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TESIS DOCTORAL

Development of DNA massive sequencing techniques and Real-Time PCR for the detection, identification and quantitation of *Phytophthora* spp. in environmental samples

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Summary

In recent years the increase of global plant trade and human movement has promoted the risk of introduction of invasive plants and exotic pathogens. Biological invasions operate globally and are considered to be the second cause of biodiversity loss after direct habitat alteration and destruction. In this context, *Phytophthora* is one of the most important and aggressive plant pathogen in agriculture and forestry. Early detection and identification of its pathways are of high importance to minimize the threat that they pose to natural ecosystems. Different molecular-based methods, including real-time PCR and Next Generation Sequencing (NGS), have been developed and applied for the detection of plant pathogens in environmental samples. These methods allow fast and accurate pathogen detection and identification even when the inoculum amount is low. Therefore, the main objective of this thesis was the development of a new method for *Phytophthora* detection in environmental samples starting from extraction of environmental DNA (eDNA) from different sources (soil, roots and water) and different ecosystems. Different studies have applied High Throughput Sequencing (HTS) for the detection of *Phytophthora* species in soil samples, but not, to date, for water.

In the Chapter 3, genus-specific primers were adapted to assess *Phytophthora* species diversity in natural ecosystems using high-throughput amplicon pyrosequencing of eDNA from soil and water environments, based in the polymorphic and widely accepted barcoding target Internal Transcribed Spacer 1 (ITS1). The assay was validated with a control reaction with DNA of pure cultures. The objectives raised and developed of this study were: a) as main objective, development and application of HTS of *Phytophthora*-specific PCR amplicons to investigate the presence of *Phytophthora* in

soil samples from different plant communities in natural forests, plantations and aquatic environments in the north of Spain; b) optimization of the conditions for *em*PCR amplification in order to obtain the best results in the pyrosequencing run; c) development of a bioinformatics pipeline for NGS data, focusing in the optimization of a barcoding threshold value to separate Molecular Operational Taxonomic Units (MOTUs).

Different score coverage threshold values were tested for optimal *Phytophthora* species separation in the bioinformatics analyses. Clustering at 99 % was the best criteria to separate most of the *Phytophthora* species. Multiple Molecular Operational Taxonomic Units (MOTUs) corresponding to 36 distinct *Phytophthora* species were amplified in the environmental samples. Pyrosequencing of amplicons from soil samples revealed low *Phytophthora* diversity (13 species) in comparison with the 35 species detected in water samples. Thirteen of the MOTUs detected in rivers and streams did not show significant matches to sequences in international sequence databases, revealing that eDNA pyrosequencing is a useful strategy to assess *Phytophthora* species diversity in natural ecosystems.

Once the technique was developed and validated, another objective was proposed in Chapter 4, focused on the oak decline. The evergreen holm oak (*Quercus ilex*) is the most representative tree species in the Iberian Peninsula and the main tree in oak-rangeland ecosystems (dehesas). Oak decline in non-calcareous soils in south-western Spain has been associated with *Phytophthora cinnamomi* for decades. However, other *Phytophthora* species such as *P. quercina* and *P. psychrophila* have been associated with *Quercus* decline in the eastern part of Spain where calcareous soils are predominant. With the aim of investigating the involvement of *Phytophthora* spp. in oak decline in eastern Spain, two forests in different geographical areas (Alcoi and

Vallivana) were selected as sampling sites. Soil and root samples were analysed in parallel by amplicon pyrosequencing and real-time PCR. Metabarcoding analyses showed *Phytophthora* species diversity, and revealed that an uncultured new *Phytophthora* taxon was the predominant species in both areas. In addition, a real-time PCR assay, based on the pyrosequencing results, was developed for the detection of this uncultured *Phytophthora* taxon, and also for the detection of *P. quercina*. Real-time assays were tested on soil and root samples, and on *Phytophthora* pure cultures. The new assays showed high specificity and were consistent with metabarcoding results. The combination of real-time PCR and amplicon pyrosequencing represented a powerful tool to be applied to the identification, detection and quantification of *Phytophthora* spp. in environmental samples.

Finally, Chapter 5 focused on the sampling strategy for water samples, because as previously seen, rivers and streams represented a hotspot for *Phytophthora* identification. Isolation of *Phytophthora* species from water sources is common and worldwide known. Usually, the most common methods used are baiting in situ and filtering of water. Both methods are successful for the isolation of aquatic species. However, only a few non-aquatic species are isolated using these methods due to the relative low inoculum available. In this study, a total of 15 rivers and streams were sampled, comprising forestry areas in the mountain, and agricultural areas in the valley. In each river and stream two different sampling methods were used: filtering, and baiting with *Rhododendron* leaves and carnation petals. Half of each filter, *Rhododendron* or carnation sample was used for *Phytophthora* isolation, and the other half was subjected to DNA extraction. DNA was extracted from the samples, amplicon libraries were generated using *Phytophthora*-specific primers, and pyrosequenced using GS Junior instrument. Six *Phytophthora* species were identified using isolation

techniques in contrast with the 25 *Phytophthora* species identified using the metabarcoding approach. A total of 8 new *Phytophthora* species, not represented in public databases, were detected on the 15 waterways sampled. The two sampling methods tested resulted highly specific for *Phytophthora* detection, but pyrosequencing of *Rhododendron* leaves resulted the most specific method (99,2 %), followed by the carnation petals (98,2 %) and filters (95,3 %). Several phylotypes which corresponded with important *Phytophthora* pathogens for forestry and agriculture were detected, including *P. cinnamomi*, *P. alni* subsp. *alni*, or *P. plurivora*. Presence of *P. cinnamomi* was significantly higher in agricultural areas than in forestry ones, and filters represented the best method for its detection. Metabarcoding approaches using the combination of baits and filters from water represented a useful approach to describe communities and could be used as a monitoring tool for pathogenic *Phytophthora* species in water ecosystems. The fast and reliable method implemented in this study gives useful insights to understand *Phytophthora* ecology and diversity.

Resumen

En los últimos años el aumento del comercio mundial de plantas y el movimiento humano ha promovido el riesgo de introducción de plantas invasoras y patógenos exóticos. Las invasiones biológicas operan a nivel mundial y se consideran como la segunda causa de pérdida de biodiversidad después de la alteración y destrucción directa del hábitat. En este contexto, *Phytophthora* es uno de los agentes patógenos más importantes y agresivos de la agricultura y la silvicultura. La detección temprana y la identificación de sus vías de propagación son de gran importancia para minimizar la amenaza que representan para los ecosistemas naturales. Se han desarrollado y aplicado diferentes métodos moleculares, incluyendo PCR en tiempo real y la secuenciación masiva de ADN, para la detección de patógenos de plantas en muestras ambientales. Estos métodos permiten una detección e identificación de patógenos rápida y precisa incluso cuando la cantidad de inóculo es baja. Por lo tanto, el objetivo principal de esta tesis fue el desarrollo de un nuevo método para la detección de *Phytophthora* en muestras ambientales a partir de la extracción de ADN ambiental (*eDNA*) de diferentes fuentes (suelo, raíces y agua) y diferentes ecosistemas. Diferentes estudios han aplicado secuenciación de alto rendimiento (*HTS*) para la detección de especies de *Phytophthora* en muestras de suelo, pero no, hasta la fecha, para el agua.

En el Capítulo 3, los cebadores específicos a nivel de género fueron adaptados para evaluar la diversidad de especies de *Phytophthora* en ecosistemas naturales utilizando la pirosecuenciación de amplicones de alto rendimiento a partir de *eDNA* de suelos y aguas, basados en la región polimórfica del espaciador interno transcrito 1 (ITS1). El ensayo se validó con una reacción de control con ADN de cultivos puros. Los objetivos planteados y desarrollados de este estudio fueron: a) como objetivo principal,

desarrollo y aplicación de *HTS* (*High Throughput Sequencing* o secuenciación de alto rendimiento) de amplicones específicos de *Phytophthora* para investigar la presencia de *Phytophthora* en muestras de suelo de diferentes comunidades vegetales en bosques naturales, plantaciones y ambientes acuáticos en el norte de España; b) optimización de las condiciones de la PCR en emulsión (*em*PCR) para obtener el mayor rendimiento en la reacción de pirosecuenciación; c) desarrollo de las herramientas para los análisis bioinformáticos de los datos generados, con especial interés en optimización de un valor umbral para separar las Unidades Taxonómicas Operativas Moleculares (*MOTU*).

Diferentes valores umbral se probaron para la separación óptima de las especies de *Phytophthora* en los análisis bioinformáticos. El agrupamiento al 99% fue el mejor criterio para separar la mayor parte de las especies de *Phytophthora*. Múltiples *MOTU*s correspondientes a 36 especies distintas de *Phytophthora* se amplificaron en las muestras ambientales. La pirosecuenciación de amplicones de muestras de suelo reveló una diversidad baja de *Phytophthora* (13 especies) en comparación con las 35 especies detectadas en muestras de agua. Trece de las *MOTU* detectadas en ríos y arroyos no mostraron coincidencias significativas con secuencias en bases de datos internacionales, lo que revela que la pirosecuenciación de *eDNA* es una estrategia útil para evaluar la diversidad de especies de *Phytophthora* en ecosistemas naturales.

Una vez que la técnica fue desarrollada y validada, otro objetivo fue propuesto en el Capítulo 4, centrado en el declive de la carrasca. La carrasca (*Quercus ilex*) es la especie arbórea más representativa de la Península Ibérica y el árbol principal de los ecosistemas de dehesas. El declive de la carrasca en suelos no calcáreos en el suroeste de España se ha asociado con *Phytophthora cinnamomi* durante décadas. Sin embargo, otras especies de *Phytophthora* como *P. quercina* y *P. psychrophila* se han asociado con el declive de *Quercus* en la parte oriental de España donde predominan los suelos

calcáreos. Con el objetivo de investigar la implicación de *Phytophthora* spp. en el declive de la carrasca en el este de España, se seleccionaron dos bosques en diferentes zonas geográficas (Alcoi y Vallivana) como lugares de muestreo. Las muestras de suelo y raíz se analizaron en paralelo mediante pirosecuenciación de amplicones y por PCR en tiempo real. Los análisis metagenómicos mostraron la diversidad de especies de *Phytophthora*, y reveló que una nueva especie de *Phytophthora* fue la especie predominante en ambas áreas. Además, se desarrolló un ensayo de PCR en tiempo real, basado en los resultados de la pirosecuenciación, para la detección de este taxón de *Phytophthora* no cultivado, y también para la detección de *P. quercina*. Los ensayos de PCR en tiempo real se aplicaron en muestras de suelo y raíz, y en cultivos puros de *Phytophthora*. Los nuevos ensayos mostraron alta especificidad y fueron consistentes con los resultados de la secuenciación masiva. La combinación de la PCR en tiempo real y la pirosecuenciación de amplicones representó una potente herramienta para la identificación, detección y cuantificación de *Phytophthora* spp. en muestras ambientales.

Por último, el capítulo 5 se centró en la estrategia de muestreo de aguas, ya que, como se ha visto anteriormente, los ríos y arroyos representaban un *hotspot* para la identificación de *Phytophthora*. El aislamiento de especies de *Phytophthora* en aguas es común y mundialmente conocido. Por lo general, los métodos más utilizados son el trampeo *in situ* y el filtrado de agua. Ambos métodos son exitosos para el aislamiento de especies acuáticas. Sin embargo, sólo unas pocas especies no acuáticas son aisladas usando estos métodos debido al nivel de inóculo relativamente bajo disponible. En este estudio, se tomaron muestras de un total de 15 ríos y arroyos, agrupando áreas forestales en la montaña, y áreas agrícolas en el valle. En cada río y arroyo se utilizaron dos métodos de muestreo diferentes: filtración y trampeo con hojas de *Rhododendron* y

pétalos de clavel. La mitad de cada filtro, *Rhododendron* o muestra de clavel se utilizó para el aislamiento de *Phytophthora*, y la otra mitad se sometió a extracción de ADN. Se extrajo ADN de las muestras, se generaron bibliotecas de amplicones usando cebadores específicos de *Phytophthora*, y se pirosecuenciaron usando el instrumento GS Junior. Se identificaron seis especies de *Phytophthora* utilizando técnicas de aislamiento en contraste con las 25 especies de *Phytophthora* identificadas mediante el método de *metabarcoding*. Un total de 8 nuevas especies de *Phytophthora*, no representadas en bases de datos públicas, fueron detectadas en los 15 ríos y arroyos muestreados. Los dos métodos de muestreo resultaron altamente específicos para la detección de *Phytophthora*, pero la pirosecuenciación de las hojas de *Rhododendron* resultó ser el método más específico (99,2%), seguido de los pétalos de clavel (98,2%) y filtros (95,3%). Se detectaron varios filotipos que correspondieron a patógenos importantes de *Phytophthora* para la silvicultura y la agricultura, incluyendo *P. cinnamomi*, *P. alni* subsp. *alni*, o *P. plurivora*. La presencia de *P. cinnamomi* fue significativamente superior en las áreas agrícolas que en las forestales, y los filtros representaron el mejor método para su detección. La aplicación de la secuenciación masiva en muestras de agua utilizando la combinación de trampas vegetales y filtros representó un método útil para describir las comunidades y podría ser utilizado como una herramienta de monitorización de especies patógenas de *Phytophthora*. El método desarrollado en este estudio proporciona una visión útil para comprender la ecología y la diversidad de *Phytophthora* en ecosistemas acuáticos.

Resum

En els últims anys, l'augment del comerç mundial de plantes i el moviment humà ha promogut el risc d'introducció de plantes invasores i patògens exòtics. Les invasions biològiques operen a nivell mundial i es consideren com a la segona causa de la pèrdua de biodiversitat després de l'alteració i destrucció directa de l'hàbitat. En aquest context, *Phytophthora* és un dels agents patògens més importants i agressius de l'agricultura i la silvicultura. La detecció temprana i la identificació de les seves vies de propagació són de gran importància per minimitzar l'amenaça que representen per als ecosistemes naturals. S'han desenvolupat i aplicat diferents mètodes moleculars, que inclouen PCR en temps real i la seqüenciació massiva d'ADN, per a la detecció de patògens de plantes en mostres ambientals. Aquests mètodes permeten una detecció i identificació de patògens ràpida i precisa fins i tot quan la quantitat d'inòcul és baixa. Per tant, l'objectiu principal d'aquesta tesi va ser el desenvolupament d'un nou mètode per a la detecció de *Phytophthora* en mostres ambientals a partir de l'extracció d'ADN ambiental (*eDNA*) de diferents orígens (sòl, arrels i aigua) i diferents ecosistemes. Diferents estudis han aplicat la seqüenciació d'elevat rendiment (*HTS*) per a la detecció d'espècies de *Phytophthora* en mostres de sòl, però no, fins a la data, per a l'aigua.

Al Capítol 3, els encebadors específics a nivell de gènere van ser adaptats per avaluar la diversitat d'espècies de *Phytophthora* en ecosistemes naturals utilitzant la piroseqüenciació d'amplicons d'elevat rendiment a partir d'*eDNA* de sòls i aigües, basats en la regió polimòrfica de l'Espaiador Intern Transcrit 1 (ITS1). L'assaig es va validar amb una reacció de control amb ADN de cultius purs. Els objectius plantejats i desenvolupats d'aquest estudi van ser: a) com a objectiu principal, desenvolupament i aplicació de seqüenciació d'elevat rendiment d'amplicons específics de *Phytophthora*

per investigar la presència de *Phytophthora* en mostres de sòl de diferents comunitats vegetals en boscos naturals, plantacions i ambients aquàtics al nord d'Espanya; b) optimització de les condicions de la PCR en emulsió (*em*PCR) per obtenir el major rendiment en la reacció de piroseqüenciació; c) desenvolupament de les eines per els anàlisis bioinformàtics de les dades generades, amb especial interés en l'optimització d'un valor umbral per separar les Unitats Taxonòmiques Operatives Moleculars (*MOTU*).

Diferents valors umbral es van provar per a la separació òptima de les espècies de *Phytophthora* en els assaigs bioinformàtics. L'agrupament al 99% va ser el millor criteri per separar la major part de les espècies de *Phytophthora*. Múltiples *MOTU*s corresponents a 36 espècies diferents de *Phytophthora* es van amplificar en les mostres ambientals. La piroseqüenciació d'amplicons de mostres de sòl va revelar una baixa diversitat de *Phytophthora* (13 espècies) en comparació amb les 35 espècies detectades en mostres d'aigua. Tretze de les MOTU detectades en rius i rierols no van mostrar coincidències significatives amb seqüències en bases de dades internacionals, el que revela que la piroseqüenciació d'*eDNA* és una estratègia útil per avaluar la diversitat de les espècies de *Phytophthora* en ecosistemes naturals.

Una vegada que la tècnica va ser desenvolupada i validada, altre objectiu va ser plantejat al Capítol 4, centrat en el declivi de la carrasca. La carrasca (*Quercus ilex*) és l'espècie arbòria més representativa de la Península Ibèrica i l'arbre principal dels ecosistemes de deheses. El declivi de la carrasca en sòls no calcaris al sud-est d'Espanya s'ha associat amb *Phytophthora cinnamomi* durant dècades. No obstant això, altres espècies de *Phytophthora* com *P. quercina* i *P. psychrophila* s'han associat amb el declivi de *Quercus* a la part oriental d'Espanya, on predominen els sòls calcaris. Amb l'objectiu d'investigar la implicació de *Phytophthora* spp. en el declivi de la carrasca a

l'est d'Espanya, es van seleccionar dos boscos en diferents zones geogràfiques (Alcoi i Vallivana) com a llocs de mostreig. Les mostres de sòl i arrels es van analitzar en paral·lel mitjançant la piroseqüenciació d'amplicons i per PCR en temps real. Els estudis metagenòmics van mostrar la diversitat d'espècies de *Phytophthora*, i va revelar que una nova espècie de *Phytophthora* va ser l'espècie predominant en ambdues àrees. A més, es va desenvolupar un assaig de PCR en temps real, basat en els resultats de la piroseqüenciació, per a la detecció d'aquest taxó de *Phytophthora* no cultivat, i també per a la detecció de *P. quercina*. Els assaigs de PCR en temps real es van aplicar en mostres de sòl i arrel, i en cultius purs de *Phytophthora*. Els nous assaigs van mostrar alta especificitat i van ser consistents amb els resultats de la seqüenciació massiva. La combinació de la PCR en temps real i la piroseqüenciació d'amplicons va representar una potent eina per a la identificació, detecció i quantificació de *Phytophthora* spp. en mostres ambientals.

Per últim, el Capítol 5 es va centrar en l'estratègia de mostreig d'aigües, ja que, com s'ha vist anteriorment, els rius i rierols representaven un *hotspot* per a la identificació de *Phytophthora*. L'aïllament d'espècies de *Phytophthora* en aigües és comú i mundialment conegut. Pel general, els mètodes més utilitzats són el trampeig *in situ* i el filtrat d'aigua. Ambdós mètodes són exitosos per a l'aïllament d'espècies aquàtiques. No obstant això, només unes poques espècies no aquàtiques són aïllades utilitzant aquests mètodes degut al nivell d'inòcul relativament baix disponible. En aquest estudi, es van prendre mostres d'un total de 15 rius i rierols, agrupant àrees forestals a la muntanya, i àrees agrícoles a la vall. En cada riu i rierol es van utilitzar dos mètodes de mostreig diferents: filtració i trampeig amb fulles de *Rhododendron* i pètals de clavell. La meitat de cada filtre, *Rhododendron* o mostra de clavell es va utilitzar per a l'aïllament de *Phytophthora*, i l'altra mitja es va sotmetre a extracció d'ADN. Es va extreure l'ADN de les mostres, es

van generar biblioteques d'amplicons utilitzant encebadors específics de *Phytophthora*, i es van piroseqüenciar utilitzant l'instrument GS Junior. Es van identificar sis espècies de *Phytophthora* utilitzant tècniques d'aïllament en contrast amb les 25 espècies de *Phytophthora* identificades mitjançant el mètode de *metabarcoding*. Un total de 8 noves espècies de *Phytophthora*, no representades en bases de dades públiques, van ser detectades en els 15 rius i arroyos mostrejats. Els dos mètodes de mostreig van resultar molt específics per a la detecció de *Phytophthora*, però la piroseqüenciació de les fulles de *Rhododendron* va resultar ser el mètode més específic (99,2%), seguit dels pètals de clavell (98,2%) i els filtres (95,3%). Es van detectar diversos filotips que corresponien a importants patògens de *Phytophthora* per a la silvicultura i l'agricultura, incloent *P. cinnamomi, P. alni* subsp. *alni,* o *P. plurivora*. La presència de *P. cinnamomi* va ser significativament superior a les àrees agrícoles que a les forestals, i els filtres van representar el millor mètode per a la seva detecció. L'aplicació de la seqüenciació massiva en mostres d'aigua mitjançant la combinació de trampes vegetals i filtres va representar un mètode útil per a descriure les comunitats i podria ser utilitzat com una eina de monitorització de les espècies patògenes de *Phytophthora*. El mètode desenvolupat en aquest estudi proporciona una visió útil per comprendre l'ecologia i la diversitat de *Phytophthora* en ecosistemes aquàtics.

Content Index

Figure Index

Table Index

Chapter 1

General Introduction

1.1. Oomycetes and the genus *Phytophthora*

Oomycetes represent a large group of terrestrial and aquatic eukaryotic organisms which group many plant and animal pathogens that produce filamentous structures (de Wit, 2015) and also include saprophytic taxa. Oomycetes, also known as water molds and downy mildews, which have colonized almost all ecosystems, cause a high range of diseases in fish and other aquatic vertebrates (Saprolegniales) and destructive diseases of plants important to agriculture, forestry, ornamental and natural ecosystems (Rossman & Palm, 2006; Jiang and Tyler, 2012). Traditionally classified in the phycomycetes or "lower fungi" (Rossman & Palm, 2006), there are several features that distinguish Oomycetes from true fungi which supported their excision into the kingdom Straminipila (Dick, 1995) (Figure 1.1), such as sexual reproduction by oospores and asexual reproduction by aquatic biflagellate zoospores, which are released to the media by sporangia (Erwin & Ribeiro, 1996). Additionally, the oomycetes cell wall contains β-glucans but not chitin as true fungi, the mycelia is coenocytic, their vegetative state is diploid, and mitochondria are characterized by tubular cristae (Erwin & Ribeiro, 1996).

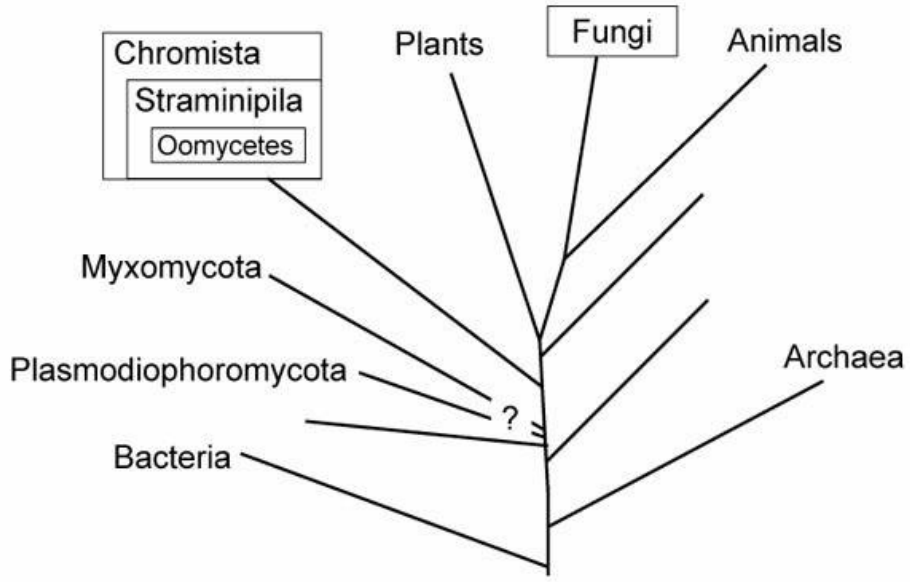

Figure 1.1. Phylogenetic tree showing the relations between oomycetes, fungi and other kingdoms. Extracted from Rossman & Palm (2006).

The plant pathogenic oomycetes group has many taxa and exhibit remarkably diverse lifestyles ranging from obligate biotroph to necrotroph (Agrios, 2011), including some genera such as *Phytophthora*, *Pythium*, *Peronospora*, *Albugo* or *Aphanomyces*.

The genus *Phytophthora* (Stramenopiles: Oomycetes: Peronosporales) was placed in the order Pythiales, which include important plant pathogenic genera as *Pythium*, but recently, molecular analyses revealed that *Phytophthora* was more closely related to Peronosporales (downy mildews and white rusts) than to Pythiales (Beakes & Sekimoto, 2009; Thines *et al*., 2009).

The genus *Phytophthora* (derived from Greek: 'plant destroyer') was initially described by Heinrich Anton de Bary (1876) with the late potato blight pathogen *Phytophthora infestans*, that was responsible for the famine in Ireland in 1845-1849. Actually there are over 150 species described with some species causing significant economic losses in agriculture, horticulture and forestry. Some species are soil-borne and others are aerial dispersed; they can affect all plant parts; some are host specific and others can affect a wide host range. For example, *P. sojae* affects soybean and has caused losses of over $2 billion in USA (Tyler, 2007); *P. cinnamomi* has altered native plant communities in Australia where it can affect over 2000 plant species (Hardman, 2005); *P. ramorum* responsible for the mortality of native oaks and tanoak in northern California and southern Oregon (Rizzo *et al*., 2002), is also responsible for the mortality of Japanese larch in the UK (Webber *et al*., 2010) and can affected a high number of ornamentals (over 130 plant species) in nurseries in Europe, the USA and Canada (Goss *et al*., 2011).

Reproduction in *Phytophthora*, as mentioned before, can be asexual or sexual. In asexual reproduction, *Phytophthora* produce asexual zoospores by the formation of a structure called sporangium, which are released to the aquatic medium at low

temperatures. Zoospores have anterior and posterior flagella, both typically attached in a ventral groove, which make them able to swim. Sexual reproduction in Oomycetes occurs between two dissimilar gametangia, a large oogonium containing one to several eggs and a smaller antheridium that fertilized the oogonium. Species can be distinguished into homothallic and heterothallic forms, where fertilization occurs in a single strain or between two strains of opposite mating types, respectively.

Traditionally the identification of *Phytophthora* was based on morphological and physiological characters such as the morphology of sporangia, oogonia and antheridia, the presence or absence of chlamydospores, as well as optimal growth temperature, colony morphology and host range. Six morphological groups were established by Waterhouse (1963) and later revised and updated by Stamps *et al.* 1990. However, as the number of species has been increasing it has been more difficult to characterize the new species due to intra-specific variation and the overlapping of some of the characters. Only skilled mycologist could distinguish the identification features, it takes a long time for the identification and in some cases the discrimination amongst species is not always conclusive (Jung & Burgess, 2009). For that reason, molecular techniques represent the faster and accurated method for *Phytophthora* identification.

Based on their ITS rRNA sequences, Cooke *et al.* (2000) stablished the first comprehensive phylogenetic classification of the genus *Phytophthora*. This molecular phylogeny, showed the relationships between 50 *Phytophthora* species, and introduced the classification into 10 clades based in the tree. Phylogenetic analysis of *Phytophthora* grew up in the last years with the addition of more taxa and the analysis of additional DNA regions (Kroon *et al*., 2004; Blair *et al*., 2008; Martin *et al*., 2014).

1.1.1. Water sources as hotspot for *Phytophthora*.

The increase of amount of *Phytophthora* inoculum is caused by the rapid production of sporangia and zoospores from infected plant tissues when the conditions are favorable and free water is available (Erwin & Ribeiro, 1996). In this context, rivers and streams play an important role in the *Phytophthora* spread and represent a hotspot for new species identification (Català *et al*., 2015; Eyre & Garbelotto, 2015; Hüberli *et al*., 2013; Oh *et al*., 2013). Free water allows a rapid dispersal of *Phytophthora* with their flagellate zoospores (Erwin & Ribeiro, 1996). However, compared with species from forests and agricultural systems, little is known about the ecology and diversity of *Phytophthora* spp. present in freshwater ecosystems (Guha Roy & Grunwald, 2014). Understanding *Phytophthora* species diversity in aquatic ecosystems represents a key parameter to analyze and prevent invasions of alien species under an ecological perspective.

Several studies were focused on the isolation of *Phytophthora* species from rivers and streams (Hüberli *et al*., 2013; Oh *et al*., 2013; Loyd *et al*., 2014; Hong *et al*., 2012). As recently reviewed, more than 25 species have been isolated from natural waterways and irrigation reservoirs (Guha Roy & Grunwald, 2014). A high proportion of the species isolated from aquatic ecosystems belong to clade 6 (according to Cooke's designation), which show a strong association with both forests and riparian ecosystems (Jung *et al*., 2011). Presence and, sometimes, dominance of clade 6 species in water surveys supported the hypothesis that they could live as saprotrophic organisms (Brasier *et al*., 2003), although some species of clade 6 can be opportunistic tree pathogens (Pérez-Sierra *et al*., 2013). Moreover, some important plant pathogens such as *P. ramorum* (Werres *et al*., 2001; Sutton *et al*., 2009; Eyre and Garbelotto, 2015),

P. cinnamomi (Kliejunas *et al*., 1976; Oh *et al*., 2013) or *P. multivora* (Hüberli *et al*., 2013) have been isolated from rivers and streams.

Water from rivers and streams is used in some regions to irrigate crops and could represent an important inoculum source and cause of plant diseases. *Phytophthora* detection in water sources is a valuable information to minimize the threat that poses to the crops and to increase the productivity.

1.2. Species identification via DNA analysis: from DNA-barcoding to metabarcoding.

DNA barcoding, the use of a standardized DNA sequence region to identify species, is a popular solution for taxonomic identification of individual specimens (Valentini *et al*., 2009). During the last years, the species identification via DNA barcoding became a fundamental part on biodiversity research, providing new insights into microbial ecology and species distribution (Anderson & Cairney, 2004; Chase & Fay, 2009; Seiffert, 2009). Different DNA regions have been stablished and accepted as DNA barcoding target in different organisms. For example, the most useful regions for species identification in kingdom Plantae are *mat*K, *rbc*L, and *trn*H-*psb*A regions (Group *et al*., 2009; Hollingsworth *et al*., 2011), 16S gene in the case of bacteria and archaea (Caporaso *et al*., 2011), COX1 for animals (Ward *et al*., 2005; Savolainen *et al*., 2005) and ITS for fungi and Oomycetes (Schoch *et al*., 2012; Robideau *et al*., 2011) (Table 1.1).

Table 1.1. Sequence distribution of different taxonomic groups. Search was performed against NCBI Database, accessed on 25th January 2017.

	Number of ITS sequences
Fungi	931017
Ascomycetes	271854
Basidiomycetes	130573
Plants	293988
Animals	118015
Oomycetes	25060
Phytophthora	13900

Internal transcribed spacers from the nuclear ribosomal operon 18S-5.8S-28S rRNA gene (nrITS) was stablished as the most suitable barcode for fungal species identification (Schoch *et al*., 2012) and Oomycetes (Robideau *et al*., 2011), and to carry out phylogenetic reconstructions of several taxonomic groups (Figure 1.2).

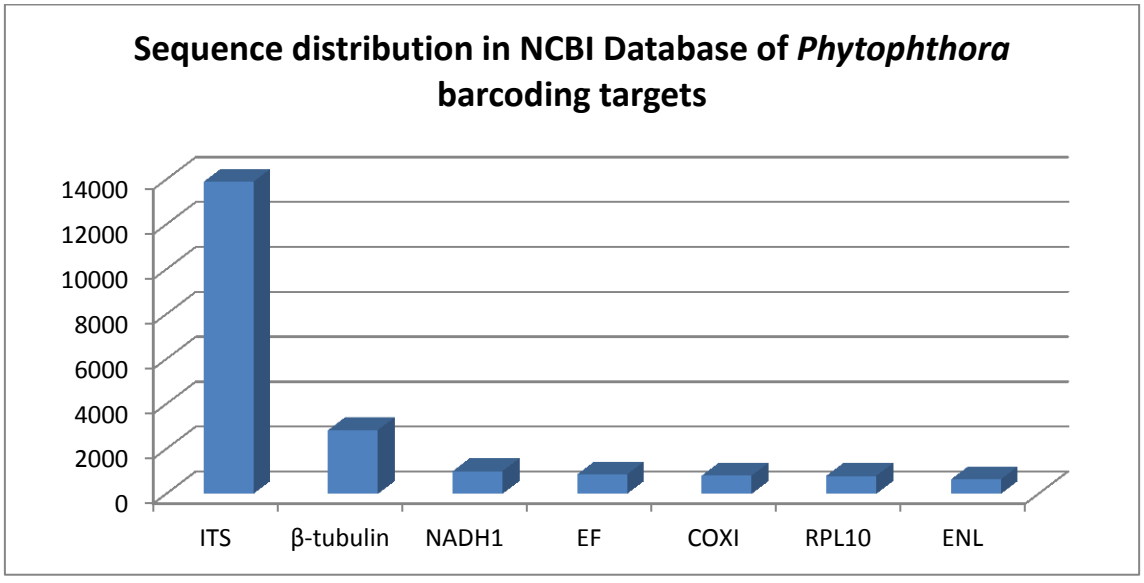

Figure 1.2. Sequence distribution of the most common DNA regions used for *Phytophthora* identification. Search was performed against NCBI Database, accessed on 25th January 2017.

The ITS regions provide attractive targets because is divided into two polymorphic regions, ITS1 and ITS2, separated by the conserved ribosomal gene 5.8S (Figure 1.3).

Figure 1.3. Representation of ribosomal DNA (rDNA) polycistron from eukaryotic organisms, including the ITS1 and ITS2 regions, separated by the 5.8S gene and flanked by the 18S (small subunit, SSU) and 28S (large subunit, LSU) genes. Extracted and modified from Begerow *et al.* (2010).

Moreover, the selection of a barcode is not only limited to the genetic variability that it offers. In fact, one of the prior considerations that should be assumed previously to the data generation is the selection of a correct barcode region (Figure 1.4), especially in taxonomic groups poorly characterized. As shown in figure 1.1 and 1.4, a first approach on the identification of a *Phytophthora* culture should be based on the amplification and sequencing of ITS region. However, this is not the optimal locus for all species, particularly those that are phylogenetically closely related (for example, some clade 1c species have identical ITS sequences and cannot be differentiated with this locus) (Martin *et al*., 2012).

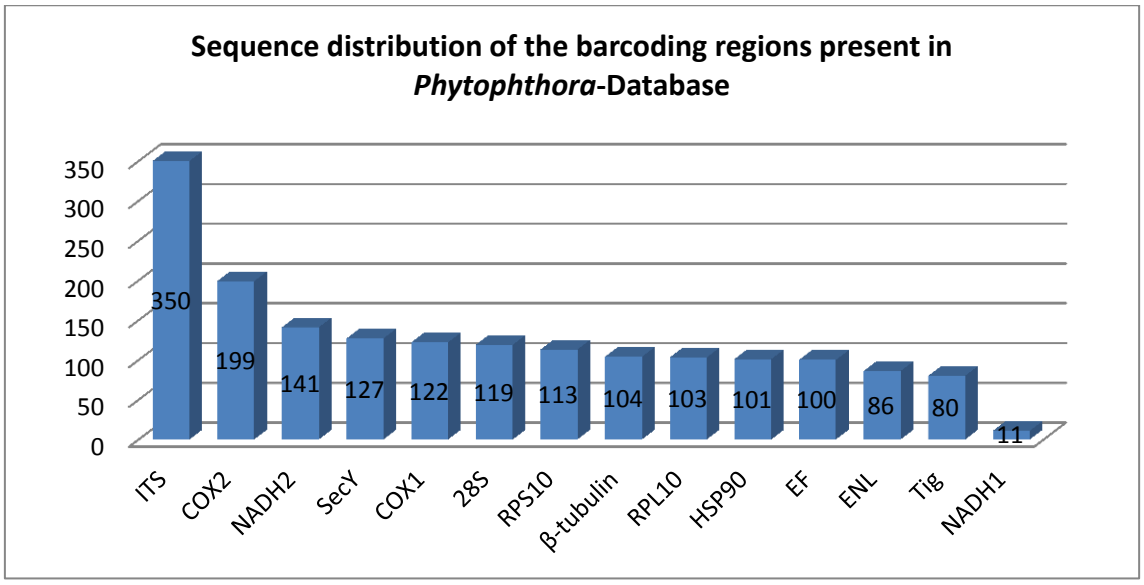

Figure 1.4. Sequence distribution of the DNA regions used for *Phytophthora* identification. Search was performed against *Phytophthora*-Database (Park *et al*., 2008), accessed on 26th January 2017.

The strategic position of 5.8S gene provides the possibility of design universal primers for Fungi or Oomycetes and to amplify separately the ITS1 or ITS2 regions. This strategy converts the ITS region as a prime target for High-Throughput DNA sequencing (HTS) of environmental samples (Nilsson *et al*., 2009), due to its length suitable for massive sequencing platforms such as Junior and Titanium Genome Sequencers or Illumina MiSeq. The approach consisting of identifying multiple species, in a single experiment, using complex or degraded environmental samples can be termed as DNA metabarcoding (Taberlet *et al*., 2012). DNA Metabarcoding approaches implementing HTS technologies using environmental DNA (eDNA) as input revealed to be as the best strategy to assess communities' species profiles. Environmental DNA can be defined as a complex mixture of genomic DNA from living cells and extracellular DNA from natural cell death from many different organisms (Taberlet *et al*., 2012), and represents the source of DNA most common in metabarcoding studies from a wide range of ecosystems (Català *et al*., 2015; Català *et al*., 2017).

Metabarcoding approaches represented the revolution of diversity studies. Recent studies have applied HTS techniques to identify *Phytophthora* communities in different ecosystems (Coince *et al*., 2013; Vannini *et al*., 2013; Prigigallo *et al*., 2016), using eDNA as the DNA source. Analysis of eDNA allows the rapid characterization of microbial communities without isolating the target organisms.

1.3. Molecular methods for *Phytophthora* detection in environmental samples

Ecological understanding of the role of *Phytophthora* species diversity and their interactions in natural ecosystems is limited by the difficulty of accurately and efficiently determining microbiome composition. In the recent years, methods for the

detection of *Phytophthora* and other plant pathogens experienced a great change. Traditionally, different methods have been used for the detection of plant pathogens using baiting techniques or isolation on selective media (Hüberli *et al*., 2013; Jung & Nechwatal, 2008; Loyd *et al*., 2014). Isolation of *Phytophthora* from environmental samples, such as plant material (roots, stems, leaves, fruits), soil or water is a time-consuming technique, and frequently produces false negatives due to the relative low presence of inoculum. Moreover, other factors are involved in the low success rate in the pathogen isolation as the selection of the bait, the presence of inhibitors in plants or the preservation state of the material. Furthermore, the different growing rate and relative proportion of several *Phytophthora* species coexisting in the same sample difficult the isolation of some species or emerging pathogens (Hüberli *et al*., 2013). In addition, identification of *Phytophthora* and other Oomycetes based on morphological characterization requires specific expertise.

Increasing economic and environmental losses caused by invasive *Phytophthora* species in natural ecosystems justifies the implementation of an efficient and rapid technique for their detection and accurate identification. In this context, molecular methods offer the possibility to detect a single target molecule in a complex mixture without the need of isolation and the posterior morphological characterization. These methods include techniques like conventional PCR, quantitative PCR, digital PCR, cloning or massive sequencing, which allow fast and accurate pathogen detection and identification even when the inoculum amount is low. From these techniques, the most used and more informative are the Real-Time PCR and those methods based on HTS.

Real-time PCR assays have been widely used for the detection and quantification of fungal and bacterial plant pathogens (Kang *et al*., 2012; Agustí-Brisach *et al*., 2014; Liu *et al*., 2015). Quantification of *Phytophthora* spp. in soil and

root samples by fluorescent probe-based quantitative real-time PCR can help gain a better understanding of potential threats to forest health. Quantitative PCR offers numerous advantages over conventional PCR techniques such as higher sensitivity and specificity, faster rate of detection, no post-PCR manipulation, minimizing the risk of contamination, and the possibility to provide quantitative results (Català *et al*., 2017). Real-time PCR represents a suitable method to assess the presence of specific plant pathogens in environmental samples. Several authors have focused on the detection of known *Phytophthora* spp. using real-time PCR: *P. infestans* (Avrova *et al*., 2003), *P. medicaginis* (Vandemark & Barker, 2003), *P. nicotianae* and *P. citrophthora* (Ippolito *et al*., 2004), *P. fragariae* (Bonants *et al*., 2004), *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* (Schena *et al*., 2006), *P. capsici* (Silvar *et al*., 2005) or *P. pseudosyringae* (Tooley *et al*., 2006). In addition, the generation of environmental data from metabarcoding studies allowed the design of specific assays for the detection of uncultured *Phytophthora* spp. never isolated (Català *et al*., 2017).

In some situations when using real-time PCR assays, healthy material or unknown environmental samples give late exponential curves, or a non-exponential increase of the fluorescence is produced. The establishment of Ct cut-off values is always a critical step in order to distinguish positive or negative samples (Català *et al*., 2017). Moreover, real-time PCR assays depend on specific oligonucleotide primers and probes, which usually do not cover all *Phytophthora* species, and thus do not provide an accurate indication of the diversity and abundance of *Phytophthora* spp. in the microbiome. In this context, metabarcoding implementing HTS technologies provides a more accurate approach to characterization of samples that would otherwise be considered negative for the presence of *Phytophthora* spp.

1.4. From Sanger to Next Generation Sequencing technologies

In 1975, Frederick Sanger developed the DNA sequencing technology based on the incorporation chain-terminating dideoxynucleotides by DNA polymerase (Sanger and Coulson, 1975). Due to the high efficiency and low radioactivity, the called as Sanger sequencing was adopted as the main sequencing technology. A few years later, in 1987, Applied Biosystems introduced the first automated DNA sequencer (ABi370), which incorporated the capillary electrophoresis, providing a faster and accurate sequencing. The ABi370 sequencer was able to detect 96 bases simultaneously and to generate a throughput of 500 Kb per day with reads up to 600 bases. The AB370 evolved to the AB3730xl sequencer which reached a read length of 900 bp and a throughput of 2,88 Mb per day. The development of automatic sequencing machines and related software in 1998 conducted Sanger sequencing to become the main technology to develop important projects like human genome (Collins *et al.*, 1998). Sanger sequencing technology was the only technology available for a quite long period of time. However, it allowed the sequencing of only one molecule at a time (either by direct sequencing of a PCR product or after cloning). This limitation was one of the challenges that allowed the birth of NGS technologies, allowing a massively parallel sequencing of DNA. In addition, the Sanger technique permitted the completion of the first human genome sequence in 2004 (International Human Genome Sequencing Consortium, 2004), which required vast amounts of time, resources and funds.

In the same year (2004) the National Human Genome Research Institute (NHGRI) initiated a funding program with the goal of reducing the cost of human genome sequencing to US$1000 in ten years (van Dijk *et al*., 2014). This stimulated the birth of next-generation sequencing (NGS) technologies, with the main advantage over

Sanger sequencing of generating thousands-to-many-millions of sequencing reactions in parallel, without the need for electrophoresis.

The first NGS technology (Figure 1.5) was released in 2005 by 454 Life Sciences (acquired later by Roche) and it was based on the pyrosequencing technology developed by Ronaghi (2001). In brief, in 454 pyrosequencing, the incorporation of a nucleotide by DNA polymerase results in the release of an inorganic pyrophosphate molecule (PPi). This release initiates a series of downstream reactions to produce light by the action of the enzyme luciferase. The amount of generated light is directly proportional to the number of nucleotides incorporated (Margulies *et al*., 2005).

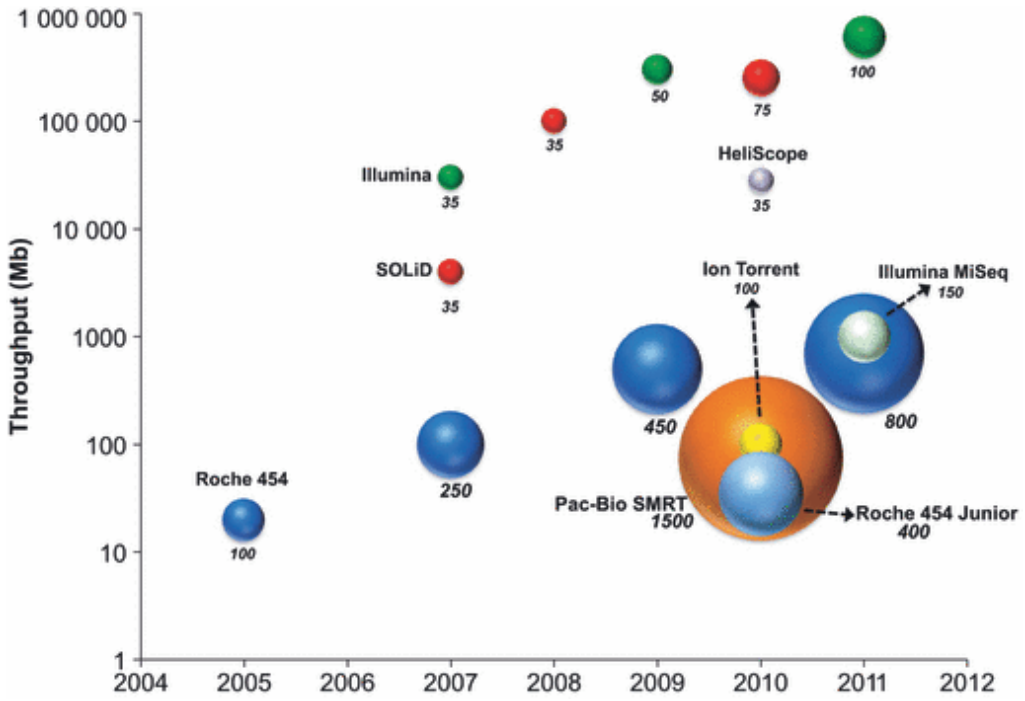

Figure 1.5. Historical development of next-generation sequencing technologies. The diameter of each bubble represents the sequencing read length of the platform [in base pairs (bp)]. Colors correspond to individual platforms. Extracted from Shokralla *et al*. (2012).

Although other sequencing technologies were released in the following years (such as Illumina, Solid or Ion Torrent, Figure 1.6), due to the length of the sequence produced by pyrosequencing technology, it became the most popular in ecological studies based on *omycs* sciences (e.g. metabarcoding, metagenomics and metatranscriptomics). NGS technologies represented the revolution of microbial ecology, opening new horizons in microbial community analysis.

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Chapter 2

Objectives

Phytophthora is one of the most important and aggressive plant pathogens in agriculture and forestry. Early detection and identification of its pathways are of high importance to minimize the threat that they pose to natural ecosystems. In this context, molecular methods allow fast and accurate pathogen detection and identification even when the inoculum amount is low. Therefore, this thesis focus on the development of a new improved method for *Phytophthora* detection in environmental samples (soil, roots and water) from different ecosystems, with the following objectives:

1. **Design and application of a *Phytophthora*-specific amplicon pyrosequening assay based on the barcoding target ITS1 to investigate the presence of *Phytophthora* in different plant communities in natural forests, plantations and aquatic environments**
2. **Assessment of *Phytophthora* spp. diversity on holm oak forests using high-throughput amplicon pyrosequencing.**
3. **Development of a real-time PCR assay based on the pyrosequencing results for the detection of uncultured or previously recorded *Phytophthora* spp. in environmental samples.**
4. **Exploration of *Phytophthora* diversity in aquatic ecosystems by high-throughput sequencing.**
5. **Comparison of two sampling methods for aquatic ecosystems: filtering and baiting with *Rhododendron* leaves and with carnation petals.**

Chapter 3

The use of genus-specific amplicon pyrosequencing to assess *Phytophthora* species diversity using eDNA from soil and water in northern Spain

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Keywords: *Phytophthora*, metabarcoding, environmental DNA, diversity.

Abstract

Phytophthora is one of the most important and aggressive plant pathogenic genus in agriculture and forestry. Early detection and identification of its pathways of infection and spread are of high importance to minimize the threat they pose to natural ecosystems. Environmental DNA (eDNA) was extracted from soil and water from forests and plantations in the north of Spain. *Phytophthora*-specific primers were adapted for use in high-throughput sequencing (HTS). Primers were tested in a control reaction containing eight *Phytophthora* species and applied to water and soil eDNA samples from northern Spain. Different score coverage threshold values were tested for optimal *Phytophthora* species separation in a custom-curated database and in the control reaction. Clustering at 99% was the optimal criteria to separate most of the *Phytophthora* species. Multiple Molecular Operational Taxonomic Units (MOTUs) corresponding to 36 distinct *Phytophthora* species were amplified in the environmental samples. Pyrosequencing of amplicons from soil samples revealed low *Phytophthora* diversity (13 species) in comparison with the 35 species detected in water samples. Thirteen of the MOTUs detected in rivers and streams showed no close match to sequences in international sequence databases, revealing that eDNA pyrosequencing is a useful strategy to assess *Phytophthora* species diversity in natural ecosystems.

Introduction

In recent years the increase of global plant trade and human movement have promoted the risk of introduction of invasive plants and exotic pathogens (Reichard & White, 2001; Jones & Baker, 2007; Brasier, 2008). Biological invasions operate globally and are considered to be the second cause of biodiversity loss after direct habitat alteration and destruction. In this context, *Phytophthora* species are of particular

importance worldwide as they are major pathogens in agriculture, horticulture and forestry causing important economic and ecological losses.

Environmental DNA (eDNA) from samples such as soil, water, air or permafrost is a complex mixture of genomic DNA from living cells and extracellular DNA from natural cell death from many different organisms (Taberlet *et al*., 2012). In recent years, the examination of the eDNA in ecological studies has been used to characterize microbial and fungal communities using high-throughput sequencing (HTS) technologies (Roesch *et al*., 2007; Acosta-Martínez *et al*., 2008; Jumpponen & Jones, 2009; Nilsson *et al*., 2009; Sogin *et al*., 2012; Coince *et al*., 2013) and to detect alien species in soil samples (Vannini *et al*., 2013). eDNA has also been used successfully to detect low density populations in freshwater environments (Jerde *et al*., 2011), lakes (Monchy *et al*., 2011; Jobard *et al*., 2012) and aquifers (Livermore & Mattes, 2013). Most studies have focused on the detection of all organisms present in environmental samples using rDNA genes (Creer & Sinniger, 2013; Davey *et al*., 2013; Nakayama *et al*., 2013; Weber *et al*., 2013) but only a few have targeted only one organism/genera (Bergmark *et al*., 2012; Li *et al*., 2013).

Traditionally, different methods have been used for the detection of *Phytophthora* species based on isolation on selective media or using different baiting techniques. However, these techniques are time-consuming, and sometimes produce false negatives due to the low inoculum available. Furthermore, identification based on morphological characteristics requires specific taxonomical expertise and a considerable effort. In contrast, different molecular-based methods have been developed and applied for the detection of *Phytophthora* spp. in environmental samples. These methods allow fast and accurate pathogen detection and identification even when the inoculum amount is low, and quantitation of specific *Phytophthora* pathogens is also possible using Real-

Time PCR assays (Schena *et al*., 2006; Tooley *et al*., 2006; Pavon *et al*., 2008; Than *et al*., 2013). A membrane-based oligonucleotide array was developed for the detection of 98 described and 15 undescribed species of *Phytophthora* (Chen *et al*., 2013). Cloning was also applied to assess the *Phytophthora* communities present in soil and water samples (Scibetta *et al*., 2012), but the low throughput of the technique lowers the probability of detecting rare or newly introduced species with low inoculum levels. Different studies have applied HTS for the detection of *Phytophthora* species in soil samples (Català *et al*., 2012a; Català *et al*., 2012b; Coince *et al*., 2013; Vannini *et al*., 2013), but not, to date, for water.

Increasing economic and environmental losses caused by invasive *Phytophthora* species in natural ecosystems justifies the implementation of an efficient and rapid technique for their detection and accurate identification. In this study, genus-specific primers were adapted to assess *Phytophthora* species diversity in natural ecosystems using high-throughput amplicon pyrosequencing of eDNA from soil and water environments, based in the polymorphic and widely accepted barcoding target Internal Transcribed Spacer 1 (ITS1), and validated with a control reaction with DNA of pure cultures. The objective of this study was to apply HTS to investigate the presence of *Phytophthora* in different plant communities in natural forests, plantations and aquatic environments in the north of Spain.

Material and Methods

Culture samples

Pure cultures of *Phytophthora gonapodyides* (PS-1512), *P.* taxon PgChlamydo (PS-1510), *P. lacustris* (PS-1513), *P. cryptogea* (PS-1584), *P. citrophthora* (PS-1544),

P. plurivora (PS-1514), *P. cambivora* (PS-1556) and *P. cinnamomi* (PS-1520) were obtained from the fungal culture collection of the Instituto Agroforestal Mediterráneo, Universitat Politècnica de València (Spain). The identities of these cultures were previously determined by sequencing of ITS region.

Sampling areas and environmental samples

Two geographical areas were studied. Villanúa (Central Pyrenees, Aragón, Spain) which was selected as a pure European silver fir (*Abies alba*) forest (42°04′13″N, 0°03′13″W) and Irati Forest (42°56′-43°00'N, 1°10′-0°58'E) located in northern Spain (Western Pyrenees, Navarra, Spain). The main vegetation type of Irati Forest is either native beech stands (*Fagus sylvatica*) or mixed beech and European silver fir (*Abies alba*) forest, although it is possible to find recent plantings of Lawson Cypress (*Chamaecyparis lawsoniana*) or Douglas fir (*Pseudotsuga menziesii*) in some areas.

Special authorization for sampling in the protected area of Irati Forest was granted by the Administración Forestal del Gobierno de Navarra. No special permit was required for sampling in Villanúa. This study did not involve endangered or protected species.

Soil samples

In Villanúa six soils from beneath European silver fir (AS) were collected in June 2012. In Irati Forest, soil samples were collected in October 2012 from 24 different locations selected according to their main vegetation community. The number of samples per vegetation type was proportional to their area in the forest: ten samples were collected from beech stands (F), six from Douglas fir (PS), five from European silver fir (AB), and three from Lawson Cypress (CH). Each sample consisted of soil

subsamples from six different points selected at random that were mixed together (approx. 3 kg of soil/sample). Samples were collected around the trees at 1 meter distance from the main trunk by digging to a depth of about 30 cm.

Water samples

In total 15 water samples were analyzed, two water samples from Villanúa (R1) and 13 water samples from rivers and streams in Irati Forest (R2) during October 2012. Samples were taken after several days of rain. Water samples (10 L) were collected from rivers and streams using plastic containers. The water was filtered in situ using a clean knap-sack sprayer to pump the water fitted with a 47 mm polypropylene Swinnex filter holder (Millipore Corp. Bedford, MA, USA) with a 5 μm pore-size autoclaved filter (cellulose acetate and cellulose nitrate mixture) (SMWP04700, Millipore, Ireland). Initially, the collected water was filtered into the sprayer through a 100 μm pore size mesh to remove any debris. Then the water was pumped using three filters per 10 L of water. Each filter was carefully removed with a pair of sterilised tweezers and cut in half with disinfested scissors. The filter was kept in a sterilized 15 ml polypropylene tube (Deltalab S.A., Barcelona, Spain). The tubes were kept in a cool-box during field sampling and in the freezer (-20°C) once in the laboratory. The sprayer was washed with 70% ethanol and rinsed with water thoroughly before sampling in each location.

DNA extraction

Each soil sample (up to 3 kg) was well mixed and homogenized by sieving (2 mm mesh size), and 50–80 g was lyophilized overnight and then crushed using FRITSCH Variable Speed Rotor Mill-PULVERISETTE 14 (ROSH, Oberstein, Germany). Samples were maintained at 5°C until DNA extraction.

Up to three subsamples were taken from each soil sample, and total genomic DNA was extracted from 300 mg of each subsample using the ZR Soil Microbe DNA MiniPrep (Zymo Research, Irvine, USA) following the manufacturer's instructions but with the final elutions in 50 µL of elution buffer instead of in a 100 µL of elution buffer.

Filters from water samples (3 filters/sample) were first frozen at −80°C and then disrupted using the TissueLyser LT compact bead mill (Qiagen, UK). DNA was extracted from each of the disrupted filters using the E.Z.N.A. Plant Kit (Omega Bio-tek, Doraville, USA) according to the manufacturer's recommendations but with the final elutions in 50 µL of elution buffer instead of in a 100 µL of elution buffer.

DNA was extracted from pure cultures using the E.Z.N.A. Plant Kit (Omega Bio-tek, Doraville, USA) according to the manufacturer's recommendations.

Control reaction

Each of the genomic DNA from cultures was diluted 10, 100 and 1000 times and amplified separately using a SYBR green real-time PCR assay with the *Phytophthora*-specific primers 18Ph2F and 5.8S-1R (Scibetta *et al*., 2012). DNAs presenting similar Cycle Threshold values were mixed together to account for differences in ITS copy number.

Amplicon library generation and 454-pyrosequencing

Amplicon libraries were generated using a nested PCR approach using a Hot Start polymerase (Dominion MBL, Córdoba, Spain). In the first PCR round the *Phytophthora*-specific primers 18Ph2F and 5.8S-1R were used (Scibetta *et al*., 2012). For the second PCR round, fusion primers were designed following the GS Junior

System Guidelines for Amplicon Experimental Design (Roche, 2010), selecting the unidirectional sequencing protocol for library construction (Lib-L chemistry for emulsion PCR, emPCR, 'One-Way Reads'). The template-specific sequence of the forward fusion primer was the universal ITS6 primer (Cooke *et al*., 2000) (5'-A-KEY-MID-ITS6–3'), while that of the reverse fusion primer was the same reverse primer used in the first PCR round (5'-B-KEY-5.8S-1R-3'), where A and B represent the pyrosequencing adaptors and the multiplex identifier (MID) was added for postsequencing sample identification. In the case of soil samples, 1 μL of genomic DNA was used in the first PCR round and in the case of water samples the genomic DNA was diluted ten times (approximately 0.2–2 ng was used as template in both DNA sources). The PCR products from the first round were diluted 10 times (soil samples) or 100 times (water samples) for the second PCR round, and in this case, 1 μL was used as a template. The PCR conditions were: 1 cycle of 95°C for 2 min, 30 cycles (1st round) or 25 cycles (2nd round) of 95°C for 20 s, 60°C for 30 s, 72°C for 30 s and a final extension of 72°C for 7 min. For the control reaction, 1 μl of genomic DNA was used in the first PCR round (30 cycles), and 1 μl of PCR product was used as template in the second round (25 cycles).

The PCR products were visualized in a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). The amplicons from soil and pure culture samples were double purified using the Agencourt AMPure XP Bead PCR Purification protocol (Beckman Coulter Genomics, MA, USA). However, in the case of water samples a different method was used with the aim to avoid non-specific products corresponding with other organisms with longer ITS1 fragments than *Phytophthora*, as well as chimeras, which could reduce the quality of the sequencing. Each PCR product from water samples was

cleaned using E-Gel SizeSelect (Invitrogen, Burlington, ON, Canada) and the bands between 290 and 450 bp were recovered for sequencing.

After purification, the amplicons were visualized in a Bioanalyzer 2100 and quantified by fluorimetry using Quant-iT PicoGreen kit (Invitrogen Molecular Probes, Eugene, Oregon, USA). The three PCR products from each soil and water samples were mixed together based on their concentration to obtain a single product. The 45 resultant amplicons from the seven areas (each area identified with an individual MID) were pooled at equal concentrations for sequencing.

Amplicon libraries were sequenced in a GS Junior 454 system (Roche 454 Life Sciences, Branford, CT, USA) by the Sequencing and Genotyping Service from the University of Valencia (Burjassot, Spain). Three different emPCR conditions were tested in three different sequencing runs. The first run was performed following Roche's standard protocol for emPCR amplification for the 'One-Way Reads' experimental design, the second run was using the same protocol but removing the larger amplicons by E-Gel SizeSelect, and in the third run, the emulsion was performed following the short length amplicon libraries protocol. Library from pure cultures was sequenced in a fourth sequencing run following the short length protocol.

Trimming and Molecular Operational Taxonomic Unit (MOTU) clustering

The sequences were first sorted into separate files according to their multiplex identifier (MID) using the sfffile script included in the Roche Newbler package (http://www.454.com/products/analysis-software/). Sequences from each SFF file were extracted using the sff_extract script (http://bioinf.comav.upv.es/sff_extract/index.html) to generate single FASTA, XML and quality files. FASTA and quality files were

combined into a single FASTQ file using a Python script (Python version 2.7, Python Software Fdn) and then opened in FastQC software (Andrews, 2010) to examine the length and quality of reads.

Reads were trimmed based on quality scores using Lucy software (Chou & Holmes, 2001). Sequence length shorter than 100 bp and low quality reads (average read quality below 20) were not considered for analysis. Sequences from the three sequencing runs were combined in a single FASTA file after quality trimming. A new Python script was created to trim the 3' end (B adaptor, key and reverse primer) and the 5' end (key, MID and forward primer). This script included a new step to remove sequences shorter than 100 bp after the second trimming.

Different score coverage threshold values were tested for MOTU clustering with a custom-curated database including 146 ITS1 sequences of described and new *Phytophthora* taxa, and the sequences of species used in the control reaction. The resultant FASTA file from each library was clustered with a length coverage threshold of 90% and a score coverage threshold of 99% (-L 0.9 -S 99) using blastclust software (Altschul *et al*., 1997). After clustering, a custom Python script was used to convert each MOTU from the output clustering list file into an individual FASTA file, sorting MOTUs in decreasing order of abundance, with a step to remove unique sequences (singletons), and then were aligned by MUSCLE (Edgar, 2004). Each alignment was manually checked in Seaview software (Gouy *et al*., 2010), and finally, a consensus sequence was generated from each MOTU with the aim to reduce homopolymer and sequencing errors.

The consensus sequences of the MOTUs were identified using the BLAST tool in the GenBank database (Altschul *et al*., 1997), the *Phytophthora* Database (Park *et al*.,

2008) and a custom-curated database comprising Sanger sequences showing a non-mixed chomatogram obtained from environmental samples and culture collections maintained at the Instituto Agroforestal Mediterráneo, Universitat Politècnica de València (Spain). A single sequence was selected at random in those MOTUs that had two reads. Consensus sequences were subjected to phylogenetic analysis to confirm the results obtained from BLAST searches.

Results

Sequencing throughput and quality control

The three different emPCR conditions tested produced 1,142 good quality reads in the first run, 14,289 (10,233 used in the present study) in the second and 140,767 reads in the third run.

The total data set from the three sequencing runs comprised 152,142 good quality sequences. The results obtained for each library were: 7,206 sequences for library 1 (R1), 47,419 for library 2 (R2), 13,543 for library 3 (CH), 19,796 for library 4 (PS), 21,982 for library 5 (AB), 26,527 for library 6 (F) and 15,669 for library 7 (AS). After trimming 151,311 sequences were considered for analysis. The average quality of the filtered sequences was of 36 and the average read length was 306 bp.

Score coverage threshold analysis

A reference database comprising 146 ITS1 sequences of described and new *Phytophthora* taxa was used to test the optimal threshold value. A FASTA file containing the ITS1 sequences was created and clustered at different threshold values. Clustering at 100%, or at 99.5%, separated the higher number of *Phytophthora* species

(Figure 3.1). Furthermore, based on ITS1 sequences it was not possible to separate 10% of the species using 100% score coverage threshold.

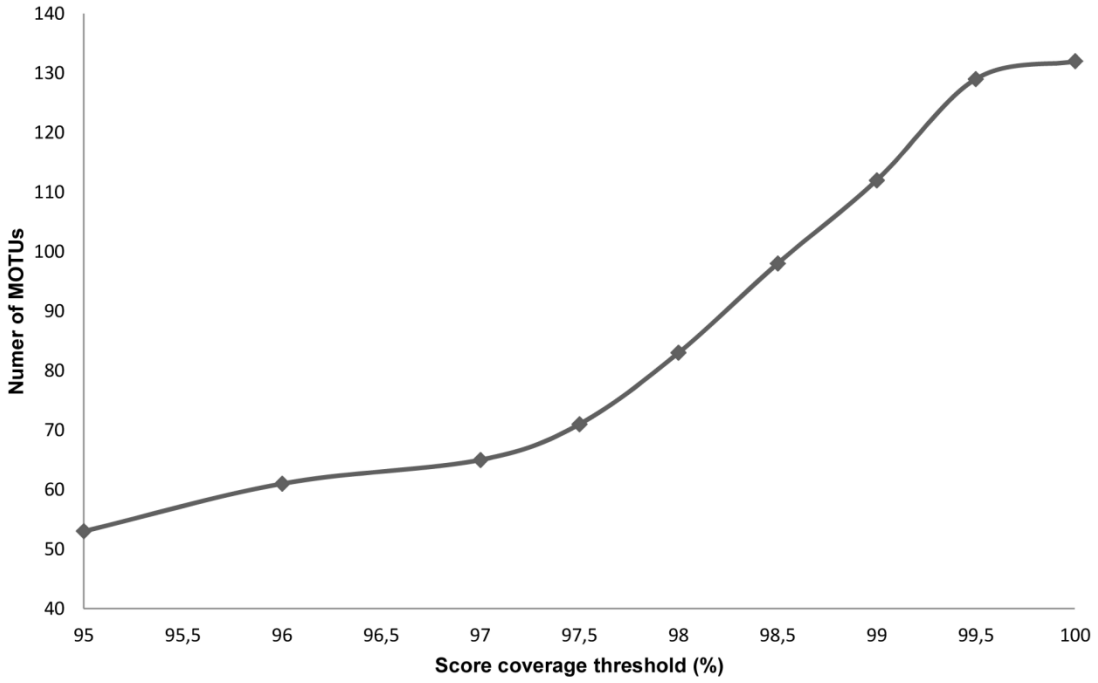

Figure 3.1. Number of MOTUs generated using different score coverage threshold values based on ITS1 using a reference database of *Phytophthora* species.

Using 99% coverage threshold allowed the separation of the highest number of *Phytophthora* species in the control reaction as shown in Figure 3.2. Clustering at 99.5% or 100% also separated the eight *Phytophthora* species. However, the number of singletons increased exponentially due to the presence of sequencing and homopolymer errors, producing a high loss of data. Therefore, for all the analysis a 99% of score coverage threshold was used for MOTU clustering.

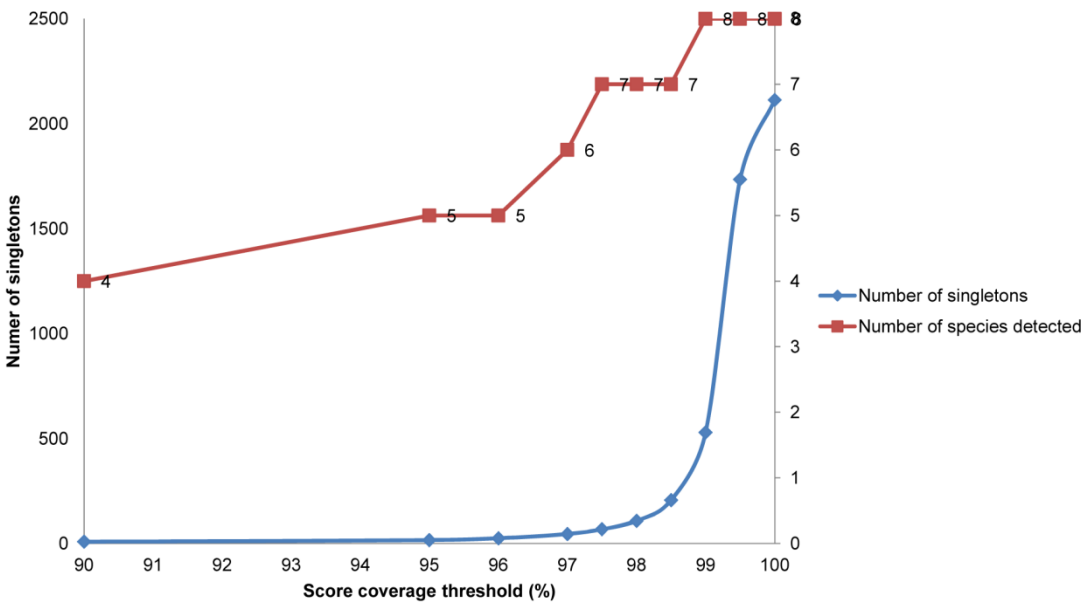

Figure 3.2. Number of *Phytophthora* species detected and singletons produced per score coverage threshold in the control reaction.

Control reaction

A total of 6,698 sequences were generated from the control reaction comprising eight *Phytophthora* species. After quality control, 6,683 sequences were considered for analysis. Applying the 99% score coverage threshold previously calculated a total of 555 MOTUs, including 529 singletons, were obtained. The MOTUs with highest number of sequences corresponded to the eight *Phytophthora* species. The identity of the consensus sequences with Sanger reads was the 100% or 99.5% in all cases. Mismatches were, in all cases, based on homopolymeric regions. The read distribution from each species is shown in Table 3.1.

Table 3.1. BLAST results of the clustered sequences from the control reaction applying a barcoding threshold value of 99%.

MOTU	Species	Number of Reads	ID with Sanger reads (%)	Error
1	***P. gonapodyides***	**1190**	**100**	-
2	***P. cryptogea***	**1025**	**100**	-
3	***P. cambivora***	**980**	**100**	-
4	***P.* taxon *PgChlamydo***	**884**	**100**	-
5	***P. colocasiae***	**879**	**99.5**	**PolyT**
6	***P. lacustris***	**604**	**99.5**	**PolyT**
7	***P. cinnamomi***	**434**	**100**	-
8	***P. plurivora***	**121**	**99.5**	**PolyT**
9	*P. gonapodyides*	3	98	PolyA, -/T, -/G, -/C
10	Chimera	2	-	-
11	*P.* taxon *PgChlamydo*	2	96	A/-, PolyA, T/-, PolyG, T/-, T/-, G/-, -/T, -/T
12	*P. gonapodyides*	2	98	PolyA, -/G, C/T, -/G
13	Chimera	2	-	-
14	*P. gonapodyides*	2	98	G/A, T/-, G/-, T/-, G/-
15	*P. cambivora*	2	97	C/T, T/C, T/A, T/A, C/T, T/A
16	*P. gonapodyides*	2	98	PolyA, A/-, PolyT, PolyA
17	*P. gonapodyides*	2	97	PolyG, T/C, C/T, T/-, T/-, G/A
18	*P. gonapodyides*	2	98	C/T, A/G, T/C, T/C
19	*P.* taxon *PgChlamydo*	2	97	A/-, -/A, T/C x3
20	*P. colocasiae*	2	98	PolyA, G/-, PolyT x2
21	*P. cryptogea*	2	99	G/A, G/T
22	*P. colocasiae*	2	98	PolyA, G/-, T/-
23	*P. colocasiae*	2	96	A/-, PolyA, T/A, A/C,-/T, PolyT
24	*P. plurivora*	2	98	PolyT, T/C, A/T
25	*P. plurivora*	2	97	PolyA, PolyT, T/C, A/T
26	*P. plurivora*	2	98	PolyT x3

Results only include MOTUs with more than two sequences. Identity and mismatches with Sanger reads are also indicated. MOTUs with higher number of sequences are in bold.

Analysis of MOTUs from soil eDNA samples

A total of 96,895 reads which passed quality control from soil samples (sampling areas CH, PS, AB, F and AS) were considered for analyses. Clustering of 96,895 reads resulted in 8,151 MOTUs, including 8,114 singletons, which were discarded for the analysis. The 37 nonsingleton MOTUs corresponded to 13 *Phytophthora* species, included in clades 1, 4, 6, 7 and 8 (Figure 3.3) from four plant communities: *A. alba*, *C. lawsoniana*, *P. menziesii* and *F. sylvatica*.

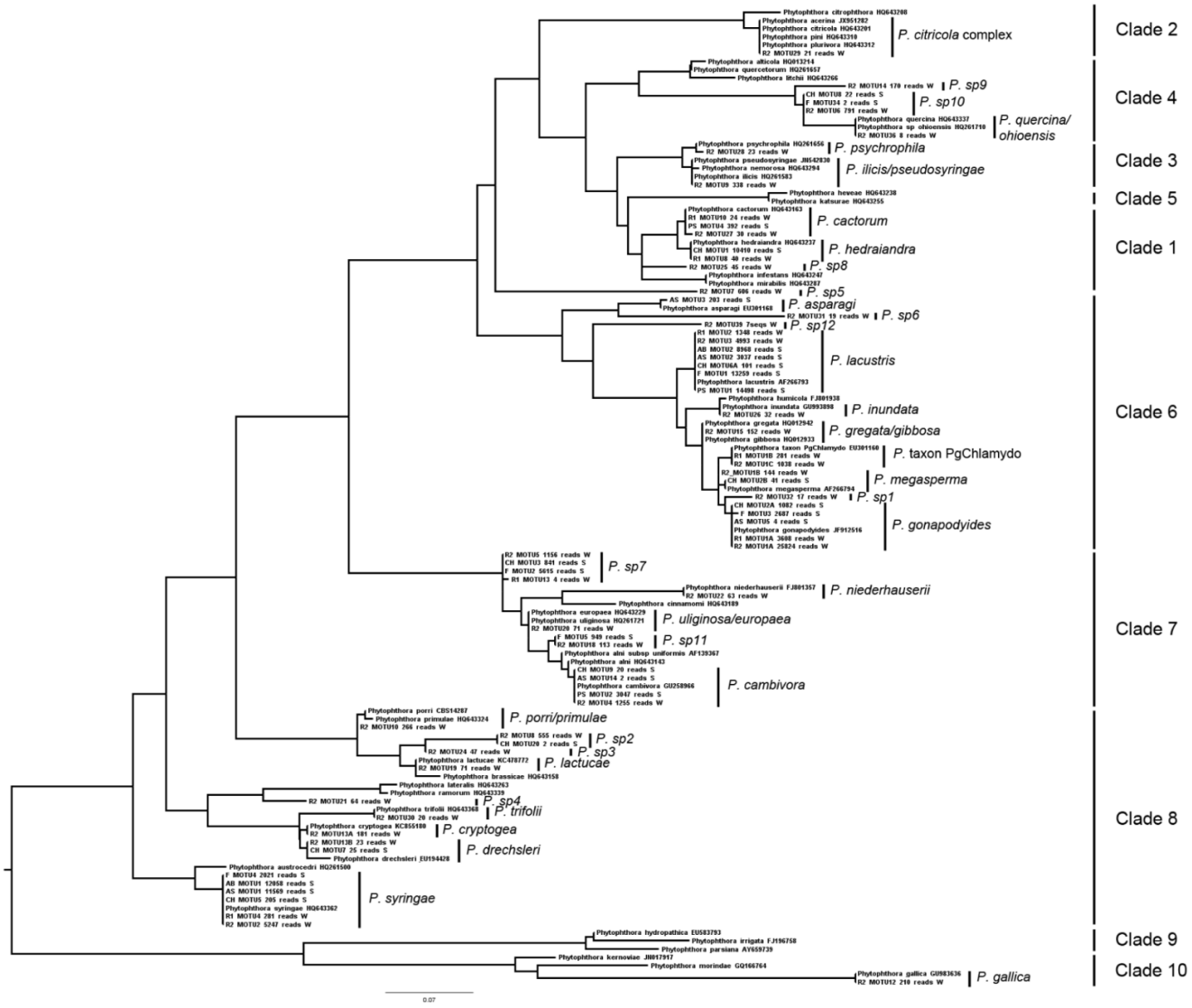

Figure 3.3. Unrooted phylogram based on nuclear ITS1 rDNA sequence analysis constructed with maximum likelihood approach.

Each of the MOTU includes library precedence (R1, R2, CH, AB, F, PS or AS), number of MOTU resulted in the clustering at 99% of each library, number of reads and source (W, water; S, soil). Vertical bars indicate the *Phytophthora* species. MOTUs corresponding with undescribed species are indicated as "sp" (from sp1-sp12).

The specificity of the technique for the detection of *Phytophthora* in soils was 98.46%. The non-*Phytophthora* MOTUs (1.54% of nonsingleton clustered reads) matched with other plant pathogen genera such as *Pythium* and *Hyaloperonospora* (Table 3.2).

Table 3.2. Read distribution of species other from *Phytophthora* based on the ITS1 after BLASTn of the consensus sequences performed against GenBank. Reads from MOTUs with more than 99% similarity are in bold.

	Villanua		Irati Forest					
Putative species detected non- *Phytophthora*	**R1**	**AS**	**R2**	**CH**	**F**	**AB**	**PS**	**Total reads per species**
Hyaloperonospora parasitica	**361**							361
Hyaloperonospora sp. 1							894	894
Hyaloperonospora sp. 2	248							248
Hyaloperonospora sp. 3			62					62
Hyaloperonospora sp. 4			117					117
Hyaloperonospora sp. 5	161							161
Hyaloperonospora sp. 6					198			198
Bremia lactucae			**5**					5
Peronospora aparines	**165**							165
Peronospora aestivalis			**232**					232
Peronospora glomerata			**150**					150
Pythium sp 1	7		2		6			15
Pythium sp 2		21						21
Pythiaceae 1	40							40
Pythiaceae 2	10							10
Pythium minus	**2**			**250**				252
Total reads per sampling area	994	21	568	250	204	0	894	

Analysis of MOTUs from water eDNA samples

A total of 54,416 reads from water eDNA from the sampling areas R1 and R2 which passed quality control were considered for analyses. Clustering of 54,416 reads resulted in 3,646 MOTUs, including 3,548 singletons, which were discarded for the analysis. The 98 nonsingleton MOTUs corresponded to 35 *Phytophthora* species (Figure 3.3) from clades 1, 2, 3, 4, 6, 7, 8 and 10 (Figure 3.3). No *Phytophthora* species were detected from clades 5 and 9. The clades with the highest number of species were clade 6 and 8 with nine species in each. Only one species from clade 2 was found, matching with a *Phytophthora citricola*-complex species. The three most abundant MOTUs in water samples were *P. gonapodyides* (29,432 reads), followed by *P. lacustris* (6,341 reads) and *P. syringae* (5,528 reads). Fifteen sequences were obtained of a *Phytophthora* species (named as *Phytophthora* sp. 13) which sequences did not match any sequences in public databases and did not cluster in any of the ITS clades

described. The closest match in GenBank was *Phytophthora* sp. REB326-69 (accession no. JX122744) with a sequence homology of 89%. This MOTU was not included in Figure 3.3 as it was too divergent.

The specificity of the water eDNA pyrosequencing assay for *Phytophthora* detection was 96.92%. The non-*Phytophthora* MOTUs (3.08% of nonsingleton clustered reads) matched with other plant pathogen genera such as *Hyaloperonospora*, *Bremia*, *Peronospora*, *Pythium* or members of an undetermined Oomycete genus not represented in GenBank (Table 3.2).

Discussion

The detection of *Phytophthora* from environmental samples using high-throughput amplicon pyrosequening of eDNA has shown high specificity and can be used hereafter to assess *Phytophthora* diversity in natural ecosystems.

Clustering at 100% was the best criteria to separate most of the species in the reference database. Other studies (Vettraino *et al*., 2012) adopted a barcoding threshold of 98% to assign most reads. However, in the current study we included a control species mixture and a 99% threshold was required to discriminate between closely related species resulting in the separation of 20% more species.

For the control reaction data, clustering at 99% of similarity was the minimum value able to separate all of the species with the minimum number of reads lost. Clustering at 99.5% or 100% resulted in the loss of reads due to the presence of homopolymer and sequencing errors that were discarded as singletons. Values below 99% were insufficient to separate *P. gonapodyides* and *P.* taxon PgChlamydo in separate MOTUs. In this study, clustering at 99% similarity allowed the separation of

most species from clade 6 found in environmental water samples and reduced the risk of false MOTUs produced by sequencing errors. Although the pooling for the control reaction was made by mixing the DNAs based on similar CT values, the number of sequences obtained of *P. plurivora* was lower than expected and this could be a potential issue to consider when interpreting the results.

The limitations of the ITS1 region for *Phytophthora* taxonomic identification are particularly evident in clade 1, in clade 2 with the *P. citricola* complex (Jung & Burgess, 2009) and in clade 6 which includes many aquatic species where many species are identical or differ by 1 bp in ITS1. Applying a similarity of 100% between sequences, it is still not possible to separate 10% of the species in the reference database, including species from clade 1 *(P. infestans*, *P. ipomoeae*, *P. mirabilis*, *P. andina*), from clade 2 (*P. capensis*, *P.* taxon emanzi) or clade 6 (*P. gibbosa*, *P. gregata*). Despite these limitations it remains a powerful tool for discrimination of most known species as well as the identification of new ones.

The conditions for emPCR amplification (2.2 million copies of template and 80 µL of Amp Primer A) used in the first and second sequencing runs resulted in light scattering into nearby wells and caused their elimination due to signal processing filtering. The third sequencing run was performed following guidelines for Short Length Libraries which reduced the amount of Amp Primer A in the Live Amp Mix from 80 to 20 µL. Although environmental ITS1 amplicons length did not match the Short Length Libraries definition (amplicons of 340 bp instead of amplicons <250 bp), the use of this protocol increased 100 fold the throughput in comparison with the first pyrosequencing run.

Similar studies based on pyrosequencing in soil samples from beech forests in France revealed the presence of only four *Pythiaceae* species (corresponding to the 0.8% of the sequences from the dataset), represented by two *Phytophthora* species: *P. plurivora* and an unidentified *Phytophthora* species from clade 7a (Coince *et al*., 2013). Their methodology for library generation was based on a nested PCR approach, where the first PCR was based on the amplification of the whole ITS region with the primer pair ITS6/ITS4 (Cooke *et al*., 2000; White *et al*., 1990) and pyrosequencing was based on the ITS6/ITS7primers (Cooke *et al*., 2000). This pyrosequencing strategy revealed lower efficacy and specificity for *Phytophthora* detection in comparison with the methodology implemented in this study. The throughput of amplification of large targets, like whole ITS region in genus *Phytophthora* (up to 840 bp), is usually a problem in environmental samples, especially in soils, with abundant humic acids inhibitors and high DNA degradation. The importance of the length of the targeted region is that short DNA fragments (less than 300–400 bp) are usually very slowly degraded which allows their detection in environmental samples (Deagle *et al*., 2006). The implementation of large-scale eDNA-based ecological studies is highly dependent on the availability of suitable short metabarcodes (Taberlet *et al*., 2012). Therefore, using in the first PCR the *Phytophthora*-specific primers as implemented, which include the ITS1 and a small portion of the 18S (amplicon length from 419 to 484 bp), rather than the whole ITS region provides better amplification results for metagenomics studies.

In contrast, other researchers (Vannini *et al*., 2013) applied the same ITS6/ITS7 primers for pyrosequencing without using a nested approach, revealing the presence of 15 *Phytophthora* species in soils from chestnut forests in Italy. Their assay was very

specific for *Phytophthora* species detection, where 78.8% of sequences (9,167 of 11,637 reads) matched *Phytophthora*.

In this study, a nested approach was used in order to yield suitable PCR products since no amplification was obtained with a single round PCR (Scibetta *et al*., 2012). Nested PCR increases sensitivity and specificity compared to non-nested PCR techniques, but also increases the risk of cross-contamination events. To further reduce the occurrence of false-positives based on cross-contamination extremely care should be taken during DNA extraction, preparation of PCR reactions and post-PCR steps.

From a methodological point of view, soil pre-processing procedures (drying, sieving, lyophilizing, crushing and weighting) are time-consuming in comparison with the water filter processing. Working with water samples allows the processing and amplicon library generation in one day. Pyrosequencing of soil samples revealed lower *Phytophthora* diversity than from water samples, where only thirteen species were detected in five different ecosystems comprising 30 sampling sites. The highest number of species was detected in three sampling sites of *Chamaecyparis* plantations where 10 of the 13 *Phytophthora* species were detected. It is especially surprising that so few species were detected in *Abies alba* soil samples from Irati Forest, where only two species, *P. lacustris* and *P. syringae* were detected. Similarly within *Pseudotsuga menziesii* associated soil samples where only three species were detected. Pyrosequencing of water eDNA from rivers and streams revealed a hidden and unexpectedly high *Phytophthora* species diversity. A total of 35 *Phytophthora* species were detected, 13 of which could represent potentially novel species. *Phytophthora* species diversity from water samples in Irati Forest comprised all species detected in soil samples from the same sampling area. However, the species detected in water and

soil samples in Villanúa were different. This could be due to the fact that a low number of samples were taken from this forest and also due to the different sampling times.

The DNA recovered from the filters is probably composed of DNA originated from living cells (mainly zoospores) and extracellular DNA originated from natural cell death in water. These could be present in the water itself or they could be have originated from washing off the soil after rain periods. Other studies (Dejean *et al*., 2011) have demonstrated that DNA fragments in freshwater ecosystems persist for less than one month. This indicates that by sampling immediately after a rain event is possible to recover the maximum amount of DNA containing the potential *Phytophthora* community from the forests and water environments. This hypothesis is congruent with the presence of several *Phytophthora* species from most clades in water eDNA, including many plant pathogenic species usually isolated from roots such as *P. cambivora*, *P. niederhauserii*, *P. plurivora*, *P. pseudosyringae* or *P. syringae*.

Most of the *Phytophthora* species isolated from irrigation reservoirs and natural waterways belong to clade 6. These species, commonly called water moulds, show a strong association with both forest and riparian ecosystems, (Brasier *et al*., 2003a; Jung *et al*., 2011; Hüberli *et al*., 2013; Guha Roy & Grunwald, 2014) and although some of them can be aggressive tree pathogens like *P. inundata* in Spain (Brasier *et al*., 2003b), it is hypothesized that they have a prevalently aquatic and saprophytic lifestyle (Hansen *et al*., 2012). In this study 73.6% of the reads belonged to clade 6, which may represent the resident community in rivers and streams. The most common species detected were *Phytophthora gonapodyides*, *P. megasperma*, *P*. taxon PgChlamydo, *P. inundata* and *P. lacustris*, which are commonly isolated by baiting from streams around the world (Reeser *et al*., 2011; Nechwatal *et al*., 2012; Huai *et al*., 2013; Hüberli *et al*., 2013; Oh *et al*., 2013).

The *Phytophthora*-specific primers (Scibetta *et al*., 2012) used in the current study showed high specificity. In the original study the amplicons were cloned and a total of 260 amplicons were obtained with a complete absence of *Pythium s. lato* species. Only a minor cross reaction with two downy mildew phylotypes was found. However, in this study using the same primers with an HTS approach a total number of 152,142 reads were obtained with 97.9% specificity to *Phytophthora*. Only a small percentage (2.1%) of non-*Phytophthora* amplification was obtained, including the genera *Hyaloperonospora*, *Bremia*, *Peronospora* and *Pythium*.

Previous techniques (baiting of environmental samples and cloning) are time consuming and costly, and often are likely to generate false negatives due to the low frequency of some *Phytophthora* species and the inability of unknown Phytophthoras to grow on some artificial media. The technique implemented here overcomes these problems and can produce results in less than one week compared to several weeks or months by conventional techniques which would allow an early response to the threats posed by these pathogens. Although similar techniques have been used in previous studies it is the first time that genus-specific amplicon pyrosequencing has been used to target only *Phytophthora* in soil and water samples, unlocking previously hidden *Phytophthora* communities.

Accession numbers

The three pyrosequencing runs were combined into a single file and deposited in GenBank-SRA under the accession number SRP027499. Consensus nucleotide sequences of each *Phytophthora* MOTU were deposited in GenBank (accession no. AF132232 to AF132286).

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Chapter 4

Metabarcoding and development of new real-time specific assays reveal *Phytophthora* species diversity in holm oak forests in eastern Spain

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Keywords: *Phytophthora*, amplicon pyrosequencing, metabarcoding, environmental DNA.

Abstract

The evergreen holm oaks (*Quercus ilex* subsp. *ilex* and *Q. ilex* subsp. *ballota*) are the most representative tree species in the Iberian Peninsula and the main tree species in oak-rangeland ecosystems (dehesas). Oak decline in western, central and southern parts of Spain has been associated with root rot caused by *Phytophthora cinnamomi* for decades. However, *Phytophthora* species such as *P. quercina* and *P. psychrophila* have recently been found associated with *Quercus* decline in eastern Spain where calcareous soils are predominant. Soil and root samples from two *Quercus* forests presenting decline symptoms in two different geographical areas in eastern Spain (Carrascar de la Font Roja and Vallivana) were analysed by amplicon pyrosequencing. Metabarcoding analysis showed *Phytophthora* species diversity, and revealed that an uncultured *Phytophthora* taxon, named provisionally *Phytophthora* taxon ballota, was the predominant species in both areas. In addition, a real-time PCR assay, based on the pyrosequencing results, was developed for the detection of this uncultured *Phytophthora* taxon, and also for the detection of *P. quercina*. TaqMan assays were tested on soil and root samples, and on *Phytophthora* pure cultures. The new assays showed high specificity and were consistent with metabarcoding results. A new real-time PCR protocol is proposed to evaluate the implication of different *Phytophthora* spp. in oak decline in eastern Spain.

Introduction

The evergreen holm oak (*Quercus ilex* subsp. *ilex* and *Q. ilex* subsp. *ballota*) is the most abundant tree species in the Iberian Peninsula and the main tree in oak-rangeland ecosystems (dehesas). Monospecific holm oak forests cover an area of about 820 000 ha in Spain, and both subspecies can also be present in mixed stands with

Quercus suber and/or *Quercus faginea* (Carevic *et al*., 2010). *Quercus ilex* subsp. *ballota* is the main species distributed in the Comunidad Valenciana region (eastern Spain), usually growing on calcareous soils. In this territory, holm oak forests do not form dehesas as in the western, central and southern areas of Spain. Some of these tree formations have social and recreational value and are legally protected Natural Parks.

The absence of natural regeneration and inappropriate livestock management are some of the most recognized problems that threaten the persistence of oak groves and dehesas (Pulido *et al*., 2001). Furthermore, oak decline in non-calcareous soils in western, central and southern parts of Spain has long been associated with root rot caused by *Phytophthora cinnamomi* (Brasier *et al*., 1993; Tuset *et al*., 1996; Sánchez *et al*., 2002; Corcobado *et al*., 2014). More recently, other *Phytophthora* spp. such as *P. quercina* and *P. psychrophila* have been associated with *Quercus* decline in calcareous soils located in eastern Spain (Pérez-Sierra *et al*., 2013).

Molecular approaches complementing isolations made from environmental samples have profoundly changed the view of *Phytophthora* diversity, providing new perspectives for future ecological studies. Recent studies have applied high-throughput sequencing techniques to identify *Phytophthora* communities in different ecosystems (Coince *et al*., 2013; Vannini *et al*., 2013; Català *et al*., 2015). Although different methodologies for library generation have been applied in these studies, all of them sampled environmental DNA (eDNA) as the DNA source. Analysis of eDNA allows the rapid characterization of microbial communities without isolating the target organisms. Moreover, one of the challenges of metabarcoding using eDNA is that it is recommended to confirm the presence of an organism/molecular operational taxonomic unit (MOTU) in a particular habitat or set of samples by different means to rule out artefacts or sample contamination. In this context, real-time PCR is a suitable method to

assess the presence of specific plant pathogens in environmental samples (Schena *et al*., 2006; Lees *et al*., 2012; Lin *et al*., 2013; Woodhall *et al*., 2013).

In recent years, real-time PCR assays have been widely used for the detection and quantification of fungal and bacterial plant pathogens (Kang *et al*., 2012; Agustí-Brisach *et al*., 2014; Liu *et al*., 2015). Quantification of *Phytophthora* spp. in soil and root samples by fluorescent probe-based quantitative real-time PCR can help gain a better understanding of potential threats to forest health. Quantitative PCR offers numerous advantages over conventional PCR techniques such as higher sensitivity and specificity, faster rate of detection, no post-PCR manipulation, minimizing the risk of contamination, and the possibility to provide quantitative results. Several authors have focused on the detection of known *Phytophthora* spp. using real-time PCR: *P. infestans* (Avrova *et al*., 2003), *P. medicaginis* (Vandemark & Barker, 2003), *P. nicotianae* and *P. citrophthora* (Ippolito *et al*., 2004), *P. fragariae* (Bonants *et al*., 2004), *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* (Schena *et al*., 2006), *P. capsici* (Silvar *et al*., 2005) or *P. pseudosyringae* (Tooley *et al*., 2006), but no one had applied this technique to investigate the presence of uncultured *Phytophthora* spp. in environmental samples.

A previous survey conducted in the Natural Park of Carrascar de la Font Roja using classical culturing methods revealed the presence of several known *Phytophthora* species in the rhizosphere of *Quercus* species showing typical symptoms of decline (Pérez-Sierra *et al*., 2013). Molecular methods that can detect organisms difficult to bait and isolate, such as high-throughput sequencing, are needed to investigate the diversity of *Phytophthora* spp. in the rhizosphere thoroughly. Considering the high ecological and social relevance of holm oak forests in eastern Spain and economic losses caused by *Phytophthora* spp. in this region, the objectives of this study were: (i) to assess *Phytophthora* spp. diversity on representative holm oak forests located in this territory

using high-throughput amplicon pyrosequencing, and (ii) to develop a real-time PCR assay based on the pyrosequencing results for the detection of uncultured or previously recorded *Phytophthora* spp. in environmental samples.

Materials and methods

Sampling areas and environmental samples

Oak forests located 230 km apart were studied in eastern Spain: the Natural Park of Carrascar de la Font Roja (Alicante province, 38°39′54.49″N, 0°32′26.72″W) and Vallivana (Castellón province, 40°33′10.93″N, 0°0′24.10″W). Although both forests are similar in appearance and composition due to the presence of monospecific calcicolous *Q. ilex* subsp. *ballota* woods, it is possible to distinguish them according to their vegetation. The Carrascar de la Font Roja forest is located in the mesomediterranean bioclimatic zone, including a small area in the supramediterranean zone (Rivas Martínez, 1987), with two main vegetation types: *Bupleuro rigidi–Querceto rotundifoliae* (mesomediterranean) and *Junipero thuriferae–Q. rotundifoliae* (supramediterranean). On the other hand, vegetation from Vallivana is included in the *Hedero helicis–Q. rotundifoliae* vegetation series (Costa *et al*., 1987).

In the Carrascar de la Font Roja forest, 13 samples were collected along the road that crosses the Natural Park: 10 of the samples were rhizosphere soils collected around trees showing decline symptoms, and 3 samples were taken from run-off ditches located in the same area. In the Vallivana forest, 10 trees with symptoms were sampled, taking rhizosphere soil samples and root samples from the same trees.

Soil samples consisted of a mix of subsamples from six different points selected at random around trees at approximately 1 m from the trunk at a depth of 30 cm. Soil

samples from run-off ditches consisted of a mix of four subsamples selected at random (c. 3 kg soil per sample). Each soil sample was well mixed and homogenized by sieving (2-mm diameter mesh size). A total of 50–80 g of soil was air dried and then crushed for further analysis using a Fritsch variable speed rotor mill-Pulverisette 14 (ROSH). Samples were maintained at 5 °C until DNA extraction.

Root samples were separated from soils immediately after sieving, washed thoroughly with water removing all soil particles attached, and surface sterilized by spraying with ethanol (96%). Roots were air dried in individual paper bags, frozen with liquid nitrogen and ground into a fine powder. All samples were stored frozen after grinding.

DNA extraction

Genomic DNA was extracted from 250 mg of crushed soil using the ZR Soil Microbe DNA MiniPrep M (Zymo Research) following the manufacturer's instructions. DNA was extracted from 50 mg of the macerated root samples using the PowerPlant DNA isolation kit (MoBio Laboratories, Inc.) following the manufacturer's instructions for removal of phenolics. Final elutions were performed in 100 μL Tris-EDTA (TE) buffer.

Amplicon library generation and 454-pyrosequencing

Amplicon libraries were generated using a nested PCR approach as described in Català *et al.* (2015). A modification was introduced in the primer design: Lib-L A pyrosequencing adaptor was attached to the reverse primer 5.8S-1R (Scibetta *et al.*, 2012) instead of to the universal ITS6 primer, and the B adaptor was attached to the ITS6 primer. In the first PCR round, 2 μL of genomic DNA (c. 0.2–2 ng) was used as

the template; in the second PCR round, 2 μL of the PCR products were used. The PCR conditions were: an initial denaturation of 95 °C for 2 min; 30 cycles (first round) or 25 cycles (second round) of 95 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s; and a final extension of 72 °C for 7 min.

The amplicons were purified twice and sequenced in a GS Junior 454 system (Roche 454 Life Sciences) by the Sequencing and Genotyping Service of the University of Valencia (Burjassot, Spain).

Sequence trimming and MOTU clustering

Reads were processed as described in Català *et al*. (2015) and clustered with a length coverage threshold of 90% and a score coverage threshold of 99% (–L 0.9 –S 99) using blastclust software (Altschul *et al*., 1997). The consensus sequences of the MOTUs were identified using the blast tool in the GenBank database (Altschul *et al*., 1997), the *Phytophthora* database (Park *et al*., 2008) and a custom-curated database.

Design of real-time primers and probes

An alignment of ITS1 sequence data of *Phytophthora* spp. isolated from holm oak forest, consensus sequences from the 454-pyrosequencing data, Sanger sequences from the eDNA samples and other non-target sequences obtained from NCBI was constructed using mega 5 software (Tamura *et al*., 2011). Primers were designed using the RealTime PCR tool (https://eu.idtdna.com/scitools/Applications/RealTimePCR/) and manually adjusted using mega 5 software. Sequences of PCR primers and double-quenched probes developed for this study are listed in Table 4.1. Real-time PCR was carried out on a Rotor-Gene Q 5plex HRM instrument (QIAGEN) using Premix Ex Taq (Probe qPCR; Takara Biotechnology (Dalian), Co., Ltd) in a final volume of 25 μL.

Reaction mixtures contained 2 µL of template eDNA, 12.5 µL Premix Ex Taq (2×) and 2.5 µL of primers-probe assay mix (500 nm of each primer and 250 nm probe). The PCR was performed following a two-step PCR: 95 °C for 30 s; then 45 cycles of 95 °C for 5 s and 60 °C for 30 s.

Table 4.1. Sequences of real-time PCR primers and double-quenched probes targeting the ITS1 rDNA region

Target	Primer/Probe name	Sequence (5′-3′)	Ampicon size
P*. taxon *ballota	taxon_ballota_F	TAGTTGGGGGTTTGCCAG	82
	taxon_ballota_R	AAACGCTCGCCATGATAGG	
	taxon_ballota_Probe	/56-FAM/CC GTC AGC A/Zen/A TCA AGC CAA CAG C/3IABkFQ/	
P. quercina	quercina_F	GGTCTTGTCTGGCGTATGG	100
	quercina_R	AGCTACTTGTTCAGACCGAAG	
	quercina_Probe	/56-FAM/GC TGT AAA A/Zen/G GCG GCG GCT GTT GC/3IABkFQ/	

Assessment of real-time PCR assay specificity and sensitivity

Specificity was tested against all the isolates obtained from the culture collection listed in Table 4.2. Assay sensitivity was determined by testing a 10-fold dilution series of *P. quercina* DNA (isolate PS-982) ranging from 2 ng/µL to 0.2 fg/µL. In the case of *P.* taxon ballota, a 10-fold dilution series from a positive environmental sample was used to test assay sensitivity. Reactions were carried out in duplicate.

Table 4.2. *Phytophthora* isolates obtained from culture collection and environmental samples used as positive and negative amplification controls in the real-time PCR assays. a) Both *P. quercina* and *P.* taxon ballota were detected via pyrosequencing. b) Only *P.* taxon ballota was detected via pyrosequencing. c) Species other than *P. quercina* and *P.* taxon ballota were detected by pyrosequencing.

Isolate	*P. quercina*	*P.* taxon *ballota*
Phytophthora cryptogea	-	-
Phytophthora cambivora	-	-
Phytophthora citrophthora	-	-
Phytophthora plurivora	-	-
Phytophthora cinnamomi PS-927	-	-
Phytophthora lacustris	-	-
Phytophthora chlamydospora	-	-
Phytophthora gonapodyides	-	-
Phytophthora palmivora PS-1464	-	-
Phytophthora citricola PS-1432	-	-
Phytophthora multivora PS-1284	-	-
Phytophthora drechsleri	-	-
Phytophthora ramorum	-	-
Phytophthora niederhauserii PS-1597	-	-
Phytophthora psychrophila PS-1030	-	-
Phytophthora asparagi	-	-
Phytophthora taxon *oaksoil*	-	-
Phytophthora tropicalis PS-1433	-	-
Phytophthora hedraiandra PS-1023	-	-
Phytophthora cactorum PS-1600	-	-
Phytophthora megasperma	-	-
Phytophthora citrophthora PS-1429	-	-
Phytophthora nicotianae PS-2227	-	-
Phytophthora alni subsp. alni PS-843	-	-
Phytophthora capsici PS-838	-	-
Phytophthora quercina PS-982	+	-
Phytophthora quercina PS-986	+	-
Phytophthora quercina PS-987	+	-
Phytophthora quercina PS-988	+	-
Phytophthora quercina PS-993	+	-
Phytophthora quercina PS-994	+	-
Phytophthora quercina PS-1000	+	-
FR4	+	+
FR6	+	+
V1	-	+
MSR5	-	-

Real-time PCR assay using environmental samples

The real-time PCR assay was validated using the same DNA samples used for pyrosequencing. In all cases, a 10-fold dilution of each DNA was included in order to test for effects of PCR inhibitors.

Statistical analysis

The counts of the number of reads from each phylotype were converted into frequencies relative to the total number of clustered reads generated within each sample. Only MOTUs represented in at least four samples were considered individually, the others were included together in groups named 'Other *Phytophthora* spp.' and 'Unknown spp.'. Analysis of variance (ANOVA) was performed using SAS v. 9.0 (SAS Institute Inc.) to determine if the frequency of an individual MOTU was affected by the sampling area (Carrascar de la Font Roja or Vallivana) or sampling material (soil or roots within the Vallivana sample). Mean frequencies were compared using the Fisher's least significant difference (LSD) procedure at $P = 0.05$. The Shannon's diversity and richness indices of MOTUs were calculated for each sample and the corresponding mean values for each sampling area.

Results

Identification of *Phytophthora* species using genus-specific 454-pyrosequencing

Twelve of the 13 DNA extractions from soil samples from Carrascar de la Font Roja yielded PCR products. In the case of the Vallivana sampling area, 8 of 10 DNA extractions from soil samples, and 5 from the 10 root samples, yielded PCR products. A total of c. 45 000 sequences were generated with ITS1 rDNA amplicon pyrosequencing from these 20 soil and 5 root samples (Table 4.3) that yielded PCR products.

Table 4.3. Read distribution per *Phytophthora* species in the 25 samples sequenced. BAL, *P.* taxon ballota; CAC, *P. cactorum*; GON, *P. gonapodyides*; NIC, *P. nicotianae*; PLU, *P. plurivora*; PSY, *P. psychrophila*; QUE, *P. quercina*; TEN, *P. tentaculata*; sp1–sp5, new phylotypes found. a FR, soil samples from Carrascar de la Font Roja; VS, soil samples from Vallivana; VSR, root samples from Vallivana. b Species detection using real-time specific PCR. '+', positive sample; '−', negative sample.

Sample	*Total reads*	CAC	NIC	QUE	QUE qPCR	PSY	GON	BAL	BAL qPCR	PLU	TEN	*SP1*	*SP2*	*SP3*	*SP4*	*SP5 Clade 9*
FR1	1859	221	4		-	481		1031	+							
FR2	2338	43			-	123		2075	+							
FR3	2026				-	502		1419	+							
FR4	2032			687	+	310		925	+							
FR5	2451			495	+			1900	+							
FR6	2340			601	+	24		1623	+							
FR7	2102				-	158		1862	+							
FR8	2173				-	1630		-	-							
FR9	2541				-	1458		47	+							
FR10	2234				-			2202	+							
FR12	3035				-			2972	+							
FR13	2885				-		2.061	682	+							
VS1	1647			503	+			651	+	80			261	28		
VS2	629				-				-						603	
VS3	1014				-	935			-	16						
VS4	1315				-			558	-		219			337		
VS5	1021				-				-			939				
VS6	969				-				-	827						
VS7	2606				-			2519	+							
VS8	1836				-	95		1623	+							
VSR1	1227				+				-							1143
VSR2	1176				-				-						1155	
VSR6	872				-				-			832				
VSR7	1256				-			1072	+			73				
VSR8	1012				-	970			+							

Pyrosequencing of soil samples from Carrascar de la Font Roja revealed the presence of six phylotypes that corresponded to five known *Phytophthora* spp. (*P. cactorum*, *P. gonapodyides*, *P. nicotianae*, *P. psychrophila* and *P. quercina*) and a phylotype that did not match any sequence in public databases (Table 4.3). This latter phylotype was detected in 11 of 12 soil samples, including both run-off ditches and rhizosphere samples, and it was provisionally named as *Phytophthora* taxon ballota. *Phytophthora* taxon ballota represented 62.5% of the clustered reads, and it was detected in 92% of the samples, followed by *P. psychrophila* (18.4% of reads in 67% of samples), *P. gonapodyides* (8% of reads in 8% of samples), *P. quercina* (7% of reads in 25% of samples), *P. cactorum* (1% of reads in 17% of samples) and *P. nicotianae* (0.016% of reads in 8% of samples).

Pyrosequencing of soil samples from Vallivana resulted in the detection of nine phylotypes that corresponded with four known *Phytophthora* spp. (*P. plurivora*, *P. psychrophila*, *P. quercina* and *P. tentaculata*) and five others that did not match with any sequence in public databases (Table 4.3). *Phytophthora* taxon ballota was detected in 50% of the soil samples, comprising 52.5% of total clustered reads, followed by *P. plurivora* and *P. psychrophila* detected in three (37.5%) and two (25%) samples, comprising 9% and 5% of clustered reads, respectively. *Phytophthora quercina* and P. *tentaculata* were detected in one sample (12.5%) each, comprising 5% and 2.1% of clustered reads, respectively. Reads from putative new species were detected in five samples (62.5%) and represented 75.9% of the clustered reads. It was not possible to include any of these in the phylogenetic genus-classification clades (Cooke *et al*., 2000).

Pyrosequencing of root samples from Vallivana revealed the presence of five phylotypes: *P. psychrophila* (one sample and 18.5% of clustered reads) and other putative new species, including *P*. taxon ballota (one sample and 20.4% of clustered

reads), a new species from clade 9 (one sample and 21.8% of clustered reads, named as sp5 in Table 4.3), and *Phytophthora* sp1 and sp4 (two and one samples representing 17.2% and 22% of clustered reads, respectively), which were also detected in soil samples (Table 4.3).

Blast searches of the unknown phylotypes against NCBI indicated that *Phytophthora* was the genus that most closely matched the sequences. Phylogenetic analyses performed to classify the unknown phylotypes indicated that *Phytophthora* sp1 and *Phytophthora* sp5 grouped with phylogenetic genus-classification clades 1 and 9 (Cooke *et al*., 2000), respectively. *Phytophthora* sp2, sp3, sp4 and *P*. taxon ballota were not grouped in any of the existing clades and neither did they cluster together in a new clade.

The consensus sequences of each of the MOTUs were aligned with all known *Phytophthora* species, new *Phytophthora* phylotypes detected in other metabarcoding studies (Català *et al*., 2014ab, 2015) and other oomycete genera. The alignments were used for the development of specific primers and probes for the detection of the species most frequently recovered in the samples studied: *P*. taxon ballota, *P. psychrophila* and *P. quercina*. The alignments revealed no substantial differences in the ITS1 region between *P. psychrophila* and the closest species in the same clade. Therefore, real-time PCR assays were developed only for *P*. taxon ballota and *P. quercina*.

Detection of *P.* taxon ballota and *P. quercina* using real-time PCR

The specificity of the assays developed was tested using *Phytophthora* isolates from the culture collection listed in Table 4.2, and with the same DNA extractions used for pyrosequencing, in separate real-time PCR reactions. Primers and probes developed for *Phytophthora* taxon ballota were highly specific, yielding a significant increase in

fluorescence (FAM) for most of the environmental samples where *P*. taxon ballota presence was confirmed by amplicon pyrosequencing (Table 4.3). *Phytophthora* taxon ballota was detected in 11 (92%) and 5 (38%) samples from Carrascar de la Font Roja and Vallivana, respectively. Sample VS4, in which *P*. taxon ballota was detected by sequencing, tested negative using the specific PCR, whereas VSR8 tested positive only using the real-time assay (Table 4.3). The correlation between 454-pyrosequencing and the real-time PCR assay results was 92%, considering both negative and positive samples.

In the case of *P. quercina*, primers and probe yielded a significant increase of FAM for the isolates tested. No amplification was detected with any other isolate tested. For the eDNAs, FR4, FR5, FR6, VS1 and VSR1 yielded a significant increase in fluorescence and were considered as positive. The presence of *P. quercina* was confirmed in four out of these five samples by amplicon sequencing (no *P. quercina* sequence was detected by 454-pyrosequencing in sample VSR1). The correlation between 454-pyrosequencing and the real-time PCR assay results was 96%.

Serial dilutions of DNA of *P. quercina* PS-982, and from an eDNA sample positive for *P*. taxon ballota (sample V1 confirmed by pyrosequencing) were used to evaluate the sensitivity of the assays. In the case of *P*. taxon ballota, an estimation of 10 to 40 copies was attributed to the last dilution that yielded a significant increase of the fluorescence, which corresponded to a 1/100 dilution of the sample. The detection limit for *P. quercina* was 0.2 fg μL−1. Standard curves showed a linear correlation between input DNA and cycle threshold (Ct) values with correlation coefficients (r2) of 0.998 (*P. quercina*; Figure 4.1) and 0.983 (*P*. taxon ballota; Figure 4.2).

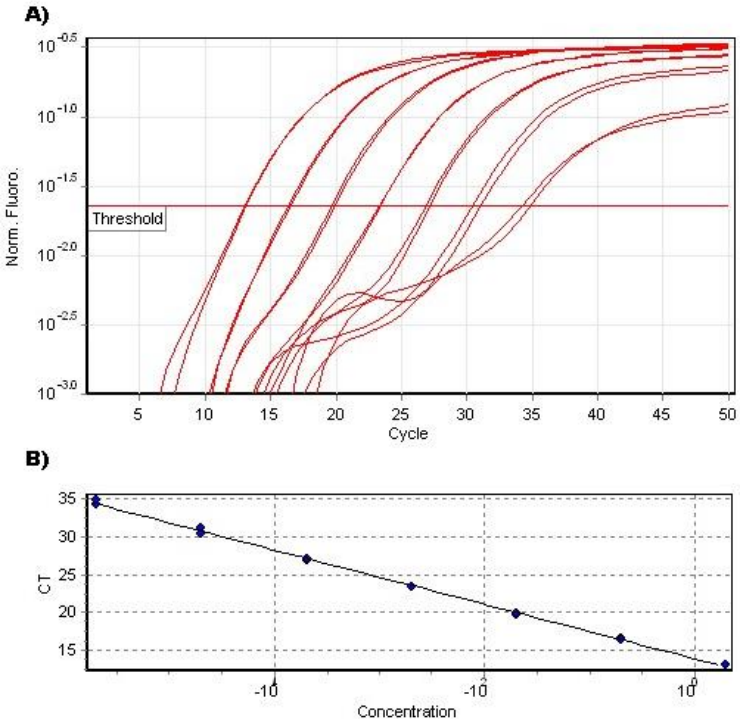

Figure 4.1. Detection limits of real-time PCR assay for *Phytophthora quercina*. Sensitivity was determined by testing a 10-fold dilution series of *P. quercina* DNA (isolate PS-982) ranging from 2 ng µL−1 to 0.2 fg µL−1 (a). Correlation coefficient (r^2) is 0.998 (b).

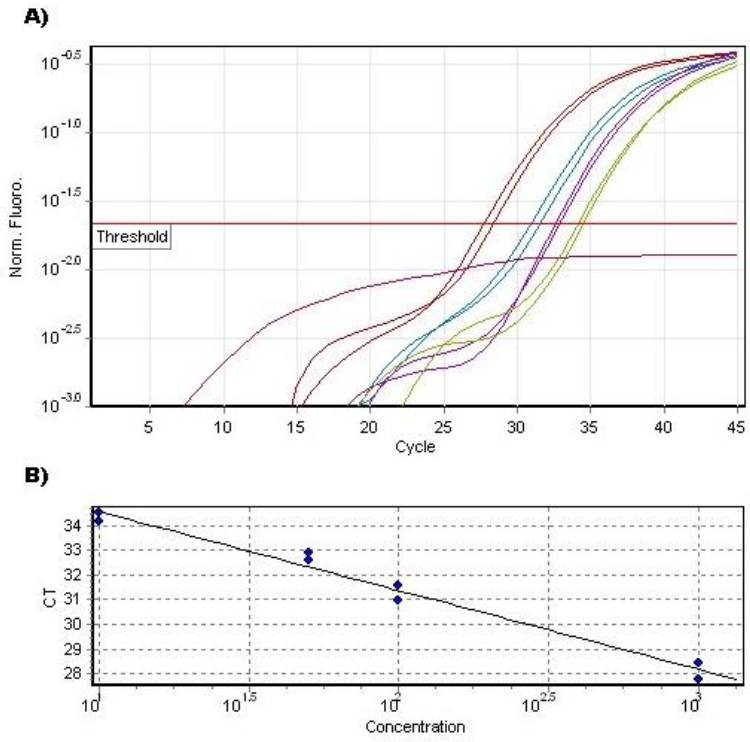

Figure 4.2. Detection limits of real-time PCR assay for *Phytophthora* taxon ballota. Serial dilutions of an environmental positive sample for *P.* taxon ballota (sample V1 confirmed by pyrosequencing) was used to determine assay sensitivity (a). Correlation coefficient (r^2) is 0.983 (b).

Effect of sampling area on *Phytophthora* spp. diversity

Phytophthora spp. diversity was analysed by comparing the mean phylotype frequency obtained between the areas studied. The effect of sampling material (soil or roots) within the Vallivana samples was not significant ($P = 0.2022$), thus these data were pooled for further analysis. However, the effect of sampling area was significant ($P < 0.001$) for *Phytophthora* spp. frequency observed. *Phytophthora* taxon ballota frequency (59.9%) was significantly higher in Carrascar de la Font Roja compared with the other species identified (Table 4.4). The frequency of *P*. taxon ballota was also significantly higher in Carrascar de la Font Roja relative to the frequency observed in Vallivana (27.1%; Table 4.4). In this sampling area the frequency of the unknown *Phytophthora* spp. was higher compared with the others (Table 4.4). Diversity was also evaluated based on Shannon diversity and richness indices. Both indices showed Carrascar de la Font Roja to have the highest *Phytophthora* spp. diversity (Table 4.4).

Table 4.4. Frequency of *Phytophthora* spp. detected and diversity and richness indices for the sampling areas studied.

Sampling area	*P. quercina*	*P. psychrophila*	*Phytophthora* taxon ballota	Other *Phytophthora* spp.	Unknown *Phytophthora* spp.	Shannon Diversity Index	Richness Index
Font Roja	6.6* ab A	20.8 b A	59.9 c A	7.1 ab A	0 a A	0.413	0.02
Vallivana	2.3 a A	14.9 ab A	27.1 ab B	8.4 a A	40.3 b B	0.217	0.01

*Least significant difference (LSD) test: means followed by the same letter do not differ significantly (P<0.05). Small letters are for comparison of means in the same row. Capital letters are for comparison of means in the same column.

Discussion

This is the first study of the diversity of *Phytophthora* spp. in declining oak forests in eastern Spain using the combination of high-throughput sequencing and real-time PCR assays. Two calcareous holm oak forests were selected for *Phytophthora* metabarcoding characterization: (i) an area previously studied using baiting methods for species isolation, and (ii) a second area never sampled before, similar in vegetation but geographically distant. To this aim, massive sequencing was applied to describe the diversity of *Phytophthora* spp. in soil and root samples. Based on the results of metabarcoding, a detection and quantification method based on real-time PCR was developed for the species most frequently sampled in order to confirm the results from pyrosequencing and quantify their presence in the environmental samples.

Survey results in the Natural Park of Carrascar de la Font Roja based on amplicon pyrosequencing revealed that 92% of the samples contained sequences identical to a single phylotype that did not match any *Phytophthora* sequence in public databases. This phylotype was provisionally named as *P*. taxon ballota due to the main *Quercus* vegetation of the areas where it was found. Furthermore, *P*. taxon ballota was recovered at a higher frequency than other phylotypes identified in the area. Interestingly, no cultures of this putative new species were obtained in previous surveys performed in the area using classical baiting methods (Pérez-Sierra *et al*., 2013). In contrast with classical methods, molecular methods for pathogen detection use the presence of nucleic acids of target organisms in any kind of sample, and are not influenced by the culturability of an organism. Molecular detection of non-culturable fungal plant pathogens in environmental samples has been reported earlier (Ioos *et al*., 2007; Zhao *et al*., 2007; Alaei *et al*., 2009).

The pyrosequencing analysis of samples from Carrascar de la Font Roja also revealed a high number of sequences from other phylotypes identified as *P. psychrophila* and *P. quercina*, in 67% and 25% of the samples studied, respectively. These results are consistent with those obtained by Pérez-Sierra *et al*. (2013), who also detected *P. quercina* and *P. psychrophila* using baiting methods in the same area. Additionally, metabarcoding results of the samples collected in Vallivana confirmed the presence of *P. psychrophila* and *P. quercina* in 23% and 8% of samples, respectively. Moreover, the mean frequency of reads identified as *P*. taxon ballota was significantly higher in Carrascar de la Font Roja compared with Vallivana, where the percentage of *Phytophthora* that could not be identified to species level was higher. The number of new phylotypes detected was higher in Vallivana, but diversity indices showed Carrascar de la Font Roja to have the highest *Phytophthora* spp. diversity. Nevertheless, these results should be confirmed through an in-depth sampling of the areas studied.

Based on the high frequency of isolation and the results of pathogenicity tests, *P. psychrophila* and *P. quercina* are considered to be the species responsible for extensive losses of fine roots and lateral small woody roots, and the bark cankers on suberized roots of declining oaks in the stands of the Natural Park of Carrascar de la Font Roja (Pérez-Sierra *et al*., 2013). A significant association of *P. quercina* with oak decline has been found also in stands located in other countries such as Italy, Austria and Turkey (Jung *et al*., 1999; Vettraino *et al*., 2002; Balci & Halmschlager, 2003a, b). However, the role of non-isolated species such as *P*. taxon ballota, and its implication in the oak decline is still unknown. In order to evaluate the involvement of *P*. taxon ballota in oak decline, efforts should concentrate on testing alternative isolation media and performing the sampling and isolation during the wet season.

The high number of reads obtained in samples from both areas studied justified the development of a specific assay to improve the detection of *P.* taxon ballota and *P. quercina* in environmental samples. This methodology will be a valuable tool to assess the presence of these species in other similar areas presenting oak decline. The development of a specific assay for *P. psychrophila* was not possible because no polymorphic region was found in order to distinguish it from *P. ilicis* and *P. pseudosyringae* based on the ITS1 barcoding tool.

The results of the current study showed that real-time PCR can be successfully implemented for the detection and quantification of *P.* taxon ballota and *P. quercina*. The DNA extraction from cultures used as negative controls included most of the *Phytophthora* species reported from Spain, isolated from nurseries and natural ecosystems. The technique was highly reproducible and the correlation with the positive samples found by amplicon pyrosequencing was very high. The species from Carrascar de la Font Roja detected by the real-time PCR assays were the same as detected in the metabarcoding approach. Using the new assay for *P.* taxon ballota in Vallivana revealed its presence in 37.5% of the soil samples tested, and in 40% of the root samples from Quercus. Detection of *P.* taxon ballota and *P. quercina* in both sampling areas was confirmed by amplicon sequencing in over 90% of the samples.

Because most previous studies investigating the composition of the forest *Phytophthora* microbiome have been performed using culture-based methods, information about *Phytophthora* spp. that are difficult to bait is still limited. Recent studies described the application of high-throughput sequencing to investigate the diversity of *Phytophthora* species in environmental samples (Burgess *et al*., 2014; Català *et al*., 2014a,b, 2015). A general finding from these studies is that many sequences in environmental metagenomes have no similarities to known species in

public databases, indicating high abundance of undescribed *Phytophthora* spp. that may be potential tree pathogens. The unexpectedly high percentage of sequences found in this study corresponding to putative new *Phytophthora* spp. revealed that Mediterranean oak environments represent a valuable habitat for species discovery.

In some situations when using real-time PCR assays, healthy material or unknown environmental samples give late exponential curves, or a non-exponential increase of the fluorescence is produced. The establishment of Ct cut-off values is always a critical step in order to distiguish positive or negative samples. Amplicon pyrosequencing provides a productive approach to characterization of samples that would otherwise be considered negative for the presence of *Phytophthora* spp. Moreover, real-time PCR assays depend on specific oligonucleotide primers and probes, which usually do not cover all *Phytophthora* species, and thus do not provide an accurate indication of the diversity and abundance of *Phytophthora* spp. in the microbiome. In this context, pyrosequencing provides a broader spectrum of taxa identification and higher detection sensitivity. As demonstrated in this study, the combination of real-time PCR and amplicon pyrosequencing represents a powerful tool to be applied to the identification, detection and quantification of *Phytophthora* spp. in environmental samples.

Accession numbers

The 25 standard flowgram format (.sff) files from the two sampling areas were combined and deposited in GenBank-SRA under the accession number SRR1772256. Consensus nucleotide sequences of each of the putative new *Phytophthora* phylotypes were deposited in GenBank (accession nos. KP691405 to KP691410).

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Chapter 5

Environmental DNA sampling methods in aquatic environments suitable for *Phytophthora* metabarcoding studies

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Abstract

The genus *Phytophthora* comprises species that are economically important plant pathogens in forestry and agriculture. Rivers and streams play an important role in the spread of *Phytophthora* and represent a hotspot for the potential discovery of new species. However, while species from forests and agricultural systems are well studied, ecology and diversity of *Phytophthora* spp. present in freshwater ecosystems are still unknown. Understanding *Phytophthora* species diversity in aquatic ecosystems is of high importance from an ecological perspective and also to prevent invasions of alien species. In this study, 15 rivers and streams in forests and agricultural areas were sampled. In each river and stream two different sampling methods were used: filtering and baiting with *Rhododendron* leaves and carnation petals. Half of each filter, *Rhododendron* or carnation sample was used for *Phytophthora* isolation, and the other half was subjected to DNA extraction. DNA was extracted from the samples, amplicon libraries were generated using *Phytophthora*-specific primers, and pyrosequenced using GS Junior instrument. Six *Phytophthora* species were identified using isolation techniques in contrast with the 25 *Phytophthora* phylotypes identified using the metabarcoding approach. A total of 8 new *Phytophthora* phylotypes, not represented in public databases, were detected on the 15 waterways sampled. The two sampling methods tested resulted highly specific for *Phytophthora* detection, but pyrosequencing of *Rhododendron* leaves resulted the most specific method (99,2 %), followed by the carnation petals (98,2 %) and filters (95,3 %). Several phylotypes which corresponded with important *Phytophthora* pathogens for forestry and agriculture were detected, including *P. cinnamomi, P. alni* subsp. *alni*, or *P. plurivora*. *P. cinnamomi*. This is the first study that uses genus-specific 454-pyrosequencing to investigate the *Phytophthora*

species diversity in aquatic ecosystems using the combination of different sampling methods.

Introduction

Rivers and streams play an important role in the water molds spread and represent a hotspot for *Phytophthora* biodiversity (Català *et al*., 2015; Eyre & Garbelotto, 2015; Hüberli *et al*., 2013; Oh *et al*., 2013). Presence of free water is a requirement for *Phytophthora* spp. to carry out its life cycle and, in most of the species, allows a rapid dispersal with their flagellate zoospores (Erwin and Ribeiro, 1996). However, compared with species from forests and agricultural systems, ecology and diversity of *Phytophthora* spp. present in freshwater ecosystems is still underestimated (Guha Roy & Grunwald, 2014). Understanding *Phytophthora* species diversity in aquatic ecosystems is interesting from an ecological perspective and also to analyze and prevent invasions of alien species. In this context, management of invasive species requires confidence in the detection methods used to assess expanding distributions (Peterson *et al*., 2014).

Several studies have focused on the isolation of *Phytophthora* species from rivers and streams (Hüberli *et al*., 2013; Oh *et al*., 2013; Loyd *et al*., 2014; Hong *et al*., 2012). As recently reviewed, more than 25 species have been isolated from natural waterways and irrigation reservoirs (Guha Roy & Grunwald, 2014). A high proportion of the species isolated from aquatic ecosystems belong to clade 6, which show a strong association with both forests and riparian ecosystems (Jung *et al*., 2011). Presence and, sometimes, dominance of clade 6 species in water surveys supported the hypothesis that they could live as saprotrophic organisms (Brasier *et al*., 2003), although some species of clade 6 can be opportunistic tree pathogens (Pérez-Sierra *et al*., 2013). Moreover,

some important plant pathogens such as *P. ramorum* (Werres *et al*., 2001; Sutton *et al*., 2009; Eyre and Garbelotto, 2015), *P. cinnamomi* (Kliejunas *et al*., 1976; Oh *et al*., 2013) or *P. multivora* (Hüberli *et al*., 2013) have been isolated from rivers and streams. The number of species recovered from water is continuously increasing with the publication of new species isolated from aquatic environments (Yang *et al*., 2014; Copes *et al*., 2015).

Baiting and filtering are current and world-wide methods used for detection of viable propagules of *Phytophthora* and other Oomycete genera such as *Pythium*, *Phytopythium* or *Halophytophthora* in streams and irrigation reservoirs (Bush *et al*., 2003; Hüberli *et al*., 2013; Parkunan *et al*., 2013; Shrestha *et al*., 2013; Yang & Hong, 2014). Isolation and plating on selective media from baits and filters are the most common techniques in order to study the diversity of *Phytophthora* spp. in aquatic ecosystems. However, isolation techniques using water samples produce a high false negative rate on the isolation of *Phytophthora* species, and especially, those species different from clade 6, which are frequently isolated from freshwater ecosystems (Hüberli *et al*., 2013).

Molecular methods allow the study of the diversity without the need of isolation the target organisms. Recent technological advances have driven a rapid development of DNA-based methods designed to facilitate detection and monitoring of species in aquatic environments, especially those based on massive sequencing technologies. These technologies offer more sensitivity in the description of microbiota as well as cost effectiveness. These methods allow the detection of organisms that would otherwise be missed using traditional isolation methods. The recently developed molecular methods based on High-Throughput Sequencing (HTS) technologies (e.g. 454-Pyrosequencing or Illumina) are profoundly changing our knowledge about microbial ecology in aquatic

ecosystems (Keshri *et al*., 2014; Wang *et al*., 2014; Català *et al*., 2015). Culture-independent studies using metabarcoding approaches provide important insights into the diversity and interactions when combined with metatranscriptomics.

In this study, *Phytophthora* diversity in aquatic ecosystems from the Basque Country (northern Spain) is explored by HTS following the methodology described in Català *et al*. (2015). In order to increase the robustness on the diversity assessment and to compare species richness, two sampling areas: forestry areas in the mountain, and agricultural areas in the valley are studied and two different sampling methods are tested: filtering and baiting with *Rhododendron* leaves and with carnation petals.

Material and Methods

Environmental samples and environmental DNA (eDNA) extraction

A total of 15 rivers and streams were sampled in October 2013, comprising forestry areas in the mountain, and agricultural areas in the valley (Table 5.1). In each river and stream two different sampling methods were used: filtering (PVA), baiting with *Rhododendron* leaves (PVARH) and with carnation petals (PVACL). A total of 10 liters of water were filtered in-situ using three filters per sample as described by Català *et al.* (2015). Half of each filter, *Rhododendron* or carnation sample was used for *Phytophthora* isolation, and the other half was stored frozen in tubes for DNA extraction. Baits (*Rhododendron* leaves and carnation petals) were placed in each of the waterways floating in mesh bags, collected after 6 days and placed in a cool-box.

Table 5.1. 454 reads generated in each of the sample included in the study, coordinates and main vegetation present in the river or stream.

Filter code	454 reads	*Rhododendron* code	454 reads	*Dianthus* code	454 reads	X	Y	Vegetation
PVA1	972	PVARH1	1537	PVACL1	907	526397	4764618	*Chamaecyparis*
PVA3	1681	PVARH3	1262	PVACL3	899	526475	4765410	*Alnus, Chamaecyparis*
PVA4	1030	PVARH4	1176	PVACL4	1550	526897	4765828	*Fagus*
PVA5	1085	PVARH5	880	PVACL5	1297	526358	4766596	*Alnus*
PVA6	1484	PVARH6	1286	PVACL6	1229	525049	4765385	*Chamaecyparis, Fagus*
PVA7	948	PVARH7	1247	PVACL7	1314	525017	4764518	*Fagus*
PVA8	1302	PVARH8	1537	PVACL8	1052	523535	4764251	*Fagus*
PVA9	1495	PVARH9	1072	PVACL9	1046	528276	4770659	*Fagus*
PVA22	2033	PVARH22	1350	PVACL22	1950	514371	4798328	Grasslands, *Alnus, Fraxinus, Salix*
PVA23	2697	PVARH23	1567	PVACL23	1759	514595	4796777	Grasslands, *Alnus, Fraxinus, Salix*
PVA24	1692	PVARH24	899	PVACL24	1446	513953	4795930	Grasslands, *Alnus, Fraxinus, Salix*
PVA25	949	PVARH25	1070	PVACL25	1729	511608	4803175	*Eucalyptus*
PVA26	1516	PVARH26	466	PVACL26	1751	507580	4807366	*Eucalyptus*
PVA27	1335	PVARH27	1762	PVACL27	1164	508478	4794215	Grasslands
PVA28	2401	PVARH28	605	PVACL28	1835	510242	4792993	*Quercus*

Filters, *Rhododendron* leaves and carnation petals from each river sampled were individually frozen with liquid nitrogen and crushed into a fine powder using a mortar and a pestle. DNA was extracted by triplicate from 50 mg of the disrupted leaves and carnation petals samples using MoBio PowerPlant DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. In the case of filter samples, DNA was extracted using MoBio PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. For all samples, final elutions were performed in 100 µL of TE buffer.

Isolation and identification of *Phytophthora* spp. from filters and baits

Half of each filter was baited with Granny Smith apples that were incubated at 25 ^{0}C on the laboratory bench. Once active growing lesions appeared on the apple, lesions were plated onto *Phytophthora* semi-selective medium P_5ARP (Erwin & Ribeiro, 1996) and on PARBPH selective culture media (Jeffers & Martin, 1986) and incubated at 25 ^{0}C in the darkness. Hyphal tips from growing colonies were transferred to PDA plates to obtain pure cultures.

Rhododendron leaves and carnation petals were recovered from each stream after 6 days. Isolations from *Rhododendron* leaves and carnation petals were carried out by plating necrotic pieces of infected tissue, after washing them in distilled water, onto PARBPH and P_5ARP mediums. Plates were incubated at 25 ^{0}C in the darkness and were checked daily for the occurrence of *Phytophthora* hyphae. Hyphal tips were transferred onto PDA to obtain pure cultures.

Pure cultures obtained from each sampling method (filtering, baiting with *Rhododendron* leaves and baiting with carnation petals) and from each site (forest and

agricultural valley) were grouped based on colony morphology on PDA. Colonies different from *Phytophthora* and *Pythium* morphologies were discarded.

Genomic DNA was extracted from pure cultures of *Phytophthora* and *Pythium* using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, USA) following the manufacturer's instructions. Internal Transcribed Spacers (ITS) from each of the isolates were amplified with primer pairs ITS6 (Cooke *et al*., 2000) and ITS4 (White *et al*., 1990), and identified using blast searches against NCBI database and *Phytophthora* Database.

Quality control of eDNA (environmental DNA), amplicon library generation and 454-pyrosequencing

Each of the genomic eDNA was diluted 10 (1/10) and 100 (1/100) times and amplified separately using a SYBR green real-time PCR assay with the *Phytophthora*-specific primers 18Ph2F and 5.8S-1R (Scibetta *et al*., 2012). Genomic eDNA or its dilution was selected according to the absence or presence of inhibition activity, respectively (Bunce *et al*., 2012). Cycle threshold values (Ct) were obtained for each sample to determine the adequate number of cycles for the first PCR round in the nested PCR.

Amplicon libraries were generated using a nested PCR approach as described in Català *et al.* (2015) with the modification in the primer design introduced in Català *et al.* (2017). In the first PCR round, 2 µl of genomic eDNA were used as template, and 2 µl of PCR products were used for the second PCR round. The PCR conditions were: an initial denaturation of 95 °C for 2 min, 95 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 7 min. Number of cycles for the first PCR round was determined according to real-time PCR assay results. For the second PCR round, 25 cycles were used in all cases.

The amplicons were purified using the Agencourt AMPure XP Bead PCR Purification protocol (Beckman Coulter Genomics, MA, USA) and pooled at equal concentrations. The emulsion PCR (emPCR) sequenced in a GS Junior 454 system (Roche 454 Life Sciences, Branford, CT, USA) by the Sequencing and Genotyping Service from the University of Valencia (Burjassot, Spain).

Trimming and Molecular Operational Taxonomic Unit (MOTU) clustering

Sequences were trimmed based on quality scores in Lucy software (Chou & Holmes, 2001) using default parameters. Adaptors and primers were removed using a Python script. Resultant FASTA file from each library was clustered with a length coverage threshold of 90% and a score coverage threshold of 99% (-L 0.9 –S 99) using blastclust software (Altschul *et al.* 1997). Consensus sequence of each MOTU was identified using the BLAST tool in the GenBank database (Altschul *et al.* 1997) and a custom-curated database. Consensus sequences were subjected to phylogenetic analyses to confirm blast searches.

Statistical analyses

The counts of the number of reads from each phylotype were converted into frequencies relative to the total number of clustered reads generated within each sample. Frequency of the total number of phylotypes identified in each sample and frequency of the non-identified *Phytophthora* spp. were also calculated. Nested analysis of variance was performed with the data using general linear model (GLM) in SAS version 9.0 (SAS Institute, Cary, NC) to estimate the effect of sampling area as main factor (forestry areas in the mountain and agricultural areas in the valley) and sampling methods nested within the main factor (filtering, baiting with *Rhododendron* leaves and baiting with

carnation petals) on the frequencies. Mean values were compared using the Fischer´s least significant difference (LSD) procedure at P=0.05.

β-diversity statistics were carried out on the most frequent *Phytophthora* species identified using the Phyloseq package (McMurdie & Holmes, 2013) in R version 3.2.2 (R Development Core Team 2014). To visualize the similarity in species composition between the samples, a multidimensional scaling (MDS) was performed of Bray–Curtis (relative abundance) indices (Oksanen, 2011). Multidimensional scaling was performed using the same package in R. Multivariate Analysis of Variance using distance matrices was performed with the function ADONIS (Dixon, 2003) in the Vegan package in R to partition the variation explained by the sampling area and sampling method on species composition.

Results

Identification of *Phytophthora* species using isolation from filters and baits

Six *Phytophthora* species were identified on filter samples, in contrast with four species isolated from *Rhododendron* (*P. chlamydospora, P. lacustris, P. gonapodyides* and *P. plurivora*) and three species detected on carnation petals (only clade 6 species).: *P. citrophthora* and *P. plurivora* were grouped in clade 2); *P. chlamydospora* and *P. lacustris* and *P. gonapodyides* in clade 6, and *P. cryptogea* in clade 8 (Figure 5.1). Most of the isolates obtained corresponded with aquatic *Phytophthora* species included in clade 6. The most frequently recovered species was *P. lacustris*, which was isolated from 69 % of the samples and from all waterways, followed by *P. plurivora* (18 % of samples), *P. chlamydospora* (11 % of samples), *P. gonapodyides* (9 % of samples), *P. citrophthora* (4 % of samples) and *P. cryptogea* (2 % of samples).

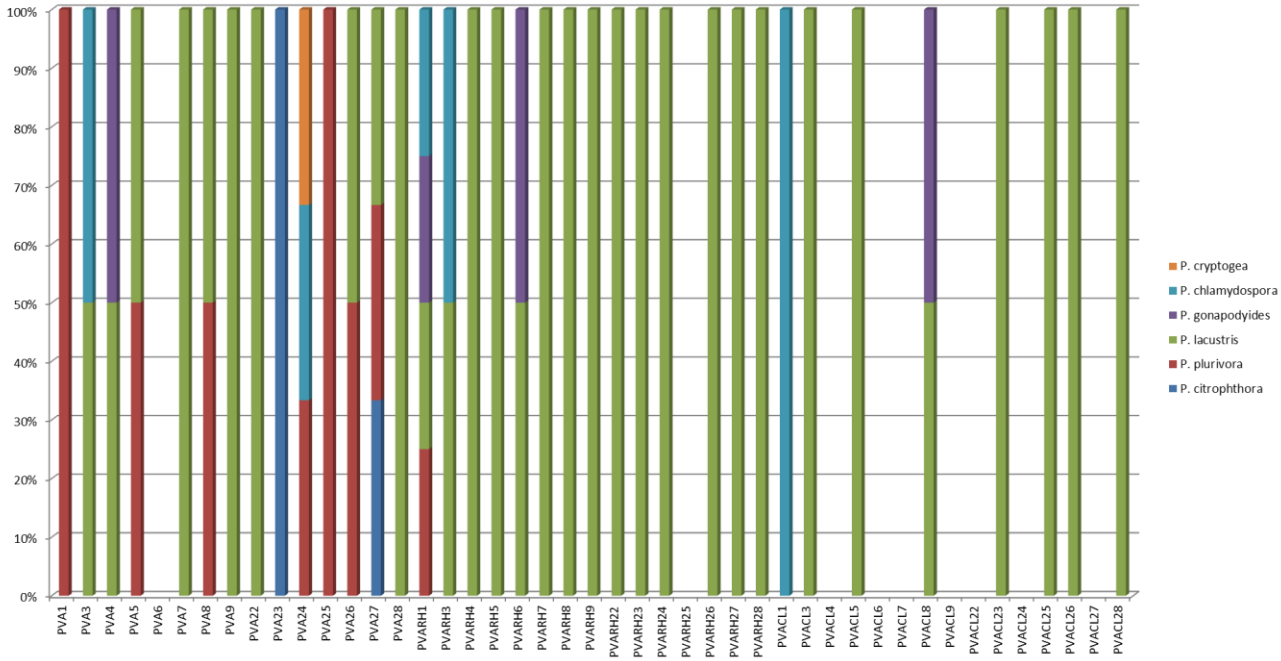

Figure 5.1. Proportion of *Phytophthora* species detected using isolation methods.

Quality control of DNA

DNA extractions from filters and *Rhododendron* leaves showed inhibition activity when using non-diluted DNA. In these samples, a 1/10 dilution of DNA was found as the optimal concentration for the first round of PCR. In the case of carnation petals, no inhibition activity was observed, thus 2 µl of genomic DNA were used for the first PCR round.

The amount of *Phytophthora* DNA, determined by using the *Phytophthora*-specific primers in the real-time PCR assay, varied between the different eDNA sources, but not between samples. Cycle threshold values (Ct), and subsequently, the number of cycles for the first PCR round was: 25 for filter samples, 20 for *Rhododendron* leaves and 12 for carnation petals.

Identification of *Phytophthora* species using 454 pyrosequencing

The specificity of the sampling for *Phytophthora* detection, in percentage of reads, was: 95.3 % for filter samples, 99.2 % for *Rhododendron* leaves and 98.2 % for carnation petals. The diversity of non-*Phytophthora* sequences was higher in the filter samples than in the baits, representing seven Oomycete genera (*Plasmopara*, *Pythium*, *Peronospora*, *Phytopythium*, *Hyaloperonospora*, *Pseudoperonospora* and *Halophytophthora*) in comparison with the two genera found in *Rhododendron* leaves samples (*Peronospora* and *Halophytophthora*) and the four genera detected on carnation petals (*Pythium*, *Phytopythium*, *Peronospora* and *Halophytophthora*). Taking into account the two sampling methods together, the specificity of the assay for *Phytophthora* detection was 97.4 %.

In total, 25 phylotypes were identified using the metabarcoding approach (Figure 5.2), which corresponded with 17 known *Phytophthora* species and 8 new *Phytophthora* phylotypes. The most frequently detected *Phytophthora* species, representing 86.3 % of the total *Phytophthora* reads, were included in clade 6: *P. lacustris* (62.8 % of reads), *P. bilorbang* (8.9 % of reads), *P. gonapodyides* (8.6 % of reads) and *P. chlamydospora* (5.9 % of reads). Besides clade 6, the most frequently detected species was *P. gallica* from clade 10 (identified in 23 samples), followed by *P. plurivora* from clade 2 (22 samples) and *P. cinnamomi* from clade 8 (12 samples). Also, as shown in Figure 5.2, other *Phytophthora* species detected were: *P. cactorum* from clade 1 (3 samples), *P. citrophthora* from clade 2 (5 samples), *P. ilicis*/*pseudosyringae* from clade 3 (6 samples, not possible to distinguish based on ITS1 data), *P. thermophila* (2 samples)

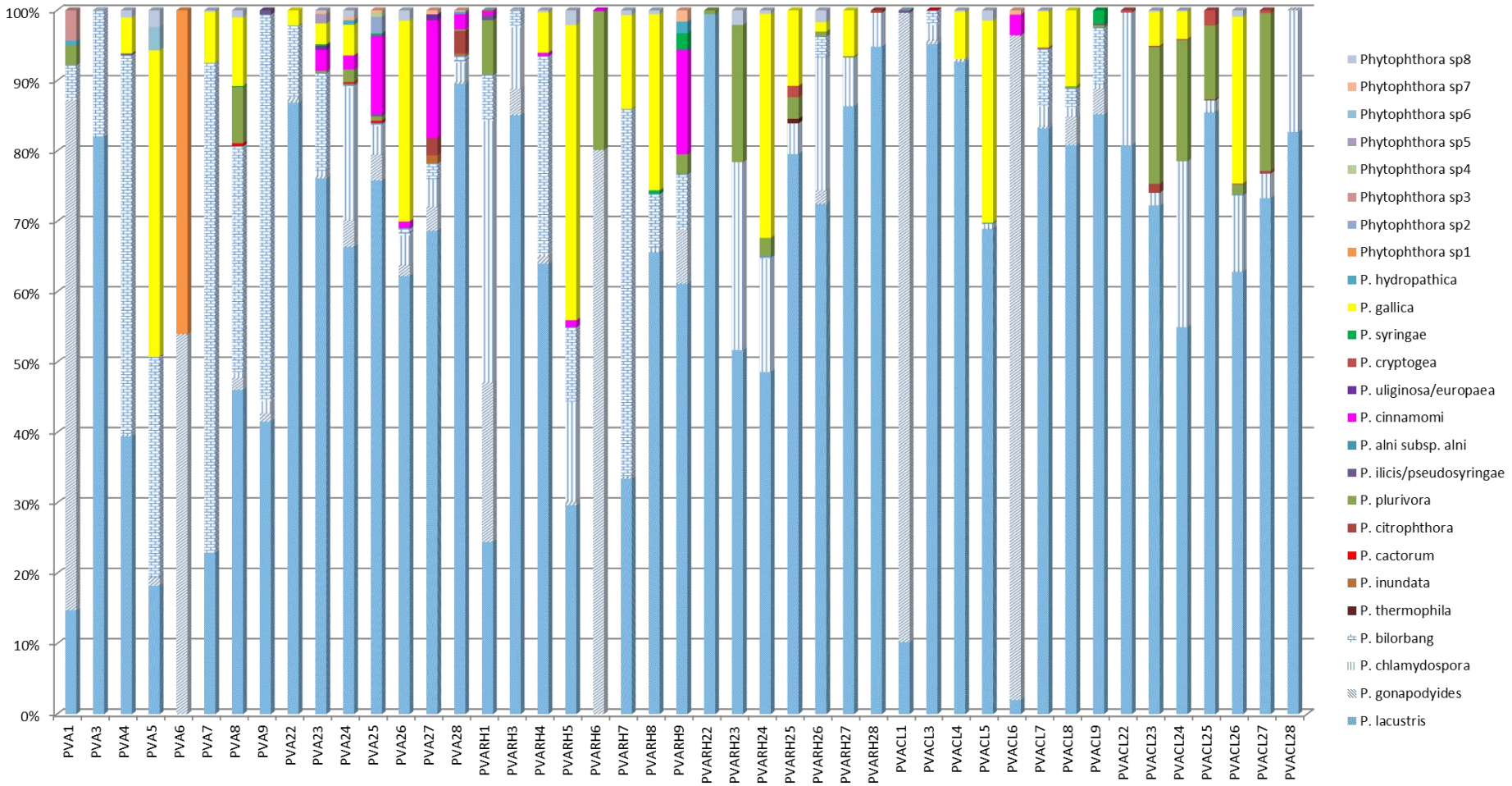

Figure 5.2. Proportion of *Phytophthora* species detected by amplicon pyrosequencing. Dominant clade 6 species are represented with blue colors.

and *P. inundata* (3 samples) from clade 6, *P. alni* subsp. *alni* (1 sample) and *P. uliginosa*/*europaea* (2 samples, not possible to distinguish based on ITS1 data) from clade 7, *P. cryptogea* (9 samples) and *P. syringae* (6 samples) from clade 8, and *P. hydropathica* from clade 10 (4 samples).

A total of 8 new *Phytophthora* phylotypes, not represented in public databases, were detected on the 15 waterways sampled using the three different sampling methods. Three phylotypes (*Phytophthora* sp1-3) were not able to be classified in any of the known phylogenetic clades (Cooke *et al*., 2000). The other five phylotypes detected were included in clades 3 (*Phytophthora* sp4), clade 4 (*Phytophthora* sp5), clade 8 (*Phytophthora* sp6), clade 9 (*Phytophthora* sp7) and clade 10 (*Phytophthora* sp8).

Effect of sampling area and sampling method on *Phytophthora* spp. diversity

Phytophthora spp. diversity observed was analyzed by comparing mean phylotype frequency obtained among the sampling areas and sampling methods studied. A significant effect of the sampling area (forestry and agricultural) was observed for the dominant species detected in clade 6. *Phytophthora lacustris* frequency (74.8 %) was significantly higher in agricultural areas than in the forests. However, *P. bilorbang* and *P. gonapodyides* frequencies (16.7 % and 18.3 %, respectively) were significantly higher in the forests that in the agricultural areas. *P. cinnamomi* was detected in both sampling areas: in forestry areas was detected in five *Rhododendron* samples and one carnation sample, but not in filter samples. In agricultural areas, *P. cinnamomi* was detected in six filter samples, but not in bait samples. Other important plant pathogens like *P. alni* subsp. *alni*, was detected on one carnation sample (2 reads) in a stream from the forestry area (baits were placed under *Alnus glutinosa*).

Regarding to the sampling area, five of the eight new phylotypes (Figure 5.2) were detected on forestry areas (*Phytophthora* sp1, *Phytophthora* sp3 and *Phytophthora* sp6 were exclusive from forestry areas), and five phylotypes (Figure 5.2) were detected on agricultural areas (*Phytophthora* sp2, *Phytophthora* sp4 and *Phytophthora* sp5 were exclusive from agricultural areas). *Phytophthora* sp7 and sp8 were detected in both areas (Figure 5.2).

All of the new phylotypes were detected by filtering but only two of them (*Phytophthora* sp7 and 8) which were detected also on *Rhododendron* leaves and carnation petals (Figure 5.2). The frequency of new *Phytophthora* phylotypes was higher in filter samples (4.4 %) than in the baits (0.5 % in *Rhododendron* leaves and 0.2 % in carnation petals).

Factors 'sampling area' and 'sampling method' had a significant effect in β-diversity (adonis: R^2 = 21% and R^2 = 12%, respectively, $P < 0.005$). Multidimensional scaling (MDS) analysis was performed to look for clustering of samples from different sampling area or sampling method. In the Bray-Curtis distance plot, the first coordinate explained 57.2% and the second coordinate explained 26.2% of the variation (Figure 5.3). The samples were clustered in three groups (Figure 5.3): group 1 (up left side) included all the samples from agricultural areas and most of the samples from forestry areas obtained from carnation petals; group 2 (down left side) included most of the samples from forestry areas obtained from *Rhododendron* leaves and filters; and group 3 (up right side) included bait (*Rhododendron* leaves and carnation petals) and filter samples from two forest locations (PV1 and PV25).

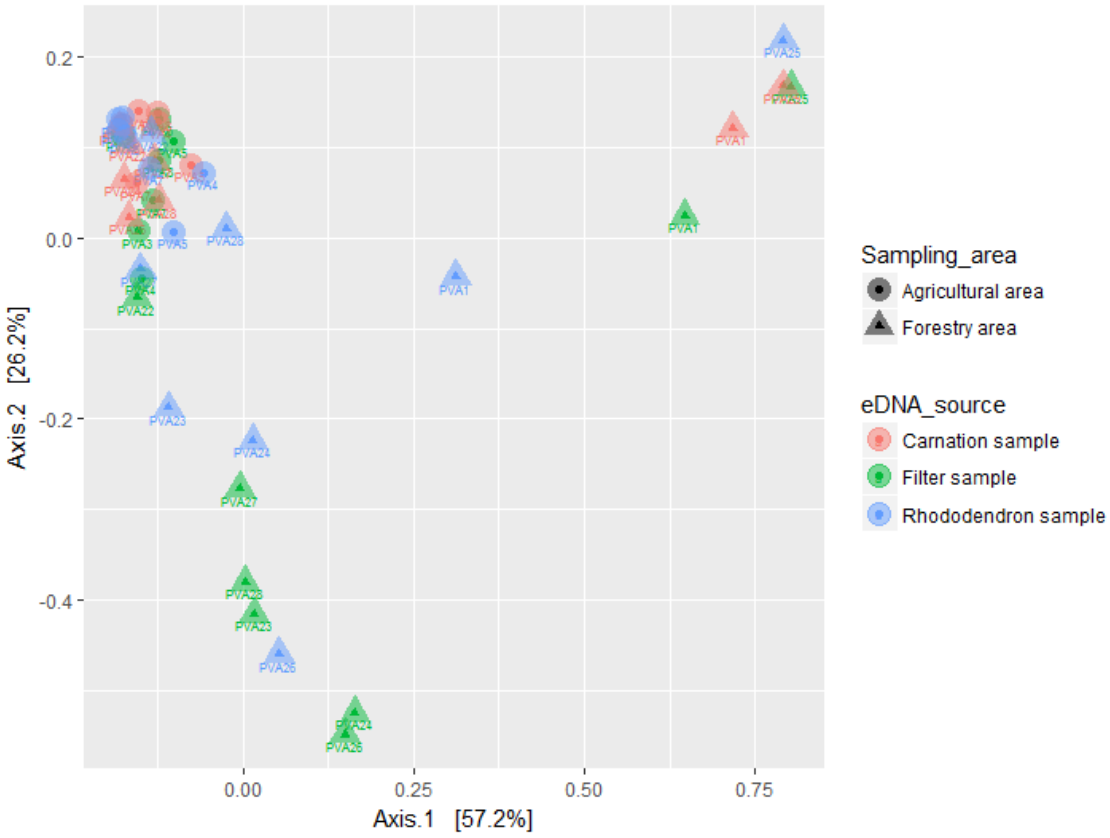

Figure 5.3. Multidimensional scaling analysis (MDS) showing similarity in community composition based on species relative abundance (Bray–Curtis distance). Three clusters were identified: group 1 (up left side) included all the samples from agricultural areas and most of the samples from forestry areas obtained from carnation baits; group 2 (down left side) included most of the samples from forestry areas obtained from Rhododendron bait and filters; and group 3 (up right side) included all bait and filter samples from two forest locations (PV1 and PV25) (Table 5.1).

Discussion

In this study genus-specific 454-pyrosequencing is applied to investigate the *Phytophthora* species diversity in aquatic ecosystems using the combination of different sampling methods. A total of 15 rivers and streams were sampled using filtering and *in-situ* baiting (*Rhododendron* leaves and carnation petals). Forty-five amplicon libraries were generated from the baits and filters, and clustering revealed 25 *Phytophthora*

phylotypes in the three eDNA sources in contrast with the six species isolated by culturing.

The two sampling methods tested resulted highly specific for *Phytophthora* detection, but pyrosequencing of *Rhododendron* leaves resulted the most specific method (99,2 %), followed by the carnation petals (98,2 %) and filters (95,3 %). As reported previously in other studies where primers were adapted for High-Throughput Sequencing and used for library generation from filter samples (Català *et al*., 2015), the specificity for *Phytophthora* detection in water samples resulted very high (96.92 %). The specificity of the assay is mainly conducted by the specificity of the primers used for library generation (Scibetta *et al*., 2012), but, as shown in the results, a small percentage of non-specific sequence recovery could be explained by the eDNA source. Sampling by filtering allowed the recovery of everything between 5-100 µm in size which was flowing in the water, with no selective process apart from size. This could be the reason to explain that filters were the sampling method which detected more *Phytophthora* diversityIn contrast, the plant material selected for this study are well-known baits for their ability to attract *Phytophthora* zoospores and are reported in a high number of studies, especially the *Rhododendron* leaves (Erwin & Ribeiro, 1996; Brasier *et al*., 2004; Bush *et al*., 2003; Themann *et al*., 2002; Pettitt *et al*., 2002; Brasier *et al*., 2010; Vettraino *et al*., 2011). *Rhododendron* leaves were more selective than filters for *Phytophthora* detection. Hüberli *et al.* (2013) used a wide range of plant baits in their study, including *Banksia attenuata*, *Pittosporum undulatum*, *Hakea laurina*, *Quercus robur*, and germinated lupin seedlings (*Lupinus angustifolius*). Multidimensional scaling analysis grouped the lower values of *Phytophthora* species diversity observed in agricultural soils with those obtained in samples from carnation

baits in forestry areas. These results demonstrate that the type of bait used can be a limiting factor when searching for species diversity.

Similar to the results obtained in this study, the plant bait used influenced the diversity of *Phytophthora* species isolated and no single bait recovered all the *Phytophthora* species from the study (Hüberli *et al*., 2013). In contrast, metabarcoding showed that many species of *Phytophthora* were present in every single sample from all sampling locations. Hüberli *et al.* (2013) also found that the durability of the bait was an important factor for isolations. At the moment of collection of the baits used in our study, a high degradation was observed in all the carnation petals, in contrast with the apparently good preservation state of the *Rhododendron* leaves. Although similar results from the metabarcoding approach were found between *Rhododendron* leaves and carnations, the preservation state was found to be an important factor for isolation. The isolation of *Phytophthora* from carnations samples was lower than using *Rhododendron*, and only eight carnation samples were positive for *Phytophthora* in comparison with the fourteen using *Rhododendron* leaves. Both baits were collected after six days for convenience during the sampling although the ideal timing for carnation petals is 48 hours.

Several phylotypes which corresponded with important *Phytophthora* pathogens for forestry and agriculture were detected, including *P. cinnamomi*, *P. alni* subsp. *alni*, or *P. plurivora*. The high detection success for *P. cinnamomi* in streams from the Basque Country contrasted to the complete absence of this pathogen in the study conducted by Català *et al.* (2015) in the closest geographical areas of Irati and Villanua. Although *P. cinnamomi* was found to be widely distributed species in the Basque Country as seen in the metabarcoding results, no isolates were obtained from filters and baits. Similar results were found by Hüberli *et al.* (2013), who conducted the survey in areas from the

southwest of Western Australia, known to be infested by *P. cinnamomi*, and no isolates were obtained. As hypothesized by Hüberli *et al.* (2013), *Phytophthora* species from clade 6 could have a competitive advantage by producing more zoospores than *P. cinnamomi* in aquatic ecosystems, which reduce the success of its isolation.

The hidden *Phytophthora* diversity in aquatic ecosystems showed by Català *et al.* (2015) using 454-Pyrosequencing was congruent with the results presented in this study. In the previous studies conducted on Spanish Pyrenees, only filters were used as sampling method, where a total of 14 new *Phytophthora* phylotypes were detected on 15 rivers and streams. In spite of baits were placed in the streams during six days in comparison with the water for filtering, which was collected in a few seconds, the diversity of new species was lower in the baits. High number of studies could miss potential new species, which could be not possible to isolate using the classical or "universal" baits like *Rhododendron* or carnations. These results emphasize the importance of including different plant baits in combination with non-selective methods like filtering in *Phytophthora* diversity studies, whether classical isolation studies or based on molecular methods.

Water from rivers and streams is used in some regions to irrigate the crops and could represent an important inoculum source and develop plant diseases. Understanding when *Phytophthora* is present in water sources is valuable information to minimize the threat that poses to the crops and to increase the productivity. In this context, high throughput sequencing technologies proved to be the revolution in culture-independent microbial studies. Metabarcoding approaches using the combination of baits and filters from water represents a useful approach to describe communities and could be used as a monitoring tool for pathogenic *Phytophthora* species in water ecosystems. The fast and

reliable method implemented in this study gives useful insights to understand *Phytophthora* ecology and diversity.

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Chapter 6

General discussion

Phytophthora is one of the most important and aggressive plant pathogenic genera in agriculture and forestry. Early detection and identification of its pathways of infection and spread are of high importance to minimize the threat they pose to natural ecosystems, that means the implementation of rapid, accurate and reliable methods for pathogen detection. For that reason, the main objective of the present thesis was the development, application and validation of molecular methods based on Next Generation Sequencing and Real-Time PCR to evaluate *Phytophthora* diversity in soil, roots and water samples from forests and natural ecosystems.

Phytophthora-specific primers described by Scibetta *et al*. (2012) were adapted for 454-Pyrosequencing. A nested approach was used in order to yield suitable PCR products. Sequencing run was performed following guidelines for Short Length Libraries which reduced the amount of Amp Primer A in the Live Amp Mix from 80 to 20 μL. The use of this protocol increased 100 fold the throughput.

Clustering at 100% was the best criteria to separate most of the species in the reference database used. Other studies (Vettraino *et al*., 2012) adopted a barcoding threshold of 98% to assign most reads. However, in the current study we included a control species mixture and a 99% threshold was required to discriminate between closely related species resulting in the separation of 20% more species. Therefore, for all the analysis a 99% of score coverage threshold was stablished as most suitable threshold value for MOTU clustering.

The *Phytophtora*-specific primers used in the current study showed high specificity, with 97.9% specificity to *Phytophthora*. Pyrosequencing of soil samples revealed lower *Phytophthora* diversity than from water samples, where only thirteen species were detected in five different ecosystems. In contrast, pyrosequencing of water

eDNA from rivers and streams revealed a hidden and unexpectedly high *Phytophthora* species diversity. A total of 35 *Phytophthora* species were detected, 13 of which could represent potentially novel species. In contrast, other researchers (Vannini *et al*., 2013) applied ITS6/ITS7 primers for pyrosequencing without using a nested approach, revealing the presence of 15 *Phytophthora* species in soils from chestnut forests in Italy. Their assay was very specific for *Phytophthora* species detection, where 78.8% of sequences (9,167 of 11,637 reads) matched *Phytophthora*.

Pyrosequencing revealed as a good strategy to detect and identify *Phytophthora* species in environmental samples, and a powerful tool for the detection of potentially new species of *Phytophthora*. After the technique was developed and applied in the Chapter 3, following Chapters were based in the same methodology in combination with other molecular techniques (qPCR), or sampling strategies.

In Chaper 4, two calcareous holm oak forests were selected for *Phytophthora* metabarcoding characterization in eastern Spain: (i) an area previously studied using baiting methods for species isolation, and (ii) a second area never sampled before, similar in vegetation but geographically distant. To this aim, massive sequencing was applied to describe the diversity of *Phytophthora* spp. in soil and root samples. Based on the results of metabarcoding, a detection and quantification method based on real-time PCR was developed for the species most frequently sampled in order to confirm the results from pyrosequencing and quantify their presence in the environmental samples.

Pyrosequencing of soil samples from Carrascar de la Font Roja (Alicante, Spain) revealed the presence of six phylotypes that corresponded to five known *Phytophthora* spp. and a phylotype that did not match any sequence in public databases, which was

detected in 92% of the samples and provisionally named as *Phytophthora* taxon ballota. Pyrosequencing of soil samples from Vallivana (Castellón, Spain) resulted in the detection of nine phylotypes that corresponded with four known *Phytophthora* spp. and five others that did not match with any sequence in public databases. *Phytophthora* taxon ballota was also detected in 50% of the soil samples. In order to confirm that this putative new species was related to the rhizosphere, and not as an organism living in the soil, analysis of root samples was also included. Pyrosequencing of root samples from Vallivana revealed the presence of five phylotypes, also including *P*. taxon ballota.

The high number of reads obtained in samples from both areas studied justified the development of a specific assay to improve the detection of *P*. taxon ballota and *P. quercina* in environmental samples. This methodology will be a valuable tool to assess the presence of these species in other similar areas presenting oak decline. The results of the current study showed that real-time PCR can be successfully implemented for the detection and quantification of P. taxon ballota and *P. quercina*. The technique was highly reproducible and the correlation with the positive samples found by amplicon pyrosequencing was very high. The species from Carrascar de la Font Roja detected by the real-time PCR assays were the same as detected in the metabarcoding approach. Using the new assay for *P*. taxon ballota in Vallivana revealed its presence in 37.5% of the soil samples tested, and in 40% of the root samples from *Quercus*. Detection of *P*. taxon ballota and *P. quercina* in both sampling areas was confirmed by amplicon sequencing in over 90% of the samples. The combination of real-time PCR and amplicon pyrosequencing represented a powerful tool to be applied to the identification, detection and quantification of *Phytophthora* spp. in environmental samples.

In some situations when using real-time PCR assays, healthy material or unknown environmental samples give late exponential curves, or a non-exponential

increase of the fluorescence is produced. The establishment of Ct cut-off values is always a critical step in order to distiguish positive or negative samples. Amplicon pyrosequencing provides a productive approach to characterization of samples that would otherwise be considered negative for the presence of *Phytophthora* spp. Moreover, real-time PCR assays depend on specific oligonucleotide primers and probes, which usually do not cover all *Phytophthora* species, and thus do not provide an accurate indication of the diversity and abundance of *Phytophthora* spp. in the microbiome. In this context, pyrosequencing provides a broader spectrum of taxa identification and higher detection sensitivity. As demonstrated in this study, the combination of real-time PCR and amplicon pyrosequencing represented a powerful tool to be applied to the identification, detection and quantification of *Phytophthora* spp. in environmental samples.

The number of species recovered from water is continuously increasing with the publication of new species isolated from aquatic environments. As revealed in Chapter 3, aquatic ecosystems revealed an unexpectedly high *Phytophthora* species diversity in comparison with soils. In this case, filtering was the single method used for sampling. The DNA recovered from the filters is probably composed of DNA originated from living cells (mainly zoospores) and extracellular DNA originated from natural cell death in water. For this reason, in Chapter 5 alternative sampling methods were tested based in the attraction of zoospores by determinate baits, making sure that DNA recovered from these baits is originated from living cells.

Chapter 5 represented the first study that used genus-specific 454-pyrosequencing to investigate the *Phytophthora* species diversity in aquatic ecosystems using the combination of different sampling methods. A total of 15 rivers and streams were sampled using filtering and *in-situ* baiting (*Rhododendron* leaves and carnation

petals). Forty-five amplicon libraries were generated from the baits and filters, and clustering revealed 25 *Phytophthora* phylotypes in the three eDNA sources. In parallel, isolations on selective media were carried out revealing the presence of only six *Phytophthora* species.

The two sampling methods tested resulted highly specific for *Phytophthora* detection, but pyrosequencing of *Rhododendron* leaves resulted the most specific method (99,2 %), followed by the carnation petals (98,2 %) and filters (95,3 %). The specificity of the assay was mainly conducted by the specificity of the primers used for library generation (Scibetta *et al*., 2012), but a small percentage of non-specific sequence recovery could be explained by the eDNA source. Sampling by filtering allowed the recovery of everything between 5-100 µm in size which was flowing in the water, with no selective process apart from size. This could be the reason to explain that filters were the sampling method which detected more *Phytophthora* diversity.

Detection of *Phytophthora* phylotypes from clade 6 represented approximately 86% of *Phytophthora* reads. As hypothesized by Hüberli *et al*. (2013), *Phytophthora* species from clade 6 could have a competitive advantage by producing more zoospores than *P. cinnamomi* in aquatic ecosystems, which reduce the success of its isolation. In spite of baits were placed in the streams during six days in comparison with the water for filtering, which was collected in a few seconds, the diversity of new species was lower in the baits. High number of studies could miss potential new species, which could be not possible to isolate using the classical or "universal" baits like *Rhododendron* or carnations. These results emphasize the importance of including different plant baits in combination with non-selective methods like filtering in *Phytophthora* diversity studies, whether classical isolation studies or based on molecular methods. Understanding when *Phytophthora* is present in water sources is

valuable information to minimize the threat that poses to the crops and to increase the productivity. Metabarcoding approaches using the combination of baits and filters from water represents a useful approach to describe communities and could be used as a monitoring tool for pathogenic *Phytophthora* species in water ecosystems.The methods developed and validated in the present study based on Next Generation Sequencing and Real-Time PCR represent valuable tools to study *Phytophthora* species diversity in natural ecosystems that can also be applied to other environments like nurseries. The fast, cost-effective and reliable methods implemented in this thesis gives useful insights to understand *Phytophthora* ecology, diversity and pathways.

References

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Chapter 7

Conclusions

1. **HTS (High Throughput Sequencing) was applied to investigate the presence of *Phytophthora* in different plant communities in natural forests, plantations and aquatic environments from Spain.**
2. **A 99% of score coverage threshold was stablished as most suitable threshold value for MOTU clustering in the genus *Phytophthora*.**
3. **The *Phytophthora*-specific primers used and adapted for pyrosequencing showed high specificity and can be used to describe *Phytophthora* species communities' in environmental samples.**
4. **Pyrosequencing of soil samples revealed lower *Phytophthora* diversity than from water samples.**
5. **The pyrosequencing analysis of samples from Holm Oak forests revealed the presence of a new *Phytophthora* phylotype, named provisionally *Phytophthora* taxon ballota, in most of the samples.**
6. **Metabarcoding results confirmed the presence of *P. psychrophila* and *P. quercina,* as shown before by other authors.**
7. **The development and application of a real-time PCR assay based on the pyrosequencing results for *P.* taxon ballota and *P. quercina* was highly reproducible and the correlation with the positive samples found by amplicon pyrosequencing was very high.**
8. **The specificity of the sampling method for *Phytophthora* detection in rivers and streams, in percentage of reads, was: 95.3 % for filter samples, 99.2 % for *Rhododendron* leaves and 98.2 % for carnation petals.**
9. ***Phytophthora* isolation from carnations was lower than using *Rhododendron,* and only eight carnation samples were positive for *Phytophthora* in comparison with the fourteen using *Rhododendron* leaves.**

The preservation state of the bait was found to be an important factor for isolation.

10. **Detection of *Phytophthora* phylotypes from clade 6 represented approximately 86% of *Phytophthora* reads.**
11. **Presence of *P. cinnamomi* was significantly higher in agricultural areas than in forestry ones, and filters represented the best method for its detection.**
12. **Pyrosequencing of filter samples resulted the best method to detect new *Phytophthora* phylotypes in the different eDNA sources.**