* parece estar asociada a la introducción de material vegetal proveniente de diferentes países (Chile y Nueva Zelanda) para establecer las repoblaciones de esta especie (Iturritxa *et al*., 2008). Cabe añadir que los valores de diversidad detectados en el País Vasco, en los dos estudios llevados a cabo, han sido inferiores a los detectados en cualquier otra población (Estados Unidos y el resto del mundo). Asimismo, no es esperable que los valores de diversidad detectados en zonas nativas de distribución sean menores a los hallados en poblaciones en los que han sido introducidos (Burgess *et al*., 2004a; Bihon *et al*., 2011a). Por un lado, los valores de diversidad hallados podrían estar relacionados con un tipo de reproducción crítica. Por otro lado, la presencia de alelos únicos puede deberse a mutaciones o al desarrollo de adaptaciones a los diferentes lugares en los que se encuentran, aportándoles beneficios significativos para su establecimiento poblacional.

Para detectar diferencias en la fisiología de estas especies que puedan estar relacionadas con el desarrollo de mayores tamaños de lesión, se ha analizado la capacidad de degradación de diferentes carbohidratos. Los patógenos safrófitos, con son *D. sapinea* y *D. scrobiculata*, están involucrados en la degradación de las paredes celulares de los hospedadores sobre los que se encuentran, permitiendo la penetración y la invasión de los tejidos (Fogarty & Kelly, 1990). En concreto, estos hongos están asociados con la degradación de la celulosa, aunque son capaces de degradar una gran cantidad de carbohidratos (Duff *et al*., 1987), como se ha detectado en este estudio. Los resultados han indicado que si bien *D. scrobiculata* no genera las mayores lesiones sobre plántulas en ensayos de 5 semanas, sí que genera las mayores degradaciones de celulosa. Los estudios con fungicidas llevados a cabo han demostrado, en el caso del benomyl, una reducida eficacia para controlar la degradación de celulosa y por tanto el crecimiento de los hongos. Su empleo para controlar diferentes enfermedades fúngicas durante muchas décadas ha

podido desencadenar una respuesta de resistencia. La aplicación de tebuconazole parece tener un mayor efecto como fungicida, ya que reduce el crecimiento de ambos hongos en un porcentaje mayor. No obstante, para el aislado de *D. scrobiculata* del País Vasco así como para los aislados de ambas especies del mundo ninguno de los dos fungicidas estudiados parece resultar efectivo para su control.

Los diferentes aislados testados han sido capaces de desarrollar lesiones de diferente magnitud sobre plántulas de *P. radiata*. Todos los estudios llevados a cabo hasta el momento también han mostrado diferentes capacidades lesivas, aunque no se han identificado los factores por los cuales unos aislados generan mayores daños que otros (Blodgett & Stanosz, 1997; Bihon *et al*., 2011a). En este caso, se ha descartado que los tipos de MAT puedan tener relación directa con la capacidad de generar daño sobre el hospedador. Se ha detectado que al igual que sucede con el crecimiento en PDA, *D. scrobiculata* parece presentar un crecimiento más lento en los ensayos de inoculación. Si bien en ensayos de 5 semanas llevados a cabo se han identificado lesiones menores para aislados de *D. scrobiculata*, en ensayos de 12 semanas parecen generar mayores lesiones que los de *D. sapinea*. Este hecho unido a la facilidad que presenta este patógeno de degradar celulosa hace pensar en que existen diferencias metabólicas en su desarrollo en comparación con *D. sapinea*.

En este sentido, resulta fundamental realizar una identificación del hongo que está presente en cada población, dado que los resultados han demostrado un comportamiento distinto para cada una de ellas. En este sentido, la detección y gestión temprana va a resultar fundamental para eliminar unos de los agentes que podrían poner en riesgo las plantaciones de *P. radiata* en el País Vasco.

Al no encontrarse catalogadas como especies de cuarentena por la EPPO (European and Mediterranean Plant Protection Organization), no hay medidas legales que regulen su gestión. En primer lugar, y como se ha descrito previamente estos patógenos pueden introducirse con material vegetal, tanto plántulas como semillas. Por lo que resultaría imprescindible desarrollar una serie de acciones que permitan identificar el material infectado antes de su establecimiento en plantaciones forestales. Por otro lado, la ausencia de fungicidas que hayan resultado eficaces en el control, especialmente de *D. scrobiculata* así como de los aislados de diferentes orígenes, hace necesaria la búsqueda de alternativas.

Las heridas producidas sobre las ramas y el tronco de los árboles actúan como vías de la entrada de estos hongos. Estas pueden derivar de actividades humanas como son las podas, por lo que estas actuaciones deben de estar restringidas lo máximo posible y llevarse a cabo en los meses de invierno conociendo el momento en el que estos hongos son menos activos (Swart *et al*., 1987). No obstante, los factores que son capaces de desarrollar daños severos e incontrolables son los de tipo meteorológico. En este contexto y ante el escenario de cambio climático en el que nos encontramos, estos fenómenos (el granizo o la sequía) van a ser cada vez más recurrentes y van a tener consecuencias importantes sobre las dinámicas de las enfermedades y su evolución va a restringir finalmente la supervivencia de las especies arbóreas más susceptibles (Garrett *et al*., 2006; Lindner *et al*., 2010; Chakraborty & Newton, 2011).

La detección de las zonas afectadas por granizo de forma precoz mediante registros en las estaciones meteorológicas, podría ayudar a identificar los pies con mayores daños y proceder a su tratamiento preventivo o eliminación, de modo que no se conviertan en foco de infección. Sin embargo, los daños derivados de las sequías no se pueden gestionar. Ante esta situación y teniendo en cuenta que *P. radiata* es uno de los más susceptibles, podría resultar necesario el establecimiento de nuevas especies (Wingfield & Swart, 1994).

En este sentido, es importante identificar otras especies que presenten tolerancias tanto a enfermedades como a condiciones climáticas desfavorables. Cambiar el manejo de grandes extensiones de monocultivo, introduciendo diferentes especies tanto coníferas como frondosas autóctonas, podría mejorar la diversidad de estas plantaciones y reducir el impacto que el brote de una determinada enfermedad pueda producir tanto a nivel ambiental como económico (Gamfeldt *et al*., 2013). La creación de bosques productivos entendidos como entornos de conservación, recreo y producción podría ser una alternativa eficaz contra la producción en monocultivos especialmente en montes de utilidad pública. Estos entornos contarían con árboles productivos de diferentes especies y edades distribuidos por las parcelas, de modo que al hacer un aprovechamiento de los mismos no amenacen la estructura del bosque. En concreto en Suecia cerca de 1 millón de hectáreas forestales se encuentran bajo esta figura (Nilson, 2018).

Estudios previos han mostrado la gran supervivencia de las esporas de estos patógenos sobre material de desecho de las talas (piñas, corteza, ramas y acículas) que incluso 5 años después actúan como fuente de inóculo para las nuevas repoblaciones de pino (Oblinger *et al.* 2011). Si bien el abandono de estos desechos es una fuente importante de nutrientes

para el suelo, también presenta un riesgo significativo para la reforestación. Para reducir la presencia de estos desechos sería interesante valorizarlos dado que de media se generan entre 23-41 ton/ha de desechos en plantaciones de *P. radiata* (Guzmán, 1984). En Chile se han introducido estos desechos para la producción de energía eléctrica por biomasa, lo que supone una diversificación del mercado.

En muchas ocasiones se mantienen estos desechos de las podas y las talas en las plantaciones para evitar los daños derivados de la erosión, especialmente en entornos, con elevadas pendientes como en el caso del País Vasco (Bertrán & Morales, 2008). Así mismo su incorporación al suelo permite recuperar algunos de los nutrientes necesarios para el desarrollo de las futuras plantaciones (Bertrán & Morales, 2008). Por lo tanto, la introducción de otras especies con diferentes tasas de crecimiento podría prevenir la erosión sin necesidad de dejar estos desechos con el riesgo que ello evitaría.

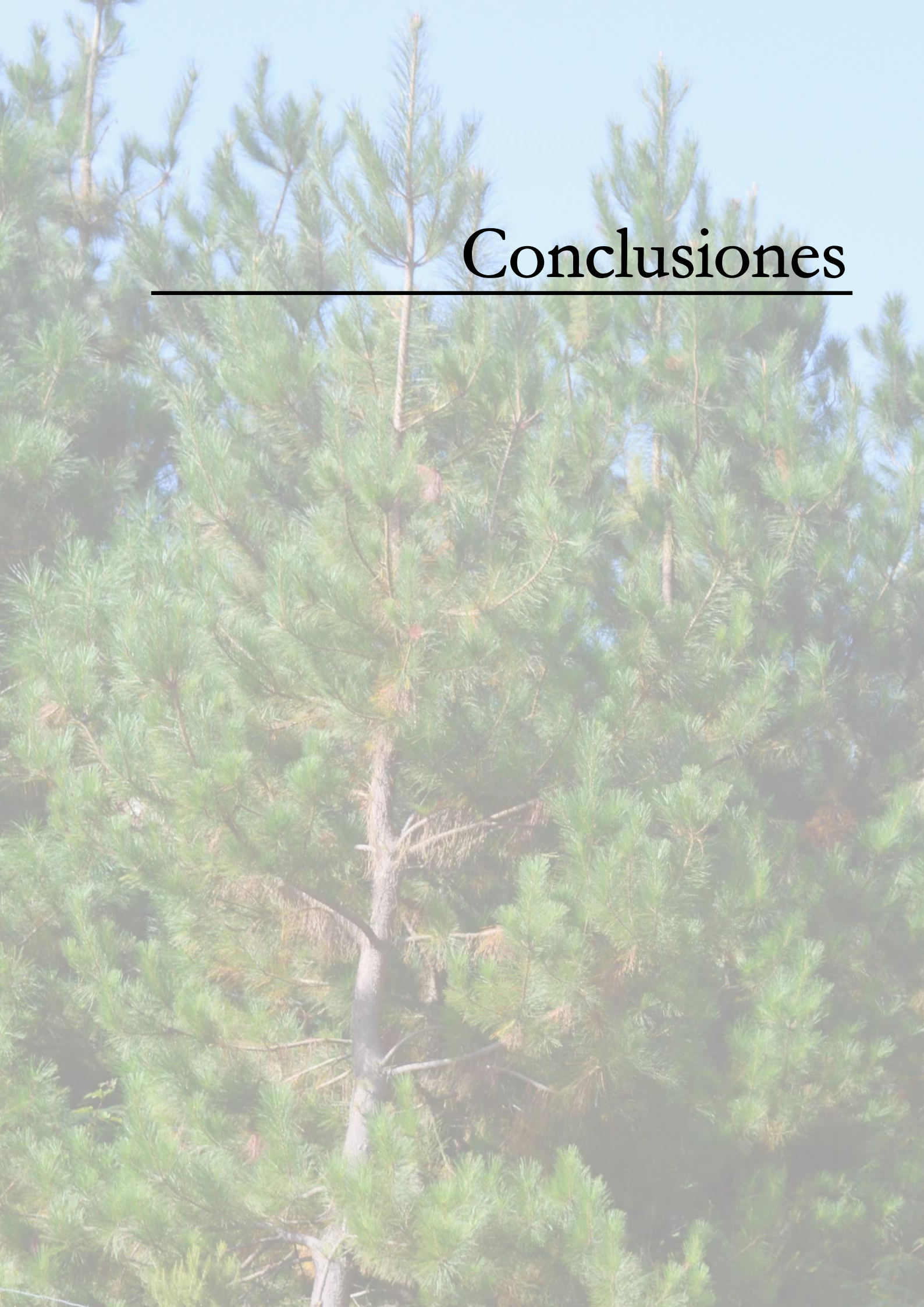
La mejora genética es una las técnicas que puede resultar vital para favorecer la adaptación ante diferentes situaciones como son; el cambio climático, el aumento de las enfermedades así como el incremento de la productividad de las masas forestales (Dennis *et al.*, 2007). A pesar de ello, los resultados de esta técnica son solo observables tras largos periodos de tiempo. Los sistemas de mejora necesitan, en el caso de *P. radiata*, de al menos 35 años para obtener los primeros resultados (Burdon *et al.*, 2008). Por lo que hasta ese momento sería recomendable llevar acabo algunas de las recomendaciones anteriormente descritas.

La importancia económica y social del sector así como la presencia de diferentes especies patógenas capaces de generar daños severos sobre las plantaciones de *P. radiata*, remarcan la importancia de mejorar la gestión forestal de esta especie. En este contexto, la introducción de nuevas especies resulta vital para la supervivencia de un sector fuertemente dominado por la elevada productividad de las plantaciones de *P. radiata*. La valorización de diferentes aspectos que engloban el forestalismo resulta fundamental para el mantenimiento sostenible de uno de los sistemas más amenazados por el cambio climático.

Los hallazgos resultantes de esta Tesis Doctoral, han permitido no solo mejorar el conocimiento sobre la enfermedad de la marchitez de los brotes a nivel del País Vasco, sino también establecer posibles similitudes entre aislados separados geográficamente. Además, se ha aportado mayor información sobre la biología y la fisiología de estos hongos patógenos. Todo ello va a permitir mejorar el manejo de esta enfermedad y por lo tanto,

aumentar la sanidad de las plantaciones forestales afectadas y reducir las pérdidas económicas derivadas.

Conclusiones



La enfermedad de la marchitez de los brotes es responsable de importantes pérdidas económicas tanto en plantaciones de *P. radiata* del País Vasco como en otras especies, especialmente, del género *Pinus* en todo el mundo. A continuación, se muestran las contribuciones más relevantes de la presente Tesis Doctoral organizadas de acuerdo a los objetivos definidos al inicio.

1. La enfermedad de la marchitez de los brotes se encuentra asociada a dos especies en las plantaciones de *P. radiata* del País Vasco: *D. sapinea* y *D. scrobiculata*. Todos los aislados estudiados son capaces de generar lesiones de diferente gravedad sobre plántulas de *P. radiata*. En concreto, *D. scrobiculata* ha mostrado ser capaz de ocasionar mayores lesiones a las registradas por *D. sapinea* en ensayos de 12 semanas.

2. La reproducción de los hongos ha resultado ser de tipo asexual para todas las muestras. Sin embargo, la distribución de los dos tipos de MATs en un ratio 1:1 indica la presencia de una reproducción de tipo críptica. La distribución de los MATs en el País Vasco no presenta correlación, lo que responde a las diferentes introducciones que se han producido de estos hongos junto con material vegetal desde otros orígenes. El tamaño de las lesiones desarrolladas no parecen tener relación con el tipo de MAT que presenta el aislado.

3. Los estudios de diversidad genética han identificado 14 haplotipos y 13 VCGs distintos para los aislados de *D. sapinea* del País Vasco. Lo que indica que ambos métodos son igualmente eficaces para determinar la diversidad poblacional. Los máximos de diversidad genotípica obtenidos son inferiores a los mostrados por aislados introducidos en otros países. La mayor diversidad a nivel territorial es debida a las diversas introducciones que se han registrado y la reducida diversidad en las plantaciones se debe a la predominancia de la reproducción asexual.

4. La distribución de genotipos y alelos entre los aislados, del País Vasco, de Estados Unidos y del mundo, sugiere que estos hongos podrían tener un origen común. A su vez, la presencia de haplotipos y alelos comunes en todas las poblaciones podría ser fundamental para el desarrollo de la enfermedad de la marchitez de los brotes. La diversidad genotípica encontrada y la presencia de alelos únicos, sugiere que estos hongos no presentan una reproducción únicamente asexual. La imposibilidad de hallar indicios de reproducción sexual (tanto a nivel físico como molecular), indica que la reproducción críptica podría resultar clave en el ciclo reproductivo de estos hongos.

Los datos de diversidad así como la presencia de alelos únicos podrían ser el resultado de una adaptación competitiva a los diferentes ecosistemas en los que se encuentran.

5. El uso de carbohidratos ha permitido detectar diferencias entre los aislados de *D. sapinea* y *D. scrobiculata*. Los resultados han mostrado la gran capacidad que presentan estos hongos para degradar distintos tipos de compuestos, afirmando su condición patogénica. A su vez, se ha identificado que si bien de forma general los aislados de *D. scrobiculata* no son los que causan las lesiones más importantes sobre plántulas en ensayos de 5 semanas, si son los que presentan mayores tasas de degradación de celulosa. Por lo que aunque actúen como endófitos, en condiciones óptimas de crecimiento podrían generar una importante reducción de la calidad de la madera.

Referencias

Adomas**,** A., Asiebu, F. O., & Stenlid, J. (2005). Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. s. l. *Molecular Plant Pathology*, *6*, 395-409.

Amezaga, I. (1996). Monterrey pine (Pinus radiata D. Don) suitability for the pine shoot beetle (*Tomicus piniperda* L.) (Coleoptera: Scolytidae). *Forest Ecology and Management*, *86*, 73-79.

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., & Daszak, P. (2004). Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends Ecology Evolution*, *19*, 535-544.

ASEMFO. (2012). *VII Estudio de inversión y empleo en el sector forestal. Años 2009-2010*. Retrieved from Asociación Nacional de Empresas Forestales, www.foresdat.es

Axelrod, D. I. (1980). History of the maritime closed-cone pines, Alta and Baja California. *University of California Publications in Geological Sciences*, *120*.

Bachi, P. R., & Peterson, J. L. (1985). Ehancement of *Spaheropsis sapinea* stem invasion of pines by water deficits. *Plant Disease*, *69*, 798-799.

Bamber, R. K., & Burley, J. (1983). *The wood properties of radiata pine*. Slough, UK: Commonwealth Agricultural Bureaux.

Bandyopadhyay, R., & Frederiksen, R. A. (1999). Contemporary global movement of emerging plant diseases. *Annals of the New York Academy of Sciences*, *894*, 28-36.

Bannister, M. H. (1962). Some variations in the growth pattern of *Pinus radiata* in New Zealand. *New Zealand Journal of Science*, *5*, 342-370.

Bengtsson, J., Nilsson, S. G., Franc, A., & Menozzi, P. (2000). Biodiversity, disturbances, ecosystem function and management of European forests. *Forest Ecology and Management*, *132*, 39-50.

Bertrán, S. J., Morales, V. E. (2008). *Potencial de biomasa forestal*. Comisión Nacional de Energía (CNE), Santiago, Chile: 56.

Bihon, W., Slippers, B., Burgess, T., Wingfield, M. J., & Wingfield, B. D. (2012a). Diverse sources of infection and cryptic recombination revealed in South African *Diplodia sapinea* populations. *Fungal Biology*, *116*, 112–120.

Bihon, W., Burgess, T., Slippers, B., Wingfield, M. J., & Wingfield, B. D. (2012b). High levels of genetic diversity and cryptic recombination is widespread in introduced *Diplodia sapinea* populations. *Australasian Plant Pathology*, *41*, 41-46.

Bihon, W., Slippers, B., Burgess, T., Wingfield, M. J., & Wingfield, B. D. (2011a). *Diplodia scrobiculata* found in the southern hemisphere. *Forest Pathology*, *41*, 175-211.

Bihon, B. W., Slippers, B., Burgess, T., Wingfield, M. J., Wingfield, B. D. (2011b). Sources of *Diplodia pinea* endophytic infections in *Pinus patula* and *P. radiata* seedlings in South Africa. Forest. Patholology, *41*, 370-375.

Bihon, W., Slippers, B., Burgess, T., Wingfield, M. J., & Wingfield, B. D. (2010). *Diplodia scrobiculata* found in the southern hemisphere. *Forest Pathology*, *41*, 175–181.

Blodgett, J. T., & Bonello, P. (2003). The aggressiveness of *Sphaeropsis sapinea* on Austrian pine varies with isolate group and site of infection. *Forest Pathology*, *33*, 15-19.

Blodgett, J. T., & Stanosz, G. R. (1999). Differences in aggressiveness of *Sphaeropsis sapinea* RAPD marker group isolates on several conifers. *Plant Disease*, *83*,853-856.

Blodgett, J. T., & Stanosz, G. R. (1997). *Sphaeropsis sapinea* morphotypes differ in aggressiveness but both infect non wounded red or jack pines. *Plant Disease*, *81*, 143-147.

Brasier, C. M., Cooke, D. E. L., & Duncan, J. M. (1999). Origin of a new *Phytophthora* pathogen through interspecific hybridization. *Proceedings of the National Academy of Sciences of the United States of America*, *96*, 5878-5883.

Brookhouser, L. W., & Peterson, G. W. (1971). Infection of Austrian, Scots, and ponderosa pines by *Diplodia pinea*. *Phytopathology*, *61*, 409-414.

Browne, F. G. (1968). *Pests and diseases of forest plantation trees: An annotated list of the principal species occurring in the British Commonwealth*. Oxford, UK: Clarendon Press.

Burdon, R. D., Carson, M. J., & Shelbourne, C. J. A. (2008). Achievements in forest tree genetic improvement in Autralia and New Zealand 10: *Pinus radiata* in New Zealand. *Autralian Forestry*, *71*, 4.

Burdon, R.D. (1994). Annual growth stages for height and diameter in *Pinus radiata*. *New Zealand Journal of Forestry Science*, *24*, 11-17.

Burdon, R. D., & Miller, J. T. (1992). Introduced forest trees in New Zealand: recognition, role, and seed source. *FRI Bulletin*, *124*.

Burdon, R. D., Firth, A., Low, C. B., & Miller, M. A. (1998). Multi-site provenance trials of *Pinus radiata* in New Zealand. Italy, Rome: Forest Genetic Resources (FAO).

Burdon, R. D. (2000). *Pinus radiata*. In Last FT (Eds.), *Ecosystems of the World. Tree Crops Ecosystems, 19*, 99-161. Amsterdam, Netherlands: Elsevier.

Burgess, T. I., Gordon, T. R., Wingfield, M. J., & Wingfield, B. D. (2004a). Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. *Mycological Research*, *108*, 1399–1406.

Burgess, T. I., Wingfield, M. J., & Wingfield, B. D. (2004b). Global distribution of *Diplodia sapinea* genotypes revealed using simple sequence repeat (SSR) markers. *Australasian Plant Pathology*, *33*, 513–519.

Burgess, T., Wingfield, B. D., & Wingfield, M. J. (2001). Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*. *Mycological Research*, *105*, 1331-1339.

Cavey, J., Passoa, S., & Kucera D. (1994). *Screening Aids for Exotic Bark Beetles in the Northeastern United States*. NA-TP-11-94. Retrieved from United States Department of Agriculture, Forest Service, http://caps.ceris.purdue.edu/screening/exotic_bark_beetles_of_northeast

Chakraborty, S., & Newton, A. C. (2011). Climate change, plant diseases and food security: an overview. *Plant Pathology*, *60*, 2-14.

Chou, C. K. S., & Mackenzie, M. (1988). Effect of pruning intensity and season on *Diplodia pinea* infection of *Pinus radiata* stem through pruning wounds. *European Journal of Forest Pathology*, *18*, 437-444.

Chou, C. K. S. (1976). A shoot dieback in *Pinus radiata* caused by *Diplodia pinea*. 1. Symptoms, disease development, and isolation of pathogen. *New Zealand Journal of Forestry Science*, *6*, 72-79.

Ciesla, W. M. (2001). *Exotic Forest Pest Information System for North America: Ips sexdentatus*. Retrieved from North American Forestry Commission, https://www.invasive.org/browse/subinfo.cfm?sub=887

Cronk, Q. C. B., & Fuller, J. L. (1995). *Plant invaders: the threat to natural ecosystems*. London, UK: Chapman & Hall, ltd.

Commonwealth Mycological Institute. (1964). *C. M. I. Descriptions of pathogenic fungi and bacteria*, 1. Kew, Surrey, England: Commonwealth Agricultural Bureaux.

Currie, D., & Toes, E. (1978). Stem volume loss due to severe *Diplodia* infection in a young *Pinus radiata* stand. *New Zealand Journal of Forestry*, *23*, 143-148.

Da Costa, E. B. (1955). Effect of blue stain on the strength of *Pinus radiata*. *Forest Products News Letter*, *209*, 1-2.

Day, K. R., & Leather, S. R. (1997). Threats to forestry by insect pests in Europe. In Watt, A. D., Stork, N. E., & Hunter M.D. (Eds.), *Forests and Insects*. London, UK: Chapman & Hall.

Denman, S., Crous, P. W., Taylor, J. E., & Kang, J. (2000). An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. *Studies in Mycology*, *45*.

de Wet, J., Burgess, T. I., Slippers, B., Presig, O., Wingfield, B. D., & Wingfield, M. J. (2003). Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and identify a new species of *Diplodia*. *Mycological Research*, *107*, 557-566.

de Wet, J., Wingfield, M. J., Coutinho, T. A., & Wingfield, B. D. (2000). Characterization of *Sphaeropsis sapinea* isolates from South Africa, Mexico and Indonesia. *Plant Disease*, *84*, 151-156.

Deniss, E. S., Ellis, J., Green, A., Llewellyn, D., Morell, M., Table, L., & Peacock, W. J. (2007). Genetic contributions to agricultural sustainability. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*, 591-609.

Desprez-Loustau, M-L., Robin, C., Buee, M., Courtecuisse, R., Garbaye, J., Suffert, F., Sache, I., & Rizzo, D. M. (2007). The fungal dimension of biological invasions. *Trends Ecology Evolution*, *22*, 472-480.

Devkota, B., & Schmidt, G. H., 1990. Larval development of *Thaumetopoea pityocampa* (Den. & Schiff.) (Lep., *Thaumetopoeidae*) from Greece as influenced by different host plants under laboratory conditions. *Journal of Applied Entomology*, *109*, 321-330.

Duff, S. J. B., Cooper, D. G., & Fuller, O. M. (1987). Effect of media composition and growth conditions on production of cellulase and β-glucosidase by a mixed fungal fermentation. *Enzyme and Microbial Technology*, *9*, 47-52.

Eldridge, K. G. (1961). Significance of *Diplodia pinea* in plantations. *Review of Applied Mycology*, *41*, 339.

Elvira-Recuenco, M., Eugenia Iturritxa, E., & Raposo, R. (2015). Impact of seed transmission on the infection and development of pitch canker disease in *Pinus radiata*. *Forests*, *6*, 3353-3368.

Eriksson, L., Boberg, J., Cech, T.L., Corcobado, T., Desprez-Lostau, M. L., Hietala, A. M., Jung, M. H., Lehtijarvi, H. T. D., Oskay, F., Slavov, S., Solheim, H., Stenlid, J., & Oliva, J. (2018). Invasive forest pathogens in Europe: Cross-country variation in public awareness but consistency in policy acceptability. *Ambio*.

EPPO. (2002). *Thaumetopoea pityocampa*. Retrieved from EPPO Global Database, https://gd.eppo.int/taxon/THAUPI

Eurostat. (2012). *Roundwoods removals by type pf wood and assortment in EU-27*. Retrieved from European Commision Statistics, http://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=for_remov&lang=en

Euskalmet. (2011). *Clasificación de territrios climáticos*. Retrieved from Agencia vasca de meteorología, http://www.euskalmet.euskadi.eus/s07-5921/es/contenidos/informacion/cla_clasificacion/es_7264/es_clasificacion.html

Evans, H. C. (1984). The genus *Mycosphaerell*a and its anamorphs *Cercoseptoria*, *Dothistroma* and *Lecanosticta* on pines. *Mycological Paper*, *153*, 102.

FAOSTAT. (2010). *Evaluación de los recursos forestales mundiales 2010*. Retrieved from Organización de las Naciones Unidas para la Agricultura y la Alimentación, http://www.fao.org/docrep/013/i1757s/i1757s.pdf

Farjon, A. 2013. *Pinus radiata*. Retrieved from the IUCN red list of threatened species, https://www.iucnredlist.org/species/42408/2977955

Flowers, J. L., Hartman, J. R. & Vaillancourt, L. J. (2006). Histology of *Diplodia pinea* in diseased and latently infected *Pinus nigra* shoots. *Forest Pathology*, *36*, 447-459.

Fogarty, W. M., & Kelly, C. T. (1990). *Microbial enzymes and biotechnology*. Elsevier Science Publishers.

Forde, M. B. (1966). *Pinus radiata* in California. *New Zealand Journal of Forestry*, *11*, 20-42.

Fuentes, V., Zapatero, L., Martínez, M. I., Alonso, E., Beitia, J. M., & Bartolomé, B. (2006). Allergy to pine processionary caterpillar (*Thaumetopoea pityocampa*) in children. *Allergologia et Immunopathologia*, *34*, 59–63.

Gamfeldt, L., Snäll, T., Bagchi, R., Jonsson, M., Gustafsson, L., Kjellander, P., Ruiz-Jaen, M. C., Fröberg, M., Stendahl, J., Philipson, C. D., Mikusiński, G., Andersson, E., Westerlund, B., Andrén, H., Moberg, F., Moen, J., Bengtsson, J. (2013). Higher levels of multiple ecosystem services are found in forests with more tree species. *Nature Communications*, *4*, 1340.

Garrett, K. A., Dendy, S. P., Frank, E. E., Rouse, M. N., & Travers, S. E. (2006). Climate change effects on plant disease: genomes to ecosystems. *Annual Review of Phytopathology*, *44*, 489-509.

Gartzia-Bengoetxea, N., & Arias-González, A. (2015). Soil degradation risks in Basque Forests. In Gartzia-Bengoetxea, N., & Arias-González, A. (Eds.), *Soil degradation risks in planted Forests* (81-96 pp). Vitoria-Gasteiz, Spain: Eusko Jaurlaritzaren Argitalpen Zerbitzua/Servicio Central de Publicaciones del Gobierno Vasco.

Gibson, I. A. S. (1972). Dothistroma blight of *Pinus radiata*. *Annual Review of Phytopathology*, *10*, 51-72.

Griffin, A.R., & Lindgren, D. (1985). Effect of inbreeding on production of filled seed in *Pinus radiata*-experimental results and a model of gene action. *Theoretical and Applied Genetics*, *71*, 334-343.

Guzmán, A. (1984). Study of wood chip production from forest residues in Chile. *Biomass*, *5*, 167-169.

Haddow, W. R., & Newman, F. S. (1942). A disease of the Scots pine (*P. sylvestris* L.) caused by the fungus *Diplodia pinea* Kickx associated with the pine spittlebug (*Aphrophora parallela* Say.) I. Symptoms and etiology. *Trans. R. Can. Inst*., *24*, 1-17.

Hanson, C., Yonavjak, L., Clarke, C., Minnemeyer, S., Boisrobert, L., Leach A., & Schleeweis, K. (2010). *Forest root diseases across the United States*. Retrieved from United States Department of Agriculture, http://www.seesouthernforests.org/files/sff/SouthernForestsfortheFuture.pdf

Henderson, L. (2001). Alien Weeds and Invasive Plants. *Plant Protection Research Institute Handbook*, *12*. Cape Town, South Africa: Paarl Printers.

Hernández-Escribano, L., Iturritxa, E., Aragonés, A., Mesanza, N., Berbegal, M., Raposo, R., & Elvira-Recuenco, M. Root infection of canker pathogens, *Fusarium circinatum* and *Diplodia sapinea*, in asymptomatic trees in *Pinus radiata* and *Pinus pinaster* plantations. *Forests* 2018, 9, 128.

IFN3. (2007). *Tercer Inventario Forestal Nacional*. Retrieved from Gobierno de España, Ministerio para la transición ecológica, https://www.miteco.gob.es/es/biodiversidad/servicios/banco-datos-naturaleza/informacion-disponible/publicacion_pdf_ifn3.aspx

IFN4. (2013). *Cuarto Inventario Forestal Nacional, Comunidad Autónoma del País Vasco*. Retrieved from Gobierno Vasco, Departamento de Desarrollo Económico y Competitividad, http://www.euskadi.eus/contenidos/informacion/inventario_forestal_2011/es_agripes/adjuntos/IFN4%20PAIS%20VASCO.pdf

Iturritxa, E., Mesanza, N., & Brenning, A. (2015). Spatial analysis of the risk of major forest diseases in Monterey pine plantations. *Plant Pathology*, *64*, 880-889.

Iturritxa, E., Ganley, R. J., Raposo, R., García-Serna, I., Mesanza, N., Kirkpatrick, S. C., & Gordon, T. R. (2013). Resistance levels of Spanish conifers against *Fusarium circinatum* and *Diplodia pinea*. *Forest Pathology*, *43*, 488-495.

Iturritxa, E., Mesanza, N., Elvira-Recuenco, M., Serrano, Y., Quintana, E., & Raposo, R. (2012). Evaluation of genetic resistance in *Pinus* to pitch canker in Spain. *Australasian Plant Pathology*, *41*, 601-607.

Iturritxa, E., García-Serna, I., & Mesanza, N. (2008). *Diplodia pinea* in forest plantations of *Pinus radiata* in northern Spain: Diversity and origin of the Spanish population. *Journal of Plant Pathology*, *90*, 366.

Iturritxa, E., & Ganley, R. (2007). Dispersión por vía aérea de esporas de *Diplodia pinea* en tres localidades de la cornisa cantábrica. *Boletín Sanidad Vegetal Plagas*, *33*, 383-390.

Jacobs, K. A., & Rehner, S. A. (1998). Comparison of Cultural and morphological characters and ITS Sequences in anamorphs of *Botryosphaeria* and related taxa. *Mycologia*, *90,* 601.

Kininmonth, J.A., & Whitehouse, L.K. (1991*). Properties and uses of radiata pine grown in New Zealand, Volume 1: basic wood properties*. Rotorua, New Zealand: Ministry of Forestry, Forest Research Institute.

Lavery, P.B. (1986). Plantation forestry with *Pinus radiata*. University of Canterbury, School of Forestry.

Lavery, P.B., & Mead, D.J. (1998). *Pinus radiata*: a narrow endemic from North America takes on the world. In Richardson, D. M. (Eds). *Ecology and Biogeography of Pinus*. Cambridge University Press.

Legat, C. E. (1930). The cultivation of exotic conifers in South Africa. *Empire Forestry Journal*, *9*, 32-63.

Libby, W. J., Bannister, M. H., & Linhart, Y. B. (1968). The Pines of Cedros and Guadalupe Islands. *Journal of Forestry*, *66*, 846-853.

Lindner, M., Maroschek, M., Netherer, S., Kremer, A., Barbati, A., Garcia-Gonzalo, J., Seidl, R., Delzon, S., Corona, P., Kolström, M., Lexer, M.J., & Marchetti, M. (2010). Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. *Forest Ecology and Management*, *259*, 698-709.

Lindsay, A.D. (1932). *Bishop pine (Pinus muricata D. Don.) in its native habitat, 11*. Canberra, Australia: Australian Commonwealth Forestry Bureau.

Loo, J. (2009). Ecological impacts of non-indigenous invasive fungi as forest pathogens. *Biological Invasions*, *11*, 81-96.

Lovett, G. M., Canham, C. D., Arthur, M. A., Weathers, K. C., & Fitzhugh, R. D. (2006). Forest ecosystem responses to exotic pests and pathogens in eastern North America. *Bioscience*, *56*, 395-405.

Luken, J. O., & Thieret, J.W. (1997). *Assessment and Management of Plant Invasions*. New York, USA: Springer-Verlag.

Maclaren, J. P. (1996). *Environmental effects of planted forests in New Zealand: the implications of continued afforestation of pasture*. Rotorua, New Zealand: New Zealand Forest Research Institute.

Madgwick, H. A. I. (1993). *Pinus radiata*-biomass, form and growth. *Pinus radiata* biomass, form and growth. *New Zealand Journal of Forestry Science*, *23*, 413-415.

MAGRAMA (Ministerio de Agricultura, Alimentación y Medio Ambiente). (2012). *Anuario Estadístico Forestal 2011*. Retrieved from Gobierno de España, Ministerio de Medio Ambiente Rural y Marino, http://www.marm.es/es/biodiversidad/ temas/montes-y-politica-forestal/estadisticas-forestales/indice_estadisticas_forestales_2011.aspx

Manion, P. D. (1990). *Tree disease concepts* (2nd ed.). Englewood Cliffs, NJ: Prentice-Hall.

Marks, G. C., & Minko, G. (1969). The pathogenicity of *Diplodia pinea* to *Pinus radiata* D. Don. *Australian Journal of Botany*, *17*, 1-12.

Marks, G. C., Smith, I. W., & Cook, I. O. (1989). Spread of *Dothistroma septospora* in plantations of *Pinus radiata* in Victoria between 1979 and 1988. *Australian Forestry*, *52*, 10-19.

Marris, B. (1969). *Pinus radiata*: a bibliography. Canberra, Australia: Forestry and Timber Bureau.

Martín-Rodrigues, N., Espinel, S., Sanchez-Zabala, J., Ortíz, A., González-Murua, C., & Duñabeitia, M. K. (2013). Spatial and temporal dynamics of the colonization of *Pinus radiata* by *Fusarium circinatum*, of conidiophora development in pith and of traumatic resin duct formation. *New Phytologist*, *198*, 1215-1227.

Mapa forestal. (2016). Retrieved from Gobierno Vasco, Departamento de Desarrollo Económico e Infraestructuras, http://www.euskadi.eus/web01-

a3estbin/es/contenidos/informacion/if_analisisdatos_mapa2016/es_def/index.shtml

McDonald, P. M., & Laacke, R. (1990). *Pinus radiata* D. Don, *Pinaceae*, Pine Family. In Burns, R. M., Honkala, B. *Silvics of North America*: *Volume 1. Conifers*. United States Department of Agriculture, Forest Service.

McDonald, B.A., & Stukenbrock, E. H. (2016). Rapid emergence of pathogens in agro-ecosystems: global threats to agricultural sustainability and food security. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*.

Mesanza, N., & Iturritxa, I. (2012). Root and butt rot caused by *Heterobasidion annosum* in Atlantic coniferous ecosystems in Spain. *Forest pathology*, *42*, 395-409.

Michel, M. (2003). El pino radiata (Pinus radiata D. Don) en la historia forestal de la Comunidad Autónoma del País Vasco: análisis de un proceso de forestalismo intensivo. Madrid, España: Universidad Politécnica de Madrid, PhD thesis.

Montero, G., & Serrada, R. (2013). *La situación de los bosques y el sector forestal en España-ISFE 2013*. Pontevedra, España: Sociedad Española de Ciencias Forestales.

Moran, R. (1996). The flora of Guadalupe Island, Mexico. *Memoirs of the Californian Academy of Sciences*, *19*.

Morse, B. A. (2005). Alien invasive species: impacts on forests and forestry. (Review of Forest Health and Biosecurity Working Paper 8). *Forest Resources Development Service Working Paper FBS/8E*. Rome, Italy: Forest Resources Division FAO.

Mundt, C. C. (2002). Use of multiline cultivars and cultivar mixtures for disease management. *Annual Review of Phytopathology*, *40*, 381-410.

Murua, J. R. (2016). *Libro blanco del sector de la madera: actividad forestal e industria de transformación de la madera; evolución reciente y perspectivas en Euskadi*. País Vasco, España: Servicio Central de Publicaciones del Gobierno Vasco.

Nicholls, T. H., & Ostry, M. E. (1990). *Sphaeropsis sapinea* cankers on stressed red and jack pines in Minnesota and Wisconsin. *Plant Disease*, *74*, 54-56.

Nilson, P. (2018). *Productive forest land*. Retrieved from Swedish University of Agricultural Sciences, https://www.slu.se/en/Collaborative-Centres-and-Projects/the-swedish-national-forest-inventory/forest-statistics/forest-statistics/productive-forest-land/

Omar, A. (2012). *Aspectos más relevantes de la sanidad forestal en Vizcaya/Bizkaia*. *Foresta*, *5*, 174-179. Retrieved from Foresta, http://www.redforesta.com/wp-content/uploads/2012/11/GF22-Aspectos-mas-relevantes-de-la-sanidad-forestal-en-Vizcaya.pdf

Oblinger, B. W., Smith, D. R., Stanosz, G. R. (2011). Red pine harvest debris as a potential source of inoculum of Diplodia shoot blight pathogens. *Forestry and Ecology Management*, *262*, 663-670.

Palmer, M. A. (1991). Isolate types of *Sphaeropsis sapinea* associated with main stem cankers and top-kill of *Pinus resinosa* in Minnesota and Wisconsin. *Plant Disease*, *75*, 507-510.

Palmer, M. A., Stewart, E. L., & Wingfield, M. J. (1987). Variation among isolates of *Sphaeropsis sapinea* in the north central United States. *Phytopathology*, *77*, 944-948.

Palmer, M. A., & Nicholls, T. H. (1985). Shoot blight and collar rot of *Pinus resinosa* caused by *Sphaeropsis sapinea* in forest tree nurseries. *Plant Disease*, *69*, 739-740.

Pehl, L., & Wulf, A. (2001). *Mycosphaerella*-needle fungi on pines-symptoms, biology and differential diagnosis. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*, *53*, 217-222.

Peterson, G. W. (1977). Infection, epidemiology, and control of Diplodia blight of Austrian, ponderosa, and Scots pines. *Phytopathology*, *67*, 511-514.

Peterson, G. W. (1981). Diplodia blight of pines. *United States Forest Service, Forest Insect & Disease Leaflet*, *161*, 7 pp.

Pimentel, D., McNair, S., Janecka, J., Wightman, J., Simmonds, C., O'Connell, C., Wong, E., Russel, L., Zern, J., Aquino, T., & Tsomondo, T. (2001). Economic and environmental threats of alien plant, animal and microbe invasions. *Agriculture, Ecosystems & Environment*, *84*, 1-20.

Pimentel, D., Zuniga, R., & Morrison, D. (2005). Update on the environmental and economic costs associated with alien invasive species in the United States. *Ecological Economics*, *52*, 273-288.

Roques, A. (2015). *Processionary moths and climate change: An update*. Dordrecht, Netherlands: Springer.

Sáenz, D., & Cantero, A. (2001). Bosque y Territorio en el País Vasco. *Euskonews & Media*, *108*, 1-26.

Santini, A., Ghelardini, L., De Pace, C., Desprez-Loustau, M. L., Capretti, P., Chandelier, A., Cech, T., Chira, D., Diamandis, S., Gaitniekis, T., Hantula, J., Holdenrieder, O., Jankovsky, L., Jung, T., Jurc, D., Kirisits, T., Kunca, A., Lygis, V., Malecka, M., Marcais, B., Schmitz, S., Schumacher, J., Solheim, H., Solla, A., Szabò, I., Tsopelas, P., Vannini, A., Vettraino, A. M., Webber, J., Woodward, S., & Stenlid, J. (2013). Biogeographical patterns and determinants of invasion by forest pathogens in Europe. *New Phytologist*, *197*,238-250.

Schoenherr, A. A. (1992). *A Natural History of California* (2nd ed.). University of California Press.

Scott, C. W. (1960). *Pinus radiata*. Rome, Italy: Forestry and Forest Products Studies FAO.

SECF. (2010). Situación de los bosques y del sector forestal en España. Informe 2010. Retrieved from Sociedad Española de Ciencias Forestales, http://secforestales.org/content/informe-isfe

Shipton, P. J. (1977). Monoculture and soilborne plant pathogens. *Annual Review of Phytopathology*, *15*, 387-407.

Sinclair, W. A., & Lyon, H. H. (2005). Disease of trees and shrubs (2nd ed.). Ithaca, United States: Cornell University Press.

Slagg, C. M., & Wright, E. (1943). Diplodia blight in coniferous seed-beds. *Phytopathology*, *33*, 390-393.

Smith, D. R., Stanosz, G. R., & Albers, J. (2014). Detection of the Diplodia shoot blight and canker pathogens from red and jack pine seeds using cultural methods. *Forest Pathology*, *37*, 61-66.

Smith, D. R., & Stanosz, G. R. (2006). A species-specific PCR assay for detection of *Diplodia pinea* and *D. scrobiculata* in dead red and jack pines with collar rot symptoms. *Plant Disease*, *90*, 307-313.

Stanosz, G. R., Swart, W. J., & Smith, D. R. (1999). RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and location. *Mycological Research*, *103*, 1193-1202.

Stenlid, J., & Oliva, J. (2016). Phenotypic interactions between tree hosts and invasive forest pathogens in the light of globalization and climate change. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*.

Sutton, B. C. (1980). *The Coelomycetes*. Surrey, England: Commonwealth Mycological Institute.

Swart, W. J., & Wingfield, M. J. (1991). Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. *Plant Disease*, *75*, 761-766.

Swart, W. J., Wingfield, M. J. & Knox-Davies, P. S. (1987). Conidial dispersal of *Sphaeropsis sapinea* in three climatic regions of South Africa. *Plant Disease*, *71*, 1038-1040.

Swart, W. J., Knox-Davies, P. S., & Wingfield, M. J. (1985). *Sphaeropsis sapinea*, with special reference to its occurrence on *Pinus* spp. in South Africa. *South African Forestry Journal*, *135*, 1-8.

Tello, M-L., Tomalak, M., Siwecki, R., Gáper, J., Motta, E., & Mateo-Sagasta, E. (2005). Biotic urban growing conditions-threats, pests and diseases. In Konijnendijk, C. C., Nilsson, K., Randrup, T. B., Schipperijn, J. (Eds.), *Urban Forests and Trees: A Reference Book*. Berlin, Germany: Springer.

van Baalen, M., & Sabelis, M. W. (1995). The dynamics of multiple infection and the evolution of virulence. *The American Naturalist*, *146*, 881-910.

Waterman, A. M. (1943). *Diplodia pinea*, the cause of a disease of hard pines. *Phytopathology*, *33*, 1018-1031.

Wang, C. G., Blanchette, R. A., Jackson, W. A., & Palmer, M. A. (1985). Differences in conidial morphology among isolates of *Sphaeropsis sapinea*. *Plant Disease*, *69*, 838-841.

Wikler, K. R., Storer, A. J., Newman, W., Gordon, T. R., & Wood, D. L. (2003). The dynamics of an introduced pathogen in a native Monterey pine (*Pinus radiata*) forest. *Forest ecology and Management*, *179*, 209-221.

Wilcox, M.D. (1983). Inbreeding depression and genetic variances estimated from self- and cross-pollinated families of *Pinus radiata*. *Silvae Genetica*, *32*, 89-96.

Wingfield, M. J., Garnas, J. R., Hajek, A., Hurley, B. P., Wilhelm de Beer, Z., & Taerum, S. J. (2016) Novel and co-evolved associations between insects and microorganisms as drivers of forest pestilence. *Biological Invasions*, *18*, 1045-1056.

Wingfield, M. J., & Swart, W. J. (1994). Integrated management of forest tree diseases in South Africa. *Forest Ecology and Management*, *65*, 11-16.

Wingfield, M. J., & Knox-Davies, P. S. (1980). Association of *Diplodia pinea* with a root disease of pines in South Africa. *Plant Disease*, *64*, 221-223.

Zhou, S., Smith, D. R.., & Stanosz, G. R. (2001). Differentiation of *Botryosphaeria* species and related anamorphic fungi using Inter Simple or Short Sequence Repeat (ISSR) fingerprinting. *Mycological Research*, *105*, 919-926.

Zhou, S., & Stanosz, G. R. (2001). Primers for Amplification of mt SSU rDNA, and a phylogenetic study of *Botryosphaeria* and associated anamorphic fungi. *Mycological Research*, *105,* 1033-1044.

Zwolinski, J. B., Swart, W. J., & Wingfield, M. J. (1990). Intensity of dieback induced by *Sphaeropsis sapinea* in relation to site conditions. *European Journal of Forest Pathology*, *20*, 167-174.

Suplementary material

Appendix A. Isolates of *Diplodia sapinea* (Dsa), *D. scrobiculata* (Dsc) used in this study, their mating types *MAT1-1-1* (1) and *MAT1-2-1* (2), locations, and hosts.

Sample ID	Species	Mating type	Location	Name of sample	Host species	Material
2	Dsa	1	P1 (Laukiniz)	P1-M1 (6-2-15)	*Pinus radiata*	Cone
3	Dsa	2	P1 (Laukiniz)	P1-M2 (6-2-15)	*Pinus radiata*	Cone
4	Dsa	2	P1 (Laukiniz)	P1-M3 (6-2-15)	*Pinus radiata*	Cone
5	Dsa	2	P1 (Laukiniz)	P1-M4 (6-2-15)	*Pinus radiata*	Cone
6	Dsa	1	P1 (Laukiniz)	P1-M5 (6-2-15)	*Pinus radiata*	Cone
7	Dsa	2	P1 (Laukiniz)	P1-M6 (6-2-15)	*Pinus radiata*	Cone
8	Dsa	2	P1 (Laukiniz)	P1-M7 (6-2-15)	*Pinus radiata*	Cone
9	Dsa	2	P1 (Laukiniz)	P1-M8 (6-2-15)	*Pinus radiata*	Cone
10	Dsa	2	P1 (Laukiniz)	P1-M9 (6-2-15)	*Pinus radiata*	Cone
11	Dsa	2	P1 (Laukiniz)	P1-M10 (6-2-15)	*Pinus radiata*	Cone
14	Dsa	2	Basque Country	PV 24 (19-2-15)	*Pinus radiata*	Cone
15	Dsa	1	Basque Country	PV 26 (19-2-15)	*Pinus radiata*	Cone
17	Dsa	1	Basque Country	P2-M1 (27-2-15)	*Pinus radiata*	Cone
18	Dsa	1	Basque Country	P2-M2 (27-2-15)	*Pinus radiata*	Cone
19	Dsa	2	Basque Country	P2-M3 (27-2-15)	*Pinus radiata*	Cone
22	Dsa	1	P2 (Sollano)	W.P. 6 (9-3-15)	*Pinus radiata*	Cone
23	Dsa	1	P2 (Sollano)	W.P. 7 (9-3-15)	*Pinus radiata*	Cone
24	Dsa	1	P2 (Sollano)	W.P. 8 (9-3-15)	*Pinus radiata*	Cone
25	Dsa	1	P2 (Sollano)	W.P. 9 (9-3-15)	*Pinus radiata*	Cone
26	Dsa	1	P2 (Sollano)	W.P. 10 (9-3-15)	*Pinus radiata*	Cone
27	Dsa	2	P2 (Sollano)	W.P. 11 (9-3-15)	*Pinus radiata*	Cone
29	Dsa	1	P2 (Sollano)	W.P. 13 (9-3-15)	*Pinus radiata*	Cone
30	Dsa	2	P2 (Sollano)	W.P. 14 (9-3-15)	*Pinus radiata*	Cone
31	Dsa	1	P2 (Sollano)	W.P. 15 (9-3-15)	*Pinus radiata*	Cone
33	Dsa	1	P2 (Sollano)	W.P. 17 (9-3-15)	*Pinus radiata*	Cone
34	Dsa	1	P2 (Sollano)	W.P. 18 (9-3-15)	*Pinus radiata*	Cone
35	Dsa	1	P2 (Sollano)	W.P. 19 (9-3-15)	*Pinus radiata*	Cone
36	Dsa	1	P2 (Sollano)	W.P. 20 (9-3-15)	*Pinus radiata*	Cone
38	Dsa	1	P2 (Sollano)	W.P. 23 (9-3-15)	*Pinus radiata*	Cone
39	Dsa	2	Basque Country	W.P. 24 (9-3-	*Pinus radiata*	Cone

				15)		
40	Dsa	2	Basque Country	W.P. 25 (9-3-15)	*Pinus radiata*	Cone
41	Dsa	2	Basque Country	N5 (2-3-15)	*Pinus nigra*	Cone
42	Dsa	1	Basque Country	M2 (2-3-15)	*Pinus nigra*	Cone
44	Dsa	2	Basque Country	N5 (23-2-15)	*Pinus nigra*	Cone
45	Dsa	2	Basque Country	Elguea 1 (7-4-15)	*Pinus radiata*	Cone
46	Dsa	2	Basque Country	Elguea 2 (7-4-15)	*Pinus radiata*	Cone
47	Dsa	1	Basque Country	Elguea 3 (7-4-15)	*Pinus radiata*	Cone
48	Dsa	1	Basque Country	Muxika 1 (13-4-15)	*Pinus radiata*	Cone
49	Dsa	1	Basque Country	Muxika 2 (13-4-15)	*Pinus radiata*	Cone
50	Dsa	1	Basque Country	Muxika 3 (13-4-15)	*Pinus radiata*	Cone
51	Dsa	2	Basque Country	Gorozika 1 (13-4-15)	*Pinus radiata*	Cone
52	Dsa	2	Basque Country	Gorozika 2 (13-4-15)	*Pinus radiata*	Cone
53	Dsa	2	Basque Country	Oiz 1 (13-4-15)	*Pinus radiata*	Cone
54	Dsa	1	Basque Country	Aulesti 1 (21-4-15)	*Pinus radiata*	Cone
55	Dsa	2	Basque Country	Agiñeko 1 (23-4-15)	*Pinus radiata*	Cone
56	Dsa	1	Basque Country	Berganzo 1 (31-3-15)	*Pinus attenuata*	Cone
57	Dsa	2	Basque Country	M1 (14-4-15)	*Pinus radiata*	Cone
60	Dsa	1	P3 (Hernani)	H 1 (13-5-15)	*Pinus radiata*	Cone
61	Dsa	2	P3 (Hernani)	H 2 (13-5-15)	*Pinus radiata*	Cone
62	Dsa	2	P3 (Hernani)	H 3 (13-5-15)	*Pinus radiata*	Cone
63	Dsa	2	P3 (Hernani)	H 4 (13-5-15)	*Pinus radiata*	Cone
64	Dsa	1	P3 (Hernani)	H 5 (13-5-15)	*Pinus radiata*	Cone
65	Dsa	1	P3 (Hernani)	H 6 (13-5-15)	*Pinus radiata*	Cone
66	Dsa	2	P3 (Hernani)	H 7 (13-5-15)	*Pinus radiata*	Cone
67	Dsa	2	P3 (Hernani)	H 8 (13-5-15)	*Pinus radiata*	Cone
68	Dsa	2	P3 (Hernani)	H 9 (13-5-15)	*Pinus radiata*	Cone
69	Dsa	1	P3 (Hernani)	H 10 (13-5-15)	*Pinus radiata*	Cone
70	Dsa	1	P3 (Hernani)	H 11 (13-5-15)	*Pinus radiata*	Cone
71	Dsa	2	P3 (Hernani)	H 12 (13-5-15)	*Pinus radiata*	Cone
72	Dsa	2	P3 (Hernani)	H 13 (13-5-15)	*Pinus radiata*	Cone
73	Dsa	2	P3 (Hernani)	H 14 (13-5-15)	*Pinus radiata*	Cone
74	Dsa	2	P3 (Hernani)	H 15 (13-5-15)	*Pinus radiata*	Cone
75	Dsa	2	P3 (Hernani)	H 16 (13-5-15)	*Pinus radiata*	Cone
76	Dsa	1	Basque Country	M1 (22-7-15)	*Pinus radiata*	Cone
77	Dsa	2	Basque Country	M2 (22-7-15)	*Pinus radiata*	Cone
78	Dsa	1	Basque Country	M3 (22-7-15)	*Pinus radiata*	Cone
79	Dsa	2	Basque Country	M4 (22-7-15)	*Pinus radiata*	Cone

80	Dsa	2	Basque Country	M5 (22-7-15)	*Pinus radiata*	Cone
81	Dsa	1	Basque Country	M6 (22-7-15)	*Pinus radiata*	Cone
82	Dsa	1	Basque Country	M7 (22-7-15)	*Pinus nigra*	Cone
83	Dsa	2	Basque Country	M8 (22-7-15)	*Pinus nigra*	Cone
84	Dsa	2	Basque Country	M9 (22-7-15)	*Pinus nigra*	Cone
86	Dsa	1	Basque Country	M1 (23-7-15)	*Pinus radiata*	Cone
87	Dsa	1	Basque Country	M2 (23-7-15)	*Pinus radiata*	Cone
89	Dsa	2	Basque Country	M4 (23-7-15)	*Pinus radiata*	Cone
90	Dsa	1	Basque Country	M5 (23-7-15)	*Pinus radiata*	Cone
91	Dsa	1	Basque Country	M6 (23-7-15)	*Pinus radiata*	Cone
92	Dsa	2	Basque Country	M7 (23-7-15)	*Pinus radiata*	Cone
93	Dsa	1	Basque Country	M8 (23-7-15)	*Pinus radiata*	Cone
94	Dsa	1	Basque Country	M1 (24-7-15)	*Pinus radiata*	Cone
95	Dsa	2	Basque Country	M2 (24-7-15)	*Pinus radiata*	Cone
96	Dsa	1	Basque Country	M3 (24-7-15)	*Pinus radiata*	Cone
97	Dsa	1	Basque Country	M4 (24-7-15)	*Pinus radiata*	Cone
98	Dsa	2	Basque Country	M5 (24-7-15)	*Pinus radiata*	Cone
99	Dsa	1	Basque Country	M6 (24-7-15)	*Pinus radiata*	Cone
100	Dsa	2	Basque Country	M1 (3-8-15)	*Pinus radiata*	Cone
101	Dsa	1	Basque Country	M2 (3-8-15)	*Pinus radiata*	Cone
104	Dsa	2	Basque Country	M5 (3-8-15)	*Pinus nigra*	Cone
105	Dsa	2	Basque Country	M6 (3-8-15)	*Pinus radiata*	Cone
106	Dsa	2	Basque Country	M7 (3-8-15)	*Pinus radiata*	Cone
108	Dsa	1	Basque Country	M9 (3-8-15)	*Pinus radiata*	Cone
109	Dsa	1	Basque Country	M1 (10-8-15)	*Pinus pinaster*	Cone
112	Dsa	2	Basque Country	M4 (10-8-15)	*Pinus radiata*	Cone
113	Dsa	1	Basque Country	M5 (10-8-15)	*Pinus nigra*	Cone
114	Dsa	2	Basque Country	M6 (10-8-15)	*Pinus radiata*	Cone
115	Dsa	2	Basque Country	M7 (10-8-15)	*Pinus radiata*	Cone
116	Dsa	1	Basque Country	M8 (10-8-15)	*Pinus radiata*	Cone
120	Dsa	2	Basque Country	M4 (10-9-15)	*Pinus halepensis*	Cone
121	Dsa	2	Basque Country	M5 (10-9-15)	*Pinus sylvestris*	Cone
123	Dsa	2	Basque Country	M7 (10-9-15)	*Pinus contorta*	Cone
124	Dsa	2	Basque Country	M1 (11-9-15)	*Pinus radiata*	Cone
125	Dsa	1	Basque Country	M2 (11-9-15)	*Pinus radiata*	Cone
127	Dsa	1	Basque Country	M4 (11-9-15)	*Pinus radiata*	Cone
128	Dsa	2	Basque Country	M1 (30-9-15)	*Pinus radiata*	Cone
129	Dsa	2	Basque Country	M2 (30-9-15)	*Pinus radiata*	Cone
131	Dsa	2	Basque Country	M4 (30-9-15)	*Pinus nigra*	Cone
132	Dsa	1	Basque Country	M5 (30-9-15)	*Pinus nigra*	Cone
133	Dsa	1	Basque Country	M1 (6-10-15)	*Pinus radiata*	Cone
134	Dsa	1	Basque Country	M2 (6-10-15)	*Pinus radiata*	Cone
135	Dsa	2	Basque Country	M3 (6-10-15)	*Pinus radiata*	Cone
137	Dsa	1	Basque Country	M5 (6-10-15)	*Pinus radiata*	Cone
138	Dsa	2	Basque Country	M1 (9-10-15)	*Pinus radiata*	Cone
141	Dsa	2	Basque Country	M1 (23-10-15)	*Pinus radiata*	Cone

142	Dsa	1	Basque Country	M2 (23-10-15)	*Pinus radiata*	Cone
143	Dsa	2	Basque Country	M3 (23-10-15)	*Pinus radiata*	Cone
144	Dsa	1	Basque Country	M4 (23-10-15)	*Pinus radiata*	Cone
145	Dsa	2	Basque Country	M5 (23-10-15)	*Pinus radiata*	Cone
146	Dsa	1	Basque Country	M6 (23-10-15)	*Pinus radiata*	Cone
147	Dsa	2	Basque Country	M1 (11-11-15)	*Pinus radiata*	Cone
148	Dsa	1	Basque Country	M2 (11-11-15)	*Pinus radiata*	Cone
149	Dsa	2	Basque Country	M3 (11-11-15)	*Pinus radiata*	Cone
150	Dsa	1	Basque Country	M1 (18-11-15)	*Pinus radiata*	Cone
151	Dsa	1	Basque Country	M2 (18-11-15)	*Pinus radiata*	Cone
152	Dsa	2	Basque Country	M3 (18-11-15)	*Pinus radiata*	Cone
154	Dsa	2	Basque Country	M1 (20-11-15)	*Pinus radiata*	Cone
155	Dsa	1	Basque Country	M2 (20-11-15)	*Pinus radiata*	Cone
156	Dsa	1	Basque Country	M3 (20-11-15)	*Pinus radiata*	Cone
157	Dsa	1	Basque Country	M4 (20-11-15)	*Pinus radiata*	Cone
158	Dsa	2	Basque Country	M5 (20-11-15)	*Pinus radiata*	Cone
159	Dsa	2	Basque Country	M1 (1-12-15)	*Pinus radiata*	Cone
160	Dsa	1	Basque Country	M2 (1-12-15)	*Pinus radiata*	Cone
161	Dsa	2	Basque Country	M3 (1-12-15)	*Pinus radiata*	Cone
162	Dsa	2	Basque Country	M4 (1-12-15)	*Pinus radiata*	Cone
163	Dsa	1	Basque Country	M1 (2-12-15)	*Pinus radiata*	Cone
164	Dsa	2	Basque Country	M2 (2-12-15)	*Pinus radiata*	Cone
165	Dsa	2	Basque Country	M3 (2-12-15)	*Pinus radiata*	Cone
166	Dsa	1	Basque Country	M4 (2-12-15)	*Pinus radiata*	Cone
169	Dsa	2	Basque Country	M3 (19-1-16)	*Pinus radiata*	Cone
170	Dsc	1	Russell County, Kentucky USA	97-16	*Pinus virginiana*	Unknown
171	Dsa	2	Barbour County, West Virginia USA	96-148	*Pinus sylvestris*	Needle
172	Dsa	2	Black Hills, South Dakota USA	04-77	*Pinus ponderosa*	Stem
173	Dsa	2	Santa Rosa, Iturbide, Nuevo León, Mexico	97-63	*Pinus culminicola*	Needle
174	Dsa	1	Idaho USA	94-156	*Pinus ponderosa*	Unknown
175	Dsa	2	Grant County, Wisconsin USA	128	*Pinus resinosa*	Unknown
176	Dsa	2	Honduras	94-177	*Pinus oocarpa*	Unknown
177	Dsa	2	Oktibbeha County, Mississippi USA	04-16	*Pinus palustris*	Unknown
178	Dsa	2	New Zealand	94-160	*Pinus radiata*	Unknown
179	Dsa	2	France	94-19	*Cedrus deodara*	Unknown
180	Dsa	1	Pennsylvania USA	92-44	*Pinus sylvestris*	Unknown
181	Dsa	1	Turkey	07-43	*Pinus nigra*	Unknown
182	Dsa	2	Gnangara	93-23	*Pinus radiata*	Unknown

			Nursery, Australia			
183	Dsa	1	Berthier Nursery, Quebec Canada	97-25	*Pinus resinosa*	Needle
184	Dsa	2	Shune, Tanzania	154	Unknown	Unknown
185	Dsa	2	Riverside County, California USA	99-5	*Pinus jeffreyi*	Unknown
186	Dsa	2	Florida USA	96-45	*Pinus elliottii*	Cone-seed
187	Dsc	2	Jackson County, Wisconsin USA	124	*Pinus bankisiana*	Unknown
188	Dsc	1	Spain	94-161	*Cedrus atlantica*	Unknown
189	Dsc	1	Tuolumne County, California USA	99-24	*Pinus sabiniana*	Unknown
190	Dsa	1	Bennington County, Vermont USA	03-15	*Pinus resinosa*	Cone
191	Dsa	1	Maui, Hawaii USA	189	*Pinus radiata*	Unknown
192	Dsa	1	Hubbard County, Minnesota USA	411	*Pinus resinosa*	Unknown
193	Dsa	2	United Kingdom	94-162	*Pinus muricata*	Unknown
194	Dsa	2	Dallas County, Texas USA	07-14	*Pinus elderica*	Cone
195	Dsc	1	Lomond Township, Ontario Canada	01-80	*Picea mariana*	Unknown
196	Dsa	1	Basque Country	M1 (14-3-16)	*Pinus radiata*	Cone
197	Dsa	2	Basque Country	M2 (14-3-16)	*Pinus radiata*	Cone
198	Dsa	2	Basque Country	M3 (14-3-16)	*Pinus radiata*	Cone
199	Dsa	1	Basque Country	M4 (14-3-16)	*Pinus radiata*	Cone
200	Dsa	2	Basque Country	M5 (14-3-16)	*Pinus radiata*	Cone
201	Dsa	1	Basque Country	M2 (18-3-16)	*Pinus radiata*	Cone
202	Dsa	2	Basque Country	M1 (22-3-16)	*Pinus radiata*	Cone
203	Dsa	2	Basque Country	M2 (22-3-16)	*Pinus radiata*	Cone
205	Dsa	2	Madagascar	96-24	*Pinus roxburghii*	Unknown
206	Dsa	2	Georgia USA	98-1	*Pinus taeda*	Needle
207	Dsc	1	Basque Country	Oihan 70S	*Pinus radiata*	Trunk
208	Dsa	1	Basque Country	Oihan 295S	*Pinus radiata*	Trunk
209	Dsa	1	Basque Country	Oihan 402S	*Pinus radiata*	Trunk
210	Dsa	2	Wisconsin USA	17-8	*Pinus banksiana*	Needle
211	Dsa	2	Wisconsin USA	17-9	*Pinus banksiana*	Needle
212	Dsa	1	Waushara County, Wisconsin USA	17-10	*Pinus resinosa*	Needle
213	Dsa	1	Portage County, Wisconsin USA	17-11	*Pinus banksiana*	Needle
214	Dsa	2	Portage County, Wisconsin USA	17-12	*Pinus resinosa*	Needle

215	Dsa	1	Wood County, Wisconsin USA	17-13	*Pinus resinosa*	Needle
216	Dsa	1	Adams County, Wisconsin USA	17-14	*Pinus resinosa*	Needle
217	Dsa	2	Marathon County, Wisconsin USA	17-01	*Pinus resinosa*	Needle
218	Dsa	1	Wallowa County, Oregon USA	13-09	*Pinus ponderosa*	Cone
219	Dsa	2	Wallowa County, Oregon USA	13-10	*Pinus ponderosa*	Needle
220	Dsa	2	Bennington County, Vermont USA	03-24	*Pinus resinosa*	Stem tip
221	Dsa	2	Sawyer County, Wisconsin USA	04-116	*Pinus banksiana*	Stem
222	Dsa	1	Wood County, Wisconsin USA	04-124	*Pinus banksiana*	Stem
223	Dsa	2	Vilas County, Wisconsin USA	04-15	*Pinus resinosa*	Unknown
224	Dsa	2	South Dakota	04-78	*Pinus ponderosa*	Unknown
225	Dsa	2	Dallas County, Texas USA	07-16	*Pinus nigra*	Cone
226	Dsa	1	Bayfield County, Wisconsin USA	07-26	*Pinus resinosa*	Needle
227	Dsa	2	Sumter County, Alabama USA	07-59	*Pinus taeda*	Cone
228	Dsa	2	Adams County, Wisconsin USA	08-15	*Pinus sylvestris*	Bark
229	Dsa	1	Flathead County, Montana USA	09-07	*Pinus ponderosa*	Unknown
230	Dsa	2	New Zealand	178	*Pinus radiata*	Unknown
231	Dsa	2	St. Louis County, Minnesota USA	217	*Pinus resinosa*	Unknown
232	Dsa	2	Pine County, Minnesota USA	191	*Pinus resinosa*	Unknown
233	Dsa	2	Monongalia County, West Virginia USA	226	*Pinus nigra*	Needle
234	Dsa	2	Jackson County, Wisconsin USA	94-107	*Pinus resinosa*	Needle
235	Dsa	2	Dane County, Wisconsin USA	94-15	*Pinus nigra*	Needle
236	Dsa	2	Northern Highland American Legion State Forest, Wisconsin USA	94-191	*Pinus banksiana*	Twig
237	Dsa	2	Foret d'Orleans France	94-25	*Pinus sylvestris*	Cone
238	Dsa	1	Marquette County, Wisconsin USA	94-22	*Pinus resinosa*	Needle

239	Dsa	1	Trempealeau County, Wisconsin USA	94-192	*Pinus resinosa*	Needle
240	Dsa	2	Fairfield County, Connecticut USA	94-41	*Pinus sylvestris*	Needle
241	Dsa	1	Adair County, Iowa USA	94-77	*Pinus nigra*	Needle
242	Dsa	1	Codington County, South Dakota USA	94-79	*Pinus sylvestris*	Needle
243	Dsa	2	Polk County, Iowa USA	94-81	*Pinus nigra*	Needle
244	Dsa	2	Lacrosse County, Wisconsin USA	94-8	*Pinus resinosa*	Needle
245	Dsa	2	Wood County, Wisconsin USA	95-42	*Pinus resinosa*	Stem
246	Dsa	1	Centre County, Pennsylvania USA	95-65	*Pinus nigra*	Needle
247	Dsa	2	Upshur County, West Virginia USA	96-142	*Pinus sylvestris*	Needle
248	Dsa	2	Lafayette County, Wisconsin USA	96-181	*Pinus resinosa*	Needle
249	Dsa	2	Mississauga, Ontario Canada	96-67	*Pinus nigra*	Unknown
250	Dsa	1	Monroe County, Wisconsin USA	96-95	*Pinus banksiana*	Needle
251	Dsa	1	Cheboygan County, Michigan USA	97-120	*Pinus resinosa*	Shoot
252	Dsa	1	Manistee National Forest, Michigan USA	97-117	*Pinus resinosa*	Unknown
253	Dsa	2	Jefferson County, West Virginia USA	97-22	*Pinus nigra*	Needle
254	Dsa	2	Stanislaus National Forest, California USA	99-4	*Pinus ponderosa*	Unknown
255	Dsa	2	Morgan County, West Virginia USA	96-160	*Pinus sylvestris*	Needle
256	Dsa	2	Marion County, Indiana USA	96-158	*Pinus nigra*	Cone
257	Dsc	2	Pine County, Minnesota USA	03-50	*Pinus banksiana*	Unknown
258	Dsc	1	Jackson County, Wisconsin USA	04-01	*Pinus banksiana*	Unknown
259	Dsc	1	Douglas County, Wisconsin USA	212	*Pinus resinosa*	Unknown

260	Dsc	2	Jackson County, Wisconsin USA	94-108	*Pinus banksiana*	Needle
261	Dsc	2	Mille Lacs County, Minnesota USA	94-194	*Pinus sylvestris*	Twig
262	Dsc	2	Waushara County, Wisconsin USA	94-45	*Pinus banksiana*	Needle
263	Dsc	2	Wood County, Wisconsin USA	95-41	*Pinus resinosa*	Stem
264	Dsc	1	St. Louis County, Minnesota USA	224	*Pinus resinosa*	Unknown
265	Dsc	2	Wadena County, Minnesota USA	407	*Pinus resinosa*	Unknown
266	Dsc	2	Adams County, Wisconsin USA	08-14	*Pinus sylvestris*	Bark
267	Dsc	2	Hubbard County, Minnesota USA	04-19	*Pinus resinosa*	Unknown
268	Dsc	1	Sauk County, Wisconsin USA	04-114	*Pinus banksiana*	Unknown
269	Dsc	2	Wood County, Wisconsin USA	95-54	*Pinus resinosa*	Needle
270	Dsc	1	Mille Lacs County, Minnesota USA	96-159	*Pinus sylvestris*	Stem
271	Dsc	1	Pine County, Minnesota USA	02-254	*Pinus resinosa*	Unknown
272	Dsc	1	Douglas County, Wisconsin USA	245	*Pinus resinosa*	Unknown
273	Dsc	2	Bath County, Virginia USA	07-01	*Pinus strobus*	Branch
274	Dsc	2	Johnson County, Illinois USA	201	*Pinus strobus*	Unknown
275	Dsc	1	Hubbard County, Minnesota USA	04-20	*Pinus resinosa*	Unknown
276	Dsc	1	Douglas County, Wisconsin USA	248	*Pinus resinosa*	Unknown
277	Dsc	1	Sumter County, Alabama USA	07-20	*Pinus taeda*	Stem
278	Dsc	1	Kern County, California USA	99-34	*Pinus sabiniana*	Unknown
279	Dsc	1	Bath County, Virginia USA	07-05	*Pinus strobus*	Stem
280	Dsc	1	Carmel Mountain Israel	96-38	*Pinus halepensis*	Unknown
281	Dsc	2	Mille Lacs County, Minnesota USA	94-112	*Pinus sylvestris*	Needle
282	Dsc	2	Monterey County, California USA	03-33	*Pinus radiata*	Cone

Appendix B. ID, Species, location, name of the sample, host species and source for each isolate used in this study.

Sample ID[a]	Species[b]	Location	Name of sample	Host species	Material
2	*Diplodia sapinea*	P1 (Laukiniz)	P1-M1 (6-2-15)	*Pinus radiata*	Cone
3	*Diplodia sapinea*	P1 (Laukiniz)	P1-M2 (6-2-15)	*Pinus radiata*	Cone
4	*Diplodia sapinea*	P1 (Laukiniz)	P1-M3 (6-2-15)	*Pinus radiata*	Cone
5	*Diplodia sapinea*	P1 (Laukiniz)	P1-M4 (6-2-15)	*Pinus radiata*	Cone
6	*Diplodia sapinea*	P1 (Laukiniz)	P1-M5 (6-2-15)	*Pinus radiata*	Cone
7	*Diplodia sapinea*	P1 (Laukiniz)	P1-M6 (6-2-15)	*Pinus radiata*	Cone
8	*Diplodia sapinea*	P1 (Laukiniz)	P1-M7 (6-2-15)	*Pinus radiata*	Cone
9	*Diplodia sapinea*	P1 (Laukiniz)	P1-M8 (6-2-15)	*Pinus radiata*	Cone
10	*Diplodia sapinea*	P1 (Laukiniz)	P1-M9 (6-2-15)	*Pinus radiata*	Cone
11	*Diplodia sapinea*	P1 (Laukiniz)	P1-M10 (6-2-15)	*Pinus radiata*	Cone
14	*Diplodia sapinea*	Basque Country	PV 24 (19-2-15)	*Pinus radiata*	Cone
15	*Diplodia sapinea*	Basque Country	PV 26 (19-2-15)	*Pinus radiata*	Cone
17	*Diplodia sapinea*	Basque Country	P2-M1 (27-2-15)	*Pinus radiata*	Cone
18	*Diplodia sapinea*	Basque Country	P2-M2 (27-2-15)	*Pinus radiata*	Cone
19	*Diplodia sapinea*	Basque Country	P2-M3 (27-2-15)	*Pinus radiata*	Cone
22	*Diplodia sapinea*	P2 (Sollano)	W.P. 6 (9-3-15)	*Pinus radiata*	Cone
23	*Diplodia sapinea*	P2 (Sollano)	W.P. 7 (9-3-15)	*Pinus radiata*	Cone
24	*Diplodia sapinea*	P2 (Sollano)	W.P. 8 (9-3-15)	*Pinus radiata*	Cone
25	*Diplodia sapinea*	P2 (Sollano)	W.P. 9 (9-3-15)	*Pinus radiata*	Cone
26	*Diplodia sapinea*	P2 (Sollano)	W.P. 10 (9-3-15)	*Pinus radiata*	Cone
27	*Diplodia sapinea*	P2 (Sollano)	W.P. 11 (9-3-15)	*Pinus radiata*	Cone
29	*Diplodia sapinea*	P2 (Sollano)	W.P. 13 (9-3-15)	*Pinus radiata*	Cone
30	*Diplodia sapinea*	P2 (Sollano)	W.P. 14 (9-3-15)	*Pinus radiata*	Cone
31	*Diplodia sapinea*	P2 (Sollano)	W.P. 15 (9-3-15)	*Pinus radiata*	Cone
33	*Diplodia sapinea*	P2 (Sollano)	W.P. 17 (9-3-15)	*Pinus radiata*	Cone
34	*Diplodia sapinea*	P2 (Sollano)	W.P. 18 (9-3-15)	*Pinus radiata*	Cone
35	*Diplodia sapinea*	P2 (Sollano)	W.P. 19 (9-3-15)	*Pinus radiata*	Cone
36	*Diplodia sapinea*	P2 (Sollano)	W.P. 20 (9-3-15)	*Pinus radiata*	Cone
38	*Diplodia sapinea*	P2 (Sollano)	W.P. 23 (9-3-15)	*Pinus radiata*	Cone
39	*Diplodia sapinea*	Basque Country	W.P. 24 (9-3-15)	*Pinus radiata*	Cone
40	*Diplodia sapinea*	P2 (Sollano)	W.P. 25 (9-3-15)	*Pinus radiata*	Cone
41	*Diplodia sapinea*	Basque Country	M1 (2-3-15)	*Pinus radiata*	Cone
42	*Diplodia sapinea*	Basque Country	M2 (2-3-15)	*Pinus nigra*	Cone
44	*Diplodia sapinea*	Basque Country	N5 (23-2-15)	*Pinus nigra*	Cone
45	*Diplodia sapinea*	Basque Country	Elguea 1 (7-4-15)	*Pinus radiata*	Cone
46	*Diplodia sapinea*	Basque Country	Elguea 2 (7-4-15)	*Pinus radiata*	Cone
47	*Diplodia sapinea*	Basque Country	Elguea 3 (7-4-15)	*Pinus radiata*	Cone
48	*Diplodia sapinea*	Basque Country	Muxika 1 (13-4-15)	*Pinus radiata*	Cone
49	*Diplodia sapinea*	Basque Country	Muxika 2 (13-4-15)	*Pinus radiata*	Cone
50	*Diplodia sapinea*	Basque Country	Muxika 3 (13-4-	*Pinus radiata*	Cone

			15)		
51	*Diplodia sapinea*	Basque Country	Gorozika 1 (13-4-15)	*Pinus radiata*	Cone
52	*Diplodia sapinea*	Basque Country	Gorozika 2 (13-4-15)	*Pinus radiata*	Cone
53	*Diplodia sapinea*	Basque Country	Oiz 1 (13-4-15)	*Pinus radiata*	Cone
54	*Diplodia sapinea*	Basque Country	Aulesti 1 (21-4-15)	*Pinus radiata*	Cone
55	*Diplodia sapinea*	Basque Country	Agiñeko 1 (23-4-15)	*Pinus insignis*	Cone
56	*Diplodia sapinea*	Basque Country	Berganzo 1 (31-3-15)	*Pinus atenuatta*	Cone
57	*Diplodia sapinea*	Basque Country	M1 (14-4-15)	*Pinus insignis*	Cone
60	*Diplodia sapinea*	P3 (Hernani)	H 1 (13-5-15)	*Pinus radiata*	Cone
61	*Diplodia sapinea*	P3 (Hernani)	H 2 (13-5-15)	*Pinus radiata*	Cone
62	*Diplodia sapinea*	P3 (Hernani)	H 3 (13-5-15)	*Pinus radiata*	Cone
63	*Diplodia sapinea*	P3 (Hernani)	H 4 (13-5-15)	*Pinus radiata*	Cone
64	*Diplodia sapinea*	P3 (Hernani)	H 5 (13-5-15)	*Pinus radiata*	Cone
65	*Diplodia sapinea*	P3 (Hernani)	H 6 (13-5-15)	*Pinus radiata*	Cone
66	*Diplodia sapinea*	P3 (Hernani)	H 7 (13-5-15)	*Pinus radiata*	Cone
67	*Diplodia sapinea*	P3 (Hernani)	H 8 (13-5-15)	*Pinus radiata*	Cone
68	*Diplodia sapinea*	P3 (Hernani)	H 9 (13-5-15)	*Pinus radiata*	Cone
69	*Diplodia sapinea*	P3 (Hernani)	H 10 (13-5-15)	*Pinus radiata*	Cone
70	*Diplodia sapinea*	P3 (Hernani)	H 11 (13-5-15)	*Pinus radiata*	Cone
71	*Diplodia sapinea*	P3 (Hernani)	H 12 (13-5-15)	*Pinus radiata*	Cone
72	*Diplodia sapinea*	P3 (Hernani)	H 13 (13-5-15)	*Pinus radiata*	Cone
73	*Diplodia sapinea*	P3 (Hernani)	H 14 (13-5-15)	*Pinus radiata*	Cone
74	*Diplodia sapinea*	P3 (Hernani)	H 15 (13-5-15)	*Pinus radiata*	Cone
75	*Diplodia sapinea*	P3 (Hernani)	H 16 (13-5-15)	*Pinus radiata*	Cone
76	*Diplodia sapinea*	Basque Country	M1 (22-7-15)	*Pinus radiata*	Cone
77	*Diplodia sapinea*	Basque Country	M2 (22-7-15)	*Pinus radiata*	Cone
78	*Diplodia sapinea*	Basque Country	M3 (22-7-15)	*Pinus radiata*	Cone
79	*Diplodia sapinea*	Basque Country	M4 (22-7-15)	*Pinus radiata*	Cone
80	*Diplodia sapinea*	Basque Country	M5 (22-7-15)	*Pinus radiata*	Cone
81	*Diplodia sapinea*	Basque Country	M6 (22-7-15)	*Pinus radiata*	Cone
82	*Diplodia sapinea*	Basque Country	M7 (22-7-15)	*Pinus nigra*	Cone
83	*Diplodia sapinea*	Basque Country	M8 (22-7-15)	*Pinus nigra*	Cone
84	*Diplodia sapinea*	Basque Country	M9 (22-7-15)	*Pinus nigra*	Cone
86	*Diplodia sapinea*	Basque Country	M1 (23-7-15)	*Pinus radiata*	Cone
87	*Diplodia sapinea*	Basque Country	M2 (23-7-15)	*Pinus radiata*	Cone
89	*Diplodia sapinea*	Basque Country	M4 (23-7-15)	*Pinus radiata*	Cone
90	*Diplodia sapinea*	Basque Country	M5 (23-7-15)	*Pinus radiata*	Cone
91	*Diplodia sapinea*	Basque Country	M6 (23-7-15)	*Pinus radiata*	Cone
92	*Diplodia sapinea*	Basque Country	M7 (23-7-15)	*Pinus radiata*	Cone
93	*Diplodia sapinea*	Basque Country	M8 (23-7-15)	*Pinus radiata*	Cone
94	*Diplodia sapinea*	Basque Country	M1 (24-7-15)	*Pinus radiata*	Cone
95	*Diplodia sapinea*	Basque Country	M2 (24-7-15)	*Pinus radiata*	Cone
96	*Diplodia sapinea*	Basque Country	M3 (24-7-15)	*Pinus radiata*	Cone

97	*Diplodia sapinea*	Basque Country	M4 (24-7-15)	*Pinus radiata*	Cone
98	*Diplodia sapinea*	Basque Country	M5 (24-7-15)	*Pinus radiata*	Cone
99	*Diplodia sapinea*	Basque Country	M6 (24-7-15)	*Pinus radiata*	Cone
100	*Diplodia sapinea*	Basque Country	M1 (3-8-15)	*Pinus radiata*	Cone

[a]All the isolates were included in the microsatellite genealogy and in the VCG study.
[b]Species designation is based on specific-species identification (Smith & Stanosz, 2006).

Appendix C. Characteristics of polymorphic SSR markers (Burgess *et al.* 2001b; Bihon *et al.*, 2011).

Locus	Sequence (5' to 3')	Annealing temp (ºC)	Fragment lenght (bp)
SS1	**6-FAM**-CAT GCA TCG ATC CTG TAG AGC CCA AGT GAT GAC CCT ATA GAG	58	324
SS5	**NED**-CCT GAG CGA CTC CAA GCT TG CTC ATT GGC TGC GAA ACG TG	62	453
SS9	**PET**-CAG CGG TTT CAT TGA AAT GCC GAC TTG TCT CCT ACC GAT TCC	62	252
SS10	**NED**-GCC AAC CCT AAT GCT TCC ATG CAG CGG CGA TTG CGG TAT GG	62	313
SS11	**VIC**-GTA ACA TTT CCC CAC GTC AGC GGA AGT ACT ACA TGG TCT TCG	58	174
SS12	**PET**-ACC ACC ACC ACC GTC AAG GAA CGC CAT CGT CGT CAC	62	107
SS13	**FAM**-GGC GTG TGT GAT GAG ATG AG GTC CTT TGT GTG TTG GGT TG	55	180
SS14	**NED**-CAC CAC CAC CAA CAC CTT G CGT GTT GGA AGC GAC GAC	58	149
SS15	**NED**-GAA TCA CG GCC GGT TTG GAG TCC AGC CTT TCC TCC TC	55	99
SS16	**VIC**-GGG GAA AAG ACG TGT TGT TGT CAG CAT CGT CGT CCC ATT AG	55	99

Appendix D. ID, Samples group, species desigation based on specific-species identification (Smith & Stanosz, 2006), location, host species and source for each isolate used in this study.

Sample ID	Sample group	Species	Location	Host species	Material
M157	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Root
M158	BC1	*D. sapinea*	Oiartzun	*Pinus radiata*	Trunk
M159	BC1	*D. sapinea*	Oiartzun	*Pinus radiata*	Trunk
M160	BC1	*D. sapinea*	Oiartzun	*Pinus radiata*	Trunk
M162	BC1	*D. sapinea*	Oiartzun	*Pinus radiata*	Trunk
M163	BC1	*D. sapinea*	Oiartzun	*Pinus radiata*	Trunk
M164	BC1	*D. sapinea*	Oiartzun	*Pinus radiata*	Trunk
M166	BC1	*D. sapinea*	Laukiniz	*Pinus radiata*	Pitch canker
M167	BC1	*D. sapinea*	Laukiniz	*Pinus radiata*	Pitch canker
M168	BC1	*D. sapinea*	Laukiniz	*Pinus radiata*	Pitch canker
M170	BC1	*D. sapinea*	Laukiniz	*Pinus radiata*	Pitch canker
M171	BC1	*D. sapinea*	Laukiniz	*Pinus radiata*	Pitch canker
M173	BC1	*D. sapinea*	Oiartzun	*Pinus radiata*	Trunk
M175	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Root
M176	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Root
M177	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Root
M178	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Root
M179	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Trunk
M180	BC1	*D. sapinea*	Oiartzun	*Pinus radiata*	Trunk
M181	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Root
M182	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Root
M183	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Root
M187	BC1	*D. sapinea*	Luiaondo	*Pinus radiata*	Trunk
M209	BC1	*D. sapinea*	Laukiniz	*Pinus radiata*	Root
M211	BC1	*D. sapinea*	Laukiniz	*Pinus radiata*	Root
M212	BC1	*D. sapinea*	Laukiniz	*Pinus radiata*	Root
M213	BC1	*D. sapinea*	Laukiniz	*Pinus radiata*	Root
M2 (3-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M5 (3-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M6 (3-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M7 (3-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M9 (3-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (10-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M4 (10-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M5 (10-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus nigra*	Cone

M6 (10-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M7 (10-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M8 (10-8-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M4 (10-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus halepensis*	Cone
M5 (10-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus sylvestris*	Cone
M7 (10-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus contorta*	Cone
M1 (11-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (11-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M4 (11-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (30-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (30-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M4 (30-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus nigra*	Cone
M5 (30-9-15)	BC2	*D. sapinea*	Basque Country	*Pinus nigra*	Cone
M1 (6-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (6-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M3 (6-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M5 (6-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (9-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (23-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (23-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M3 (23-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M4 (23-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M5 (23-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M6 (23-10-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (11-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (11-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M3 (11-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (18-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (18-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M3 (18-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (20-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (20-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M3 (20-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M4 (20-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M5 (20-11-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (1-12-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (1-12-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M3 (1-12-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M4 (1-12-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (2-12-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (2-12-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone

M3 (2-12-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M4 (2-12-15)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M3 (19-1-16)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (14-3-16)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (14-3-16)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M3 (14-3-16)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M4 (14-3-16)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M5 (14-3-16)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (18-3-16)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M1 (22-3-16)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
M2 (22-3-16)	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Cone
Oihan 70S	BC2	*D. scrobiculata*	Basque Country	*Pinus radiata*	Trunk
Oihan 295S	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Trunk
Oihan 402S	BC2	*D. sapinea*	Basque Country	*Pinus radiata*	Trunk
U1	USA 1	*D. sapinea*	F. Gilbert Hills State Forest, Foxborough, Massachusetts, USA	*Pinus resinosa*	Cone
U2	USA 1	*D. sapinea*	Vermont Institute of Natural Science, Washington, Vermont, USA	*Pinus resinosa*	Cone
U3	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U4	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U5	USA 1	*D. sapinea*	Harold Parker State Forest, Andover, Massachusetts, USA	*Pinus resinosa*	Cone
U6	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U7	USA 1	*D. sapinea*	Vermont Institute of Natural Science, Washington, Vermont, USA	*Pinus resinosa*	Cone
U8	USA 1	*D. sapinea*	Vermont Institute of Natural Science, Washington, Vermont, USA	*Pinus resinosa*	Cone
U9	USA 1	*D. sapinea*	Harold Parker State Forest, Andover, Massachusetts, USA	*Pinus resinosa*	Cone
U10	USA 1	*D. sapinea*	F. Gilbert Hills State Forest, Foxborough, Massachusetts, USA	*Pinus resinosa*	Cone
U11	USA 1	*D. sapinea*	Upper Cold River Road, Shrewsbury, Vermont, USA	*Pinus resinosa*	Cone
U12	USA 1	*D. sapinea*	Acadia National Park,	*Pinus resinosa*	Cone

			Hancock/Knox, Maine, USA		
U13	USA 1	*D. sapinea*	Upper Cold River Road, Shrewsbury, Vermont, USA	*Pinus resinosa*	Cone
U14	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U15	USA 1	*D. sapinea*	Marlborough-Sudbury State Forest, Hudson, Massachusetts, USA	*Pinus resinosa*	Cone
U16	USA 1	*D. sapinea*	Upper Cold River Road, Shrewsbury, Vermont, USA	*Pinus resinosa*	Cone
U17	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U18	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U19	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U20	USA 1	*D. sapinea*	Upper Cold River Road, Shrewsbury, Vermont, USA	*Pinus resinosa*	Cone
U21	USA 1	*D. sapinea*	Marlborough-Sudbury State Forest, Hudson, Massachusetts, USA	*Pinus resinosa*	Cone
U22	USA 1	*D. sapinea*	Meadowsend Timberlands Ltd., Washington, Vermont, USA	*Pinus resinosa*	Cone
U23	USA 1	*D. sapinea*	F. Gilbert Hills State Forest, Foxborough, Massachusetts, USA	*Pinus resinosa*	Cone
U24	USA 1	*D. sapinea*	Marlborough-Sudbury State Forest, Hudson, Massachusetts, USA	*Pinus resinosa*	Cone
U25	USA 1	*D. sapinea*	Marlborough-Sudbury State Forest, Hudson, Massachusetts, USA	*Pinus resinosa*	Cone
U26	USA 1	*D. sapinea*	Meadowsend Timberlands Ltd., Washington, Vermont, USA	*Pinus resinosa*	Cone
U27	USA 1	*D. sapinea*	Harold Parker State Forest, Andover, Massachusetts, USA	*Pinus resinosa*	Cone
U28	USA 1	*D. sapinea*	Meadowsend Timberlands Ltd., Washington,	*Pinus resinosa*	Cone

			Vermont, USA		
U29	USA 1	*D. sapinea*	F. Gilbert Hills State Forest, Foxborough, Massachusetts, USA	*Pinus resinosa*	Cone
U30	USA 1	*D. sapinea*	Vermont Institute of Natural Science, Washington, Vermont, USA	*Pinus resinosa*	Cone
U31	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U32	USA 1	*D. sapinea*	Marlborough-Sudbury State Forest, Hudson, Massachusetts, USA	*Pinus resinosa*	Cone
U33	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U34	USA 1	*D. sapinea*	Harold Parker State Forest, Andover, Massachusetts, USA	*Pinus resinosa*	Cone
U35	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U36	USA 1	*D. sapinea*	Harold Parker State Forest, Andover, Massachusetts, USA	*Pinus resinosa*	Cone
U37	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U38	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U39	USA 1	*D. sapinea*	F. Gilbert Hills State Forest, Foxborough, Massachusetts, USA	*Pinus resinosa*	Cone
U40	USA 1	*D. sapinea*	Harold Parker State Forest, Andover, Massachusetts, USA	*Pinus resinosa*	Cone
U41	USA 1	*D. sapinea*	Vermont Institute of Natural Science, Washington, Vermont, USA	*Pinus resinosa*	Cone
U42	USA 1	*D. sapinea*	Meadowsend Timberlands Ltd., Washington, Vermont, USA	*Pinus resinosa*	Cone
U43	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U44	USA 1	*D. sapinea*	Acadia National Park, Hancock/Knox, Maine, USA	*Pinus resinosa*	Cone
U45	USA 1	*D. sapinea*	Meadowsend Timberlands Ltd.,	*Pinus resinosa*	Cone

			Washington, Vermont, USA		
U46	USA 1	*D. sapinea*	Marlborough-Sudbury State Forest, Hudson, Massachusetts, USA	*Pinus resinosa*	Cone
U47	USA 1	*D. sapinea*	Vermont Institute of Natural Science, Washington, Vermont, USA	*Pinus resinosa*	Cone
U48	USA 1	*D. sapinea*	Meadowsend Timberlands Ltd., Washington, Vermont, USA	*Pinus resinosa*	Cone
U49	USA 1	*D. sapinea*	Upper Cold River Road, Shrewsbury, Vermont, USA	*Pinus resinosa*	Cone
U50	USA 1	*D. sapinea*	Upper Cold River Road, Shrewsbury, Vermont, USA	*Pinus resinosa*	Cone
97-16	USA 2	*D. sapinea*	Russell County, Kentucky USA	*Pinus virginiana*	Unknown
96-148	USA 2	*D. sapinea*	Barbour County, West Virginia USA	*Pinus sylvestris*	Needle
04-77	USA 2	*D. sapinea*	Black Hills, South Dakota USA	*Pinus ponderosa*	Stem
94-156	USA 2	*D. sapinea*	Idaho USA	*Pinus ponderosa*	Unknown
128	USA 2	*D. sapinea*	Grant County, Wisconsin USA	*Pinus resinosa*	Unknown
04-16	USA 2	*D. sapinea*	Starkville, Mississippi USA	*Pinus palustris*	Unknown
92-44	USA 2	*D. sapinea*	Pennsylvania USA	*Pinus sylvestris*	Unknown
99-5	USA 2	*D. sapinea*	Riverside County, California USA	*Pinus jeffreyi*	Unknown
96-45	USA 2	*D. sapinea*	Florida USA	*Pinus elliottii*	Cone-seed
124	USA 2	*D. sapinea*	Jackson County, Wisconsin USA	*Pinus bankisiana*	Unknown
99-24	USA 2	*D. sapinea*	Tuolumne County, California USA	*Pinus sabiniana*	Unknown
03-15	USA 2	*D. sapinea*	Bennington County, Vermont USA	*Pinus resinosa*	Cone
189	USA 2	*D. sapinea*	Maui, Hawaii USA	*Pinus radiata*	Unknown
411	USA 2	*D. sapinea*	Hubbard County, Minnesota USA	*Pinus resinosa*	Unknown
07-14	USA 2	*D. sapinea*	Dallas County, Texas USA	*Pinus elderica*	Cone
98-1	USA 2	*D. sapinea*	Georgia USA	*Pinus taeda*	Needle
17-02	USA 2	*D. sapinea*	Wisconsin USA	*Pinus banksiana*	Needle
17-03	USA 2	*D. sapinea*	Wisconsin USA	*Pinus banksiana*	Needle
17-04	USA 2	*D. sapinea*	Waushara County, Wisconsin USA	*Pinus resinosa*	Needle
17-05	USA 2	*D. sapinea*	Portage County,	*Pinus*	Needle

			Wisconsin USA	*banksiana*	
17-06	USA 2	*D. sapinea*	Portage County, Wisconsin USA	*Pinus resinosa*	Needle
17-07	USA 2	*D. sapinea*	Wood County, Wisconsin USA	*Pinus resinosa*	Needle
17-08	USA 2	*D. sapinea*	Adams County, Wisconsin USA	*Pinus resinosa*	Needle
17-01	USA 2	*D. sapinea*	Marathon County, Wisconsin USA	*Pinus resinosa*	Needle
13-09	USA 2	*D. sapinea*	Wallowa County, Oregon USA	*Pinus ponderosa*	Cone
13-10	USA 2	*D. sapinea*	Wallowa County, Oregon, USA	*Pinus ponderosa*	Needle
03-24	USA 2	*D. sapinea*	Bennington County, Vermont USA	*Pinus resinosa*	Stem tip
04-116	USA 2	*D. sapinea*	Sawyer County, Wisconsin USA	*Pinus banksiana*	Stem
04-124	USA 2	*D. sapinea*	Wood County, Wisconsin USA	*Pinus banksiana*	Stem
04-15	USA 2	*D. sapinea*	Vilas County, Wisconsin USA	*Pinus resinosa*	Unknown
04-78	USA 2	*D. sapinea*	South Dakota	*Pinus ponderosa*	Unknown
07-16	USA 2	*D. sapinea*	Dallas County, Texas USA	*Pinus nigra*	Cone
07-26	USA 2	*D. sapinea*	Bayfield County, Wisconsin USA	*Pinus resinosa*	Needle
07-59	USA 2	*D. sapinea*	Sumter County, Alabama USA	*Pinus taeda*	Cone
08-15	USA 2	*D. sapinea*	Adams County, Wisconsin USA	*Pinus sylvestris*	Bark
09-07	USA 2	*D. sapinea*	Flathead County, Montana USA	*Pinus ponderosa*	Unknown
217	USA 2	*D. sapinea*	St. Louis County, Minnesota USA	*Pinus resinosa*	Unknown
191	USA 2	*D. sapinea*	Pine County, Minnesota USA	*Pinus resinosa*	Unknown
226	USA 2	*D. sapinea*	Monongalia County, West Virginia USA	*Pinus nigra*	Needle
94-107	USA 2	*D. sapinea*	Jackson County, Wisconsin USA	*Pinus resinosa*	Needle
94-15	USA 2	*D. sapinea*	Dane County, Wisconsin USA	*Pinus nigra*	Needle
94-191	USA 2	*D. sapinea*	Northern Highland American Legion State Forest, Wisconsin USA	*Pinus banksiana*	Twig
94-22	USA 2	*D. sapinea*	Marquette County, Wisconsin USA	*Pinus resinosa*	Needle
94-192	USA 2	*D. sapinea*	Trempealeau County, Wisconsin USA	*Pinus resinosa*	Needle
94-41	USA 2	*D. sapinea*	Fairfield County, Connecticut USA	*Pinus sylvestris*	Needle
94-77	USA 2	*D. sapinea*	Adair County, Iowa USA	*Pinus nigra*	Needle

94-79	USA 2	*D. sapinea*	Codington County, South Dakota USA	*Pinus sylvestris*	Needle
94-81	USA 2	*D. sapinea*	Polk County, Iowa USA	*Pinus nigra*	Needle
94-8	USA 2	*D. sapinea*	Lacrosse County, Wisconsin USA	*Pinus resinosa*	Needle
95-42	USA 2	*D. sapinea*	Wood County, Wisconsin USA	*Pinus resinosa*	Stem
95-65	USA 2	*D. sapinea*	Centre County, Pennsylvania USA	*Pinus nigra*	Needle
96-142	USA 2	*D. sapinea*	Upshur County, West Virginia USA	*Pinus sylvestris*	Needle
96-181	USA 2	*D. sapinea*	Lafayette County, Wisconsin USA	*Pinus resinosa*	Needle
96-67	USA 2	*D. sapinea*	Monroe County, Wisconsin USA	*Pinus nigra*	Unknown
96-95	USA 2	*D. sapinea*	Cheboygan County, Michigan USA	*Pinus banksiana*	Needle
97-117	USA 2	*D. sapinea*	Manistee National Forest, Michigan USA	*Pinus resinosa*	Unknown
97-22	USA 2	*D. sapinea*	Jefferson County, West Virginia USA	*Pinus nigra*	Needle
99-4	USA 2	*D. sapinea*	Stanislaus National Forest, California USA	*Pinus ponderosa*	Unknown
96-160	USA 2	*D. sapinea*	Morgan County, West Virginia USA	*Pinus sylvestris*	Needle
96-158	USA 2	*D. sapinea*	Marion County, Indiana USA	*Pinus nigra*	Cone
03-50	USA 2	*D. scrobiculata*	Pine County, Minnesota USA	*Pinus banksiana*	Unknown
04-01	USA 2	*D. scrobiculata*	Jackson County, Wisconsin USA	*Pinus banksiana*	Unknown
212	USA 2	*D. scrobiculata*	Douglas County, Wisconsin USA	*Pinus resinosa*	Unknown
94-108	USA 2	*D. scrobiculata*	Jackson County, Wisconsin USA	*Pinus banksiana*	Needle
94-194	USA 2	*D. scrobiculata*	Mille Lacs County, Minnesota USA	*Pinus sylvestris*	Twig
94-45	USA 2	*D. scrobiculata*	Waushara County, Wisconsin USA	*Pinus banksiana*	Needle
95-41	USA 2	*D. scrobiculata*	Wood County, Wisconsin USA	*Pinus resinosa*	Stem
224	USA 2	*D. scrobiculata*	St. Louis County, Minnesota USA	*Pinus resinosa*	Unknown
407	USA 2	*D. scrobiculata*	Wadena County, Minnesota USA	*Pinus resinosa*	Unknown
08-14	USA 2	*D. scrobiculata*	Adams County, Wisconsin USA	*Pinus sylvestris*	Bark
04-19	USA 2	*D. scrobiculata*	Hubbard County, Minnesota USA	*Pinus resinosa*	Unknown
04-114	USA 2	*D. scrobiculata*	Sauk County, Wisconsin USA	*Pinus banksiana*	Unknown
95-54	USA 2	*D. scrobiculata*	Wood County, Wisconsin USA	*Pinus resinosa*	Needle

96-159	USA 2	*D. scrobiculata*	Mille Lacs County, Minnesota USA	*Pinus sylvestris*	Stem
02-254	USA 2	*D. scrobiculata*	Pine County, Minnesota USA	*Pinus resinosa*	Unknown
245	USA 2	*D. scrobiculata*	Douglas County, Wisconsin USA	*Pinus resinosa*	Unknown
07-01	USA 2	*D. scrobiculata*	Bath County, Virginia USA	*Pinus strobus*	Branch
201	USA 2	*D. scrobiculata*	Johnson County, Illinois USA	*Pinus strobus*	Unknown
04-20	USA 2	*D. scrobiculata*	Hubbard County, Minnesota USA	*Pinus resinosa*	Unknown
248	USA 2	*D. scrobiculata*	Douglas County, Wisconsin USA	*Pinus resinosa*	Unknown
07-20	USA 2	*D. scrobiculata*	Sumter County, Alabama USA	*Pinus taeda*	Stem
99-24	USA 2	*D. scrobiculata*	Kern County, California USA	*Pinus sabiniana*	Unknown
07-05	USA 2	*D. scrobiculata*	Bath County, Virginia USA	*Pinus strobus*	Stem
94-112	USA 2	*D. scrobiculata*	Mille Lacs County, Minnesota USA	*Pinus sylvestris*	Needle
03-33	USA 2	*D. scrobiculata*	Monterey County, California USA	*Pinus radiata*	Cone
97-63	W	*D. sapinea*	Facultad de Ciencias Forestales, Nuevo León, Mexico	*Pinus culminicola*	Needle
94-177	W	*D. sapinea*	Honduras	*Pinus oocarpa*	Unknown
94-160	W	*D. sapinea*	New Zealand	*Pinus radiata*	Unknown
94-19	W	*D. sapinea*	France	*Cedrus deodara*	Unknown
07-43	W	*D. sapinea*	Turkey	*Pinus nigra*	Unknown
93-23	W	*D. sapinea*	Gnangara Nursery, Australia	*Pinus radiata*	Unknown
97-25	W	*D. sapinea*	Berthier nuersery, Quebec Canada	*Pinus resinosa*	Needle
154	W	*D. sapinea*	Shune, Tanzania	Unknown	Unknown
94-161	W	*D. sapinea*	Spain	*Cedrus atlantica*	Unknown
94-162	W	*D. sapinea*	United Kingdom	*Pinus muricata*	Unknown
01-80	W	*D. sapinea*	Lomond Township, Ontario Canada	*Picea mariana*	Unknown
96-24	W	*D. sapinea*	Madagascar	*Pinus roxburghii*	Unknown
178	W	*D. sapinea*	New Zealand	*Pinus radiata*	Unknown
94-25	W	*D. sapinea*	Foret d'Orleans, France	*Pinus sylvestris*	Cone
96-67	W	*D. sapinea*	Mississauga, Ontario Canada	*Pinus nigra*	Unknown
96-38	W	*D. scrobiculata*	Carmel Mountain, Israel	*Pinus halepensis*	Unknown

Appendix E. ID, species desigation based on specific-species identification (Smith & Stanosz, 2006), location, name of the sample, host species and source for each isolate used in this study.

Sample ID	Species	Location	Name of sample	Host species	Material
2	*D. sapinea*	P1 (Laukiniz)	P1-M1 (6-2-15)	*Pinus radiata*	Cone
3	*D. sapinea*	P1 (Laukiniz)	P1-M2 (6-2-15)	*Pinus radiata*	Cone
4	*D. sapinea*	P1 (Laukiniz)	P1-M3 (6-2-15)	*Pinus radiata*	Cone
5	*D. sapinea*	P1 (Laukiniz)	P1-M4 (6-2-15)	*Pinus radiata*	Cone
6	*D. sapinea*	P1 (Laukiniz)	P1-M5 (6-2-15)	*Pinus radiata*	Cone
7	*D. sapinea*	P1 (Laukiniz)	P1-M6 (6-2-15)	*Pinus radiata*	Cone
8	*D. sapinea*	P1 (Laukiniz)	P1-M7 (6-2-15)	*Pinus radiata*	Cone
9	*D. sapinea*	P1 (Laukiniz)	P1-M8 (6-2-15)	*Pinus radiata*	Cone
10	*D. sapinea*	P1 (Laukiniz)	P1-M9 (6-2-15)	*Pinus radiata*	Cone
11	*D. sapinea*	P1 (Laukiniz)	P1-M10 (6-2-15)	*Pinus radiata*	Cone
14	*D. sapinea*	Basque Country	PV 24 (19-2-15)	*Pinus radiata*	Cone
15	*D. sapinea*	Basque Country	PV 26 (19-2-15)	*Pinus radiata*	Cone
17	*D. sapinea*	Basque Country	P2-M1 (27-2-15)	*Pinus radiata*	Cone
18	*D. sapinea*	Basque Country	P2-M2 (27-2-15)	*Pinus radiata*	Cone
19	*D. sapinea*	Basque Country	P2-M3 (27-2-15)	*Pinus radiata*	Cone
22	*D. sapinea*	P2 (Sollano)	W.P. 6 (9-3-15)	*Pinus radiata*	Cone
23	*D. sapinea*	P2 (Sollano)	W.P. 7 (9-3-15)	*Pinus radiata*	Cone
24	*D. sapinea*	P2 (Sollano)	W.P. 8 (9-3-15)	*Pinus radiata*	Cone
26	*D. sapinea*	P2 (Sollano)	W.P. 10 (9-3-15)	*Pinus radiata*	Cone
27	*D. sapinea*	P2 (Sollano)	W.P. 11 (9-3-15)	*Pinus radiata*	Cone
29	*D. sapinea*	P2 (Sollano)	W.P. 13 (9-3-15)	*Pinus radiata*	Cone
30	*D. sapinea*	P2 (Sollano)	W.P. 14 (9-3-15)	*Pinus radiata*	Cone
31	*D. sapinea*	P2 (Sollano)	W.P. 15 (9-3-15)	*Pinus radiata*	Cone
33	*D. sapinea*	P2 (Sollano)	W.P. 17 (9-3-15)	*Pinus radiata*	Cone
34	*D. sapinea*	P2 (Sollano)	W.P. 18 (9-3-15)	*Pinus radiata*	Cone
35	*D. sapinea*	P2 (Sollano)	W.P. 19 (9-3-15)	*Pinus radiata*	Cone
36	*D. sapinea*	P2 (Sollano)	W.P. 20 (9-3-15)	*Pinus radiata*	Cone
38	*D. sapinea*	P2 (Sollano)	W.P. 23 (9-3-15)	*Pinus radiata*	Cone
39	*D. sapinea*	Basque Country	W.P. 24 (9-3-	*Pinus radiata*	Cone

			15)		
40	*D. sapinea*	Basque Country	W.P. 25 (9-3-15)	*Pinus radiata*	Cone
41	*D. sapinea*	Basque Country	N5 (2-3-15)	*Pinus nigra*	Cone
42	*D. sapinea*	Basque Country	M2 (2-3-15)	*Pinus nigra*	Cone
44	*D. sapinea*	Basque Country	N5 (23-2-15)	*Pinus nigra*	Cone
45	*D. sapinea*	Basque Country	Elguea 1 (7-4-15)	*Pinus radiata*	Cone
46	*D. sapinea*	Basque Country	Elguea 2 (7-4-15)	*Pinus radiata*	Cone
47	*D. sapinea*	Basque Country	Elguea 3 (7-4-15)	*Pinus radiata*	Cone
48	*D. sapinea*	Basque Country	Muxika 1 (13-4-15)	*Pinus radiata*	Cone
49	*D. sapinea*	Basque Country	Muxika 2 (13-4-15)	*Pinus radiata*	Cone
50	*D. sapinea*	Basque Country	Muxika 3 (13-4-15)	*Pinus radiata*	Cone
51	*D. sapinea*	Basque Country	Gorozika 1 (13-4-15)	*Pinus radiata*	Cone
52	*D. sapinea*	Basque Country	Gorozika 2 (13-4-15)	*Pinus radiata*	Cone
53	*D. sapinea*	Basque Country	Oiz 1 (13-4-15)	*Pinus radiata*	Cone
54	*D. sapinea*	Basque Country	Aulesti 1 (21-4-15)	*Pinus radiata*	Cone
55	*D. sapinea*	Basque Country	Agiñeko 1 (23-4-15)	*Pinus radiata*	Cone
56	*D. sapinea*	Basque Country	Berganzo 1 (31-3-15)	*Pinus attenuata*	Cone
57	*D. sapinea*	Basque Country	M1 (14-4-15)	*Pinus radiata*	Cone
60	*D. sapinea*	P3 (Hernani)	H 1 (13-5-15)	*Pinus radiata*	Cone
61	*D. sapinea*	P3 (Hernani)	H 2 (13-5-15)	*Pinus radiata*	Cone
63	*D. sapinea*	P3 (Hernani)	H 4 (13-5-15)	*Pinus radiata*	Cone
64	*D. sapinea*	P3 (Hernani)	H 5 (13-5-15)	*Pinus radiata*	Cone
65	*D. sapinea*	P3 (Hernani)	H 6 (13-5-15)	*Pinus radiata*	Cone
66	*D. sapinea*	P3 (Hernani)	H 7 (13-5-15)	*Pinus radiata*	Cone
68	*D. sapinea*	P3 (Hernani)	H 9 (13-5-15)	*Pinus radiata*	Cone
69	*D. sapinea*	P3 (Hernani)	H 10 (13-5-15)	*Pinus radiata*	Cone
70	*D. sapinea*	P3 (Hernani)	H 11 (13-5-15)	*Pinus radiata*	Cone
71	*D. sapinea*	P3 (Hernani)	H 12 (13-5-15)	*Pinus radiata*	Cone
72	*D. sapinea*	P3 (Hernani)	H 13 (13-5-15)	*Pinus radiata*	Cone
73	*D. sapinea*	P3 (Hernani)	H 14 (13-5-15)	*Pinus radiata*	Cone
75	*D. sapinea*	P3 (Hernani)	H 16 (13-5-15)	*Pinus radiata*	Cone
76	*D. sapinea*	Basque Country	M1 (22-7-15)	*Pinus radiata*	Cone
77	*D. sapinea*	Basque Country	M2 (22-7-15)	*Pinus radiata*	Cone
78	*D. sapinea*	Basque Country	M3 (22-7-15)	*Pinus radiata*	Cone
79	*D. sapinea*	Basque Country	M4 (22-7-15)	*Pinus radiata*	Cone
80	*D. sapinea*	Basque Country	M5 (22-7-15)	*Pinus radiata*	Cone
81	*D. sapinea*	Basque Country	M6 (22-7-15)	*Pinus radiata*	Cone

82	*D. sapinea*	Basque Country	M7 (22-7-15)	*Pinus nigra*	Cone
83	*D. sapinea*	Basque Country	M8 (22-7-15)	*Pinus nigra*	Cone
84	*D. sapinea*	Basque Country	M9 (22-7-15)	*Pinus nigra*	Cone
86	*D. sapinea*	Basque Country	M1 (23-7-15)	*Pinus radiata*	Cone
87	*D. sapinea*	Basque Country	M2 (23-7-15)	*Pinus radiata*	Cone
89	*D. sapinea*	Basque Country	M4 (23-7-15)	*Pinus radiata*	Cone
90	*D. sapinea*	Basque Country	M5 (23-7-15)	*Pinus radiata*	Cone
91	*D. sapinea*	Basque Country	M6 (23-7-15)	*Pinus radiata*	Cone
92	*D. sapinea*	Basque Country	M7 (23-7-15)	*Pinus radiata*	Cone
93	*D. sapinea*	Basque Country	M8 (23-7-15)	*Pinus radiata*	Cone
94	*D. sapinea*	Basque Country	M1 (24-7-15)	*Pinus radiata*	Cone
95	*D. sapinea*	Basque Country	M2 (24-7-15)	*Pinus radiata*	Cone
96	*D. sapinea*	Basque Country	M3 (24-7-15)	*Pinus radiata*	Cone
97	*D. sapinea*	Basque Country	M4 (24-7-15)	*Pinus radiata*	Cone
98	*D. sapinea*	Basque Country	M5 (24-7-15)	*Pinus radiata*	Cone
99	*D. sapinea*	Basque Country	M6 (24-7-15)	*Pinus radiata*	Cone
100	*D. sapinea*	Basque Country	M1 (3-8-15)	*Pinus radiata*	Cone
101	*D. sapinea*	Basque Country	M2 (3-8-15)	*Pinus radiata*	Cone
104	*D. sapinea*	Basque Country	M5 (3-8-15)	*Pinus nigra*	Cone
105	*D. sapinea*	Basque Country	M6 (3-8-15)	*Pinus radiata*	Cone
106	*D. sapinea*	Basque Country	M7 (3-8-15)	*Pinus radiata*	Cone
108	*D. sapinea*	Basque Country	M9 (3-8-15)	*Pinus radiata*	Cone
109	*D. sapinea*	Basque Country	M1 (10-8-15)	*Pinus pinaster*	Cone
112	*D. sapinea*	Basque Country	M4 (10-8-15)	*Pinus radiata*	Cone
113	*D. sapinea*	Basque Country	M5 (10-8-15)	*Pinus nigra*	Cone
114	*D. sapinea*	Basque Country	M6 (10-8-15)	*Pinus radiata*	Cone
115	*D. sapinea*	Basque Country	M7 (10-8-15)	*Pinus radiata*	Cone
116	*D. sapinea*	Basque Country	M8 (10-8-15)	*Pinus radiata*	Cone
120	*D. sapinea*	Basque Country	M4 (10-9-15)	*Pinus halepensis*	Cone
121	*D. sapinea*	Basque Country	M5 (10-9-15)	*Pinus sylvestris*	Cone
123	*D. sapinea*	Basque Country	M7 (10-9-15)	*Pinus contorta*	Cone
124	*D. sapinea*	Basque Country	M1 (11-9-15)	*Pinus radiata*	Cone
125	*D. sapinea*	Basque Country	M2 (11-9-15)	*Pinus radiata*	Cone
127	*D. sapinea*	Basque Country	M4 (11-9-15)	*Pinus radiata*	Cone
128	*D. sapinea*	Basque Country	M1 (30-9-15)	*Pinus radiata*	Cone
129	*D. sapinea*	Basque Country	M2 (30-9-15)	*Pinus radiata*	Cone
131	*D. sapinea*	Basque Country	M4 (30-9-15)	*Pinus nigra*	Cone
132	*D. sapinea*	Basque Country	M5 (30-9-15)	*Pinus nigra*	Cone
133	*D. sapinea*	Basque Country	M1 (6-10-15)	*Pinus radiata*	Cone
134	*D. sapinea*	Basque Country	M2 (6-10-15)	*Pinus radiata*	Cone
135	*D. sapinea*	Basque Country	M3 (6-10-15)	*Pinus radiata*	Cone
137	*D. sapinea*	Basque Country	M5 (6-10-15)	*Pinus radiata*	Cone
138	*D. sapinea*	Basque Country	M1 (9-10-15)	*Pinus radiata*	Cone

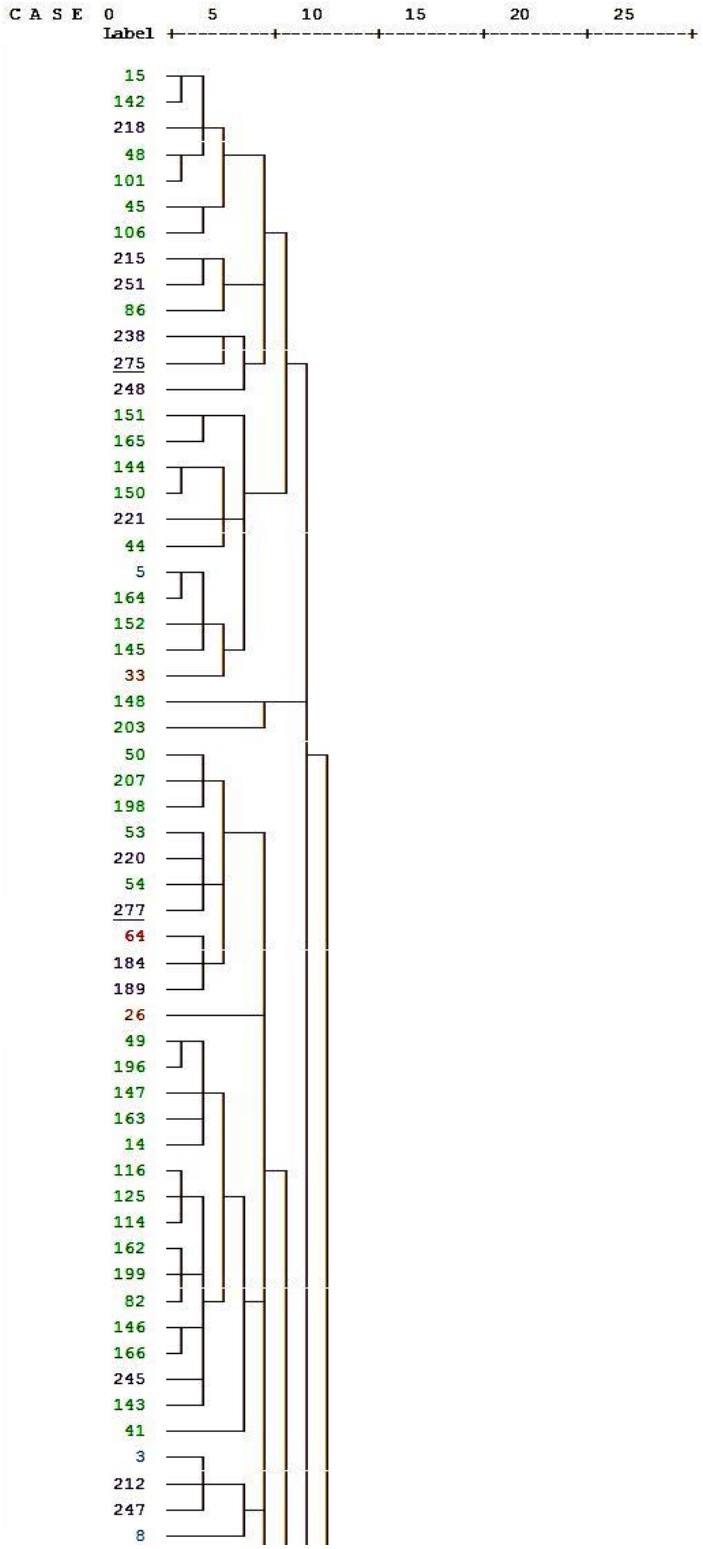
141	*D. sapinea*	Basque Country	M1 (23-10-15)	*Pinus radiata*	Cone
142	*D. sapinea*	Basque Country	M2 (23-10-15)	*Pinus radiata*	Cone
143	*D. sapinea*	Basque Country	M3 (23-10-15)	*Pinus radiata*	Cone
144	*D. sapinea*	Basque Country	M4 (23-10-15)	*Pinus radiata*	Cone
145	*D. sapinea*	Basque Country	M5 (23-10-15)	*Pinus radiata*	Cone
146	*D. sapinea*	Basque Country	M6 (23-10-15)	*Pinus radiata*	Cone
147	*D. sapinea*	Basque Country	M1 (11-11-15)	*Pinus radiata*	Cone
148	*D. sapinea*	Basque Country	M2 (11-11-15)	*Pinus radiata*	Cone
149	*D. sapinea*	Basque Country	M3 (11-11-15)	*Pinus radiata*	Cone
150	*D. sapinea*	Basque Country	M1 (18-11-15)	*Pinus radiata*	Cone
151	*D. sapinea*	Basque Country	M2 (18-11-15)	*Pinus radiata*	Cone
152	*D. sapinea*	Basque Country	M3 (18-11-15)	*Pinus radiata*	Cone
154	*D. sapinea*	Basque Country	M1 (20-11-15)	*Pinus radiata*	Cone
155	*D. sapinea*	Basque Country	M2 (20-11-15)	*Pinus radiata*	Cone
156	*D. sapinea*	Basque Country	M3 (20-11-15)	*Pinus radiata*	Cone
157	*D. sapinea*	Basque Country	M4 (20-11-15)	*Pinus radiata*	Cone
158	*D. sapinea*	Basque Country	M5 (20-11-15)	*Pinus radiata*	Cone
159	*D. sapinea*	Basque Country	M1 (1-12-15)	*Pinus radiata*	Cone
160	*D. sapinea*	Basque Country	M2 (1-12-15)	*Pinus radiata*	Cone
161	*D. sapinea*	Basque Country	M3 (1-12-15)	*Pinus radiata*	Cone
162	*D. sapinea*	Basque Country	M4 (1-12-15)	*Pinus radiata*	Cone
163	*D. sapinea*	Basque Country	M1 (2-12-15)	*Pinus radiata*	Cone
164	*D. sapinea*	Basque Country	M2 (2-12-15)	*Pinus radiata*	Cone
165	*D. sapinea*	Basque Country	M3 (2-12-15)	*Pinus radiata*	Cone
166	*D. sapinea*	Basque Country	M4 (2-12-15)	*Pinus radiata*	Cone
169	*D. sapinea*	Basque Country	M3 (19-1-16)	*Pinus radiata*	Cone
170	*D. scrobiculata*	Russell County, Kentucky USA	97-16	*Pinus virginiana*	Unknown
175	*D. sapinea*	Grant County, Wisconsin USA	128	*Pinus resinosa*	Unknown
176	*D. sapinea*	Honduras	94-177	*Pinus oocarpa*	Unknown
181	*D. sapinea*	Turkey	07-43	*Pinus nigra*	Unknown
184	*D. sapinea*	Shune, Tanzania	154	*Unknown*	Unknown
185	*D. sapinea*	Riverside County, California USA	99-5	*Pinus jeffreyi*	Unknown
187	*D. scrobiculata*	Jackson County, Wisconsin USA	124	*Pinus bankisiana*	Unknown
188	*D. scrobiculata*	Spain	94-161	*Cedrus atlantica*	Unknown
189	*D. scrobiculata*	Tuolumne County, California USA	99-24	*Pinus sabiniana*	Unknown
190	*D. sapinea*	Bennington County, Vermont USA	03-15	*Pinus resinosa*	Cone
191	*D. sapinea*	Maui, Hawaii USA	189	*Pinus radiata*	Unknown
194	*D. sapinea*	Dallas County, Texas USA	07-14	*Pinus elderica*	Cone
195	*D.*	Lomond Township,	01-80	*Picea mariana*	Unknown

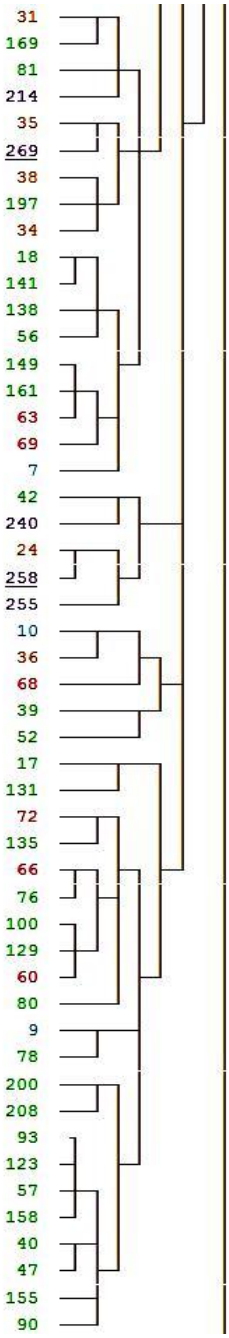
	scrobiculata	Ontario Canada			
196	*D. sapinea*	Basque Country	M1 (14-3-16)	*Pinus radiata*	Cone
197	*D. sapinea*	Basque Country	M2 (14-3-16)	*Pinus radiata*	Cone
198	*D. sapinea*	Basque Country	M3 (14-3-16)	*Pinus radiata*	Cone
199	*D. sapinea*	Basque Country	M4 (14-3-16)	*Pinus radiata*	Cone
200	*D. sapinea*	Basque Country	M5 (14-3-16)	*Pinus radiata*	Cone
201	*D. sapinea*	Basque Country	M2 (18-3-16)	*Pinus radiata*	Cone
202	*D. sapinea*	Basque Country	M1 (22-3-16)	*Pinus radiata*	Cone
203	*D. sapinea*	Basque Country	M2 (22-3-16)	*Pinus radiata*	Cone
206	*D. sapinea*	Georgia USA	98-1	*Pinus taeda*	Needle
207	*D. scrobiculata*	Basque Country	Oihan 70S	*Pinus radiata*	Trunk
208	*D. sapinea*	Basque Country	Oihan 295S	*Pinus radiata*	Trunk
209	*D. sapinea*	Basque Country	Oihan 402S	*Pinus radiata*	Trunk
210	*D. sapinea*	Wisconsin USA	17-02	*Pinus banksiana*	Needle
211	*D. sapinea*	Wisconsin USA	17-03	*Pinus banksiana*	Needle
212	*D. sapinea*	Waushara County, Wisconsin USA	17-04	*Pinus resinosa*	Needle
213	*D. sapinea*	Portage County, Wisconsin USA	17-05	*Pinus banksiana*	Needle
214	*D. sapinea*	Portage County, Wisconsin USA	17-06	*Pinus resinosa*	Needle
215	*D. sapinea*	Wood County, Wisconsin USA	17-07	*Pinus resinosa*	Needle
216	*D. sapinea*	Adams County, Wisconsin USA	17-08	*Pinus resinosa*	Needle
217	*D. sapinea*	Marathon County, Wisconsin USA	17-01	*Pinus resinosa*	Needle
218	*D. sapinea*	Wallowa County, Oregon USA	13-09	*Pinus ponderosa*	Cone
219	*D. sapinea*	Wallowa County, Oregon, USA	13-10	*Pinus ponderosa*	Needle
220	*D. sapinea*	Bennington County, Vermont USA	03-24	*Pinus resinosa*	Stem tip
221	*D. sapinea*	Sawyer County, Wisconsin USA	04-116	*Pinus banksiana*	Stem
222	*D. sapinea*	Wood County, Wisconsin USA	04-124	*Pinus banksiana*	Stem
223	*D. sapinea*	Vilas County, Wisconsin USA	04-15	*Pinus resinosa*	Unknown
224	*D. sapinea*	South Dakota	04-78	*Pinus ponderosa*	Unknown
225	*D. sapinea*	Dallas County, Texas USA	07-16	*Pinus nigra*	Cone
226	*D. sapinea*	Bayfield County, Wisconsin USA	07-26	*Pinus resinosa*	Needle
227	*D. sapinea*	Sumter County, Alabama USA	07-59	*Pinus taeda*	Cone
228	*D. sapinea*	Adams County, Wisconsin USA	08-15	*Pinus sylvestris*	Bark

230	*D. sapinea*	New Zealand	178	*Pinus radiata*	Unknown
232	*D. sapinea*	Pine County, Minnesota USA	191	*Pinus resinosa*	Unknown
234	*D. sapinea*	Jackson County, Wisconsin USA	94-107	*Pinus resinosa*	Needle
235	*D. sapinea*	Dane County, Wisconsin USA	94-15	*Pinus nigra*	Needle
236	*D. sapinea*	Northern Highland American Legion State Forest, Wisconsin USA	94-191	*Pinus banksiana*	Twig
237	*D. sapinea*	Foret d'Orleans France	94-25	*Pinus sylvestris*	Cone
238	*D. sapinea*	Marquette County, Wisconsin USA	94-22	*Pinus resinosa*	Needle
239	*D. sapinea*	Trempealeau County, Wisconsin USA	94-192	*Pinus resinosa*	Needle
240	*D. sapinea*	Fairfield County, Connecticut USA	94-41	*Pinus sylvestris*	Needle
241	*D. sapinea*	Adair County, Iowa USA	94-77	*Pinus nigra*	Needle
242	*D. sapinea*	Codington County, South Dakota USA	94-79	*Pinus sylvestris*	Needle
243	*D. sapinea*	Polk County, Iowa USA	94-81	*Pinus nigra*	Needle
244	*D. sapinea*	Lacrosse County, Wisconsin USA	94-8	*Pinus resinosa*	Needle
245	*D. sapinea*	Wood County, Wisconsin USA	95-42	*Pinus resinosa*	Stem
246	*D. sapinea*	Centre County, Pennsylvania USA	95-65	*Pinus nigra*	Needle
247	*D. sapinea*	Upshur County, West Virginia USA	96-142	*Pinus sylvestris*	Needle
248	*D. sapinea*	Lafayette County, Wisconsin USA	96-181	*Pinus resinosa*	Needle
250	*D. sapinea*	Monroe County, Wisconsin USA	96-67	*Pinus nigra*	Unknown
251	*D. sapinea*	Cheboygan County, Michigan USA	96-95	*Pinus banksiana*	Needle
252	*D. sapinea*	Manistee National Forest, Michigan USA	97-117	*Pinus resinosa*	Unknown
253	*D. sapinea*	Jefferson County, West Virginia USA	97-22	*Pinus nigra*	Needle
254	*D. sapinea*	Stanislaus National Forest, California USA	99-4	*Pinus ponderosa*	Unknown
255	*D. sapinea*	Morgan County, West Virginia USA	96-160	*Pinus sylvestris*	Needle
256	*D. sapinea*	Marion County, Indiana USA	96-158	*Pinus nigra*	Cone
258	*D. scrobiculata*	Jackson County, Wisconsin USA	04-01	*Pinus banksiana*	Unknown
259	*D. scrobiculata*	Douglas County, Wisconsin USA	212	*Pinus resinosa*	Unknown
260	*D.*	Jackson County,	94-108	*Pinus*	Needle

	scrobiculata	Wisconsin USA		*banksiana*	
261	*D. scrobiculata*	Mille Lacs County, Minnesota USA	94-194	*Pinus sylvestris*	Twig
262	*D. scrobiculata*	Waushara County, Wisconsin USA	94-45	*Pinus banksiana*	Needle
263	*D. scrobiculata*	Wood County, Wisconsin USA	95-41	*Pinus resinosa*	Stem
264	*D. scrobiculata*	St. Louis County, Minnesota USA	224	*Pinus resinosa*	Unknown
265	*D. scrobiculata*	Wadena County, Minnesota USA	407	*Pinus resinosa*	Unknown
267	*D. scrobiculata*	Hubbard County, Minnesota USA	04-19	*Pinus resinosa*	Unknown
268	*D. scrobiculata*	Sauk County, Wisconsin USA	04-114	*Pinus banksiana*	Unknown
269	*D. scrobiculata*	Wood County, Wisconsin USA	95-54	*Pinus resinosa*	Needle
270	*D. scrobiculata*	Mille Lacs County, Minnesota USA	96-159	*Pinus sylvestris*	Stem
271	*D. scrobiculata*	Pine County, Minnesota USA	02-254	*Pinus resinosa*	Unknown
272	*D. scrobiculata*	Douglas County, Wisconsin USA	245	*Pinus resinosa*	Unknown
273	*D. scrobiculata*	Bath County, Virginia USA	07-01	*Pinus strobus*	Branch
274	*D. scrobiculata*	Johnson County, Illinois USA	201	*Pinus strobus*	Unknown
275	*D. scrobiculata*	Hubbard County, Minnesota USA	04-20	*Pinus resinosa*	Unknown
277	*D. scrobiculata*	Sumter County, Alabama USA	07-20	*Pinus taeda*	Stem
280	*D. scrobiculata*	Carmel Mountain Israel	96-38	*Pinus halepensis*	Unknown
281	*D. scrobiculata*	Mille Lacs County, Minnesota USA	94-112	*Pinus sylvestris*	Needle
282	*D. scrobiculata*	Monterey County, California USA	03-33	*Pinus radiata*	Cone

Appendix F. Phylogenetic tree showing the relatedness off all samples using SAS 9.1.3. service pack 3 (2008). Isolates from different sample sets were identified with different colours: blue (P1), orange (P2), red (P3), green (BC) and purple (W). Species were also identified as *Diplodia sapinea* and *Diplodia scrobiculata* (stress).

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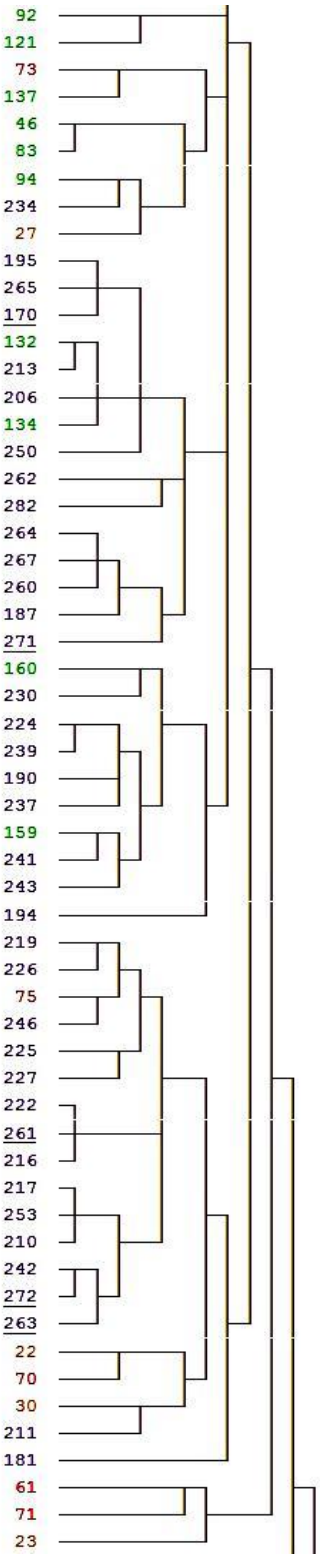
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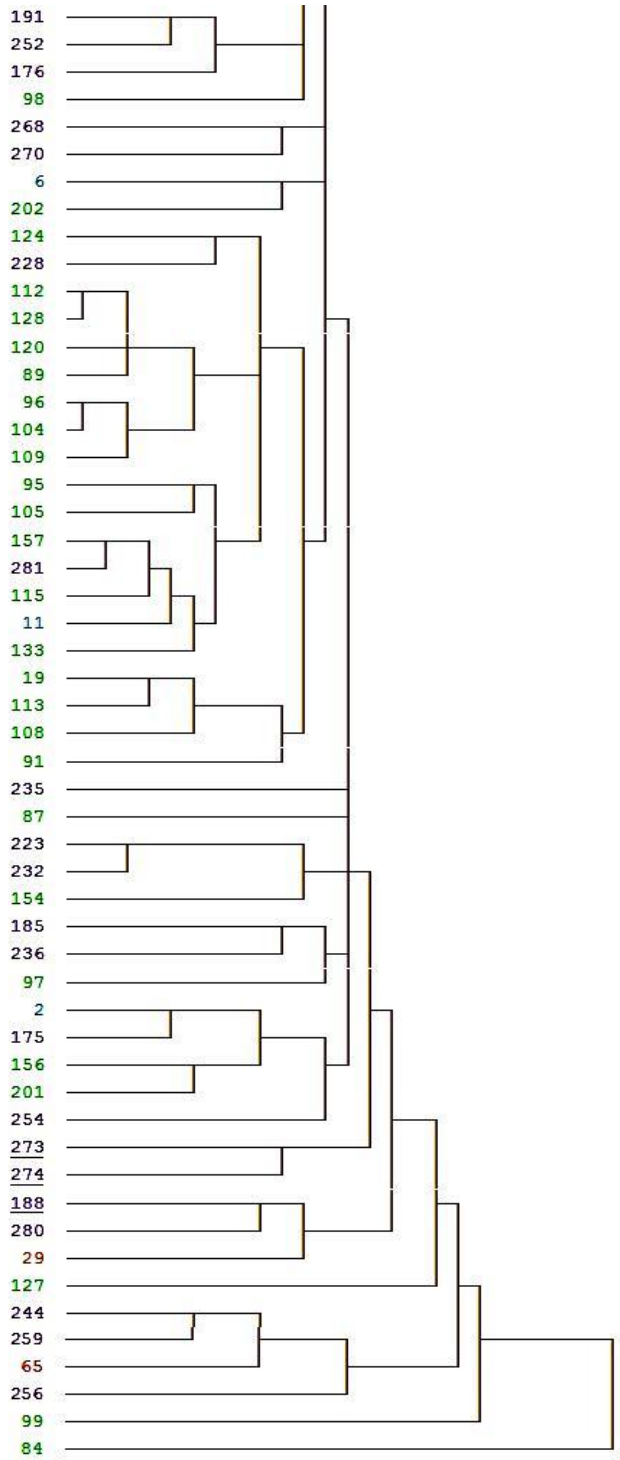
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