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METAGENOMIC STRATEGIES TO ASSESS GENETIC DIVERSITY IN *PHYTOPHTHORA* SPECIES

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What we know is a drop, what we don't know is an ocean.

Isaac Newton

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CHAPTER 1

GENERAL INTRODUCTION

BACKGROUND

Introduction to *Oomycetes*

The *Oomycetes*, also known as water molds, form a distinct group of fungus-like eukaryotic microorganisms, which are saprophytes or parasites of diverse hosts in marine, freshwater and terrestrial environments (Margulis & Schwartz, 2000). Although they resemble fungi in mycelial growth and mode of nutrition, distinct morphological characteristics place them in the kingdom of Chromista or Stramenopila with brown and golden algae and diatoms (Harper *et al.*, 2005; Richards *et al.*, 2006). Moreover, phylogenetic analyses have confirmed the assertions of earlier systematists that the *Oomycetes* are different from fungi (Rossman & Palm, 2006; Fry & Grünwald, 2010). Indeed, fungi appear more closely related to animals and *Oomycetes* are more closely related to algae and to green plants (Fig. 1).

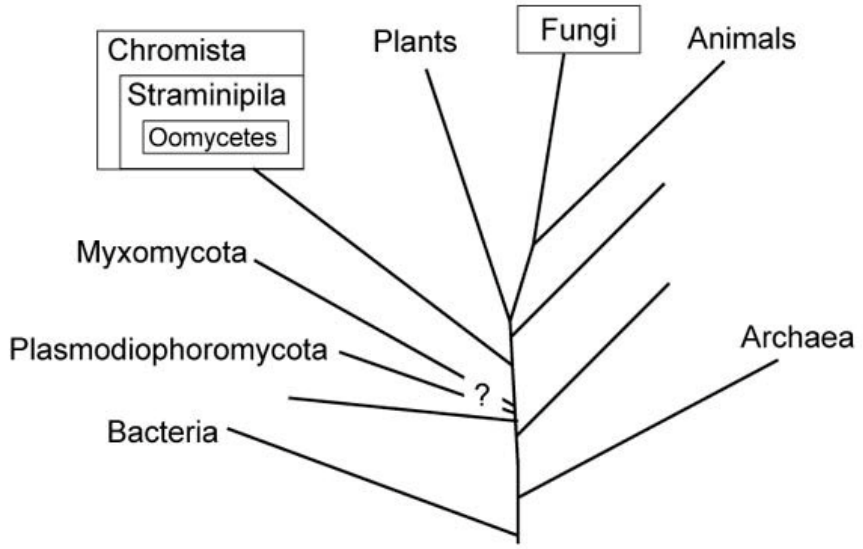

Figure 1. Phylogenetic tree illustating the approximate relationship between oomycetes and fungi. [Reproduced from Rossman & Palm (2006)].

Several features distinguish *Oomycetes* from fungi; *i.e.*, septa in the hyphae are rare, resulting in coenocytic hyphae; the cell wall contains cellulose and β-glucans but do not contain chitin, which occurs in the true fungi; the vegetative state of *Oomycetes* is diploid, whereas true fungi are haploid or

dikaryotic; the mitochondria are characterized by tubular cristae and protoplasmic and nuclear-associated microtubules, while the true fungi have flattened mitochondrial cristae (Aronson *et al.*, 1967; Alexopoulos *et al.*, 1997; Kortekamp, 2005).

One of the most distinguishing characteristics of *Oomycetes* is the production of zoospores by the formation of a structure called “sporangium” that arises on a specialized hypha named “sporangiophore”. Sporangia differ among various *Oomycetes* with respect to the shape, its mode of germination, and the structure of the sporangiophores. They may be terminal or intercalary (within a hyphal filament), bulbous or not, and if terminal, caducous (sporangia detach readily) or not. The mechanism for germination of sporangia is often species-specific and the type of germination is influenced most strongly by environmental conditions (Erwin & Ribeiro, 1996; Belli, 2011).

Zoospores have a tinsel-type anterior flagellum and a whiplash-type posterior flagellum, both typically attached in a ventral groove, which make them able to swim. In some species, the ability to produce zoospores has been lost, and sporangia are thought to have evolved into structures that germinate directly to produce germ tubes. In this case, the sporangia are sometimes termed “conidia” or "sporangioconidia". In yet other species, sporangia can germinate to produce either conidia or zoospores, according to environmental conditions (Erwin & Ribeiro, 1996; Belli, 2011).

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. Depending on the location of the antheridium, it is possible to distinguish between the paragynous and the amphigynous arrangement. Species can be distinguished into homothallic and heterothallic forms, where fertilization occur in a single strain or between two strains of opposite mating types, respectively. In both cases, the fertilized oogonium develops into a thick-walled oospore. These are resting structures and after a period of dormancy (often of apparently diverse and undefined durations), can germinate to produce hyphae, which may immediately produce a sporangium (Erwin & Ribeiro, 1996; Belli, 2011).

Some taxa also produce thick-walled survival structures called chlamydospores, which represent asexual resting spores with a surprising degree of intraspecific variation in structure, wall thickness and tolerance of extreme environments (Erwin & Ribeiro, 1996; Belli, 2011). General understanding of the relationships among *Oomycetes* is evolving rapidly as researchers gather additional information, particularly from molecular analyses. The rapid evolution of these techniques and analysis of DNA sequence are providing new criteria for assessing relationships (Cooke *et al.*, 2000; Lamour & Kamoun, 2009). The analysis of probable relationships among the major genera of *Oomycetes* is reported in Fig. 2. While *Pythium*, *Phytophthora* and *Peronospora* appear related, the relationship of these organisms with the other taxa remains problematic (Cooke *et al.*, 2000). *Oomycetes* counts hundred organisms that have colonized many ecological niches with a worldwide distribution. They can be found in terrestrial and marine environments. They have not only colonised the deserts of Iran (Abbasi & Mohammadi, 2009), but also the arctic regions of the world, including Antarctica (Hughes *et al.*, 2003; Bridge *et al.*, 2008). Nonetheless, the *Oomycetes* remain relatively poorly known compared to the true fungi.

More than 60% of the known species are plant pathogens, which threaten natural and managed ecosystems (Thines & Kamoun, 2010; Kamoun *et al.*, 2014). In particular, the order *Peronosporales* contains three families of plant pathogens. In the family Peronosporaceae, *Plasmopara*, *Peronospora*, *Pseudoperonospora*, *Sclerospora*, and *Bremia* are obligate parasites that cause serious foliar diseases known as downy mildews on many host plants such as grape, broccoli, onion, cucurbits, sorghum, and lettuce (Ash, 2000; Colucci & Holmes, 2010). The family *Pythiaceae* contains obligate and nonobligate parasites, and includes the important pathogen genera *Pythium* and *Phytophthora*. *Pythium* species cause a variety of diseases including root rots of numerous plant species, *Pythium* blight of turf, and damping-off, which involves seed rot and pre- and post-emergence seedling death.

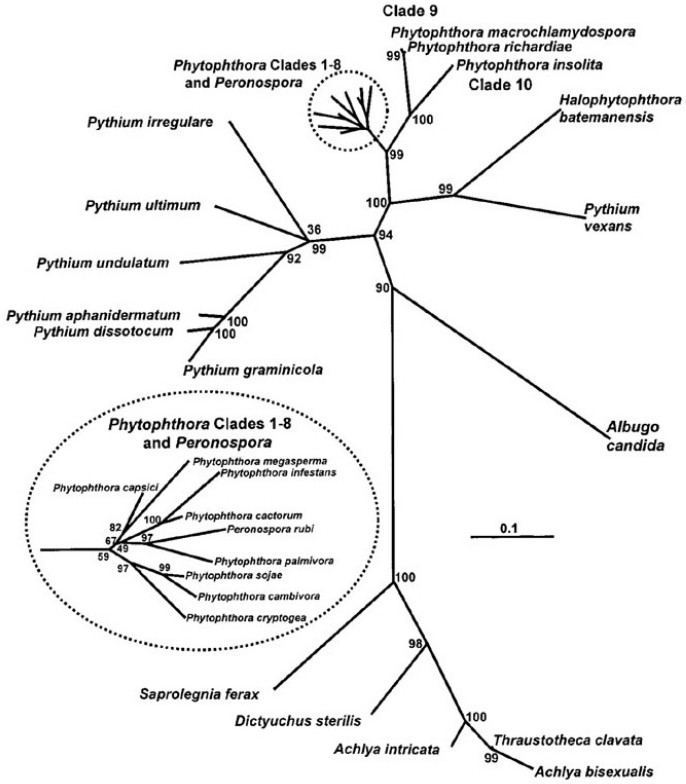

Figure 2. Phylogenetic analysis among related oomycetes obtained by DNA distance-based analysis of the combined 5.8S subunit and ITS2 regions of the rDNA. The numbers at the branch points are bootstrap values, percentages that indicate how consistently the data support given branching points. The inset shows the details of the relationships among eight *Phytophthora* species and a *Peronospora* species. [Reproduced from Cooke *et al.* (2000)].

Phytophthora species cause late blight of potato and tomato, foliar blights on peppers and cucurbits, and root or stem rots of many plant species. One member of this genus has greatly influenced history, namely *P.* infestans, the cause of the late blight of potatoes. As a result of the famine in Ireland caused by this plant pathogen, more than 1 million people died and another 1.5 million emigrated (Fig. 3; Alexopoulos *et al.*, 1997; Schumann & D'Arcy, 2000; Allen *et al.*, 2004; Babadoost, 2005; Brooks, 2005; Gallup *et al.*, 2006; Dorrance *et al.*, 2007; Parke & Lucas, 2008). According to a recent survey to query the community for their ranking of plant-pathogenic oomycete species based on scientific and economic importance the Top 10 species and their ranking are: (1) *P. infestans*; (2, tied) *Hyaloperonospora arabidopsidis*; (2, tied) *P. ramorum*; (4) *P. sojae*; (5) *P. capsici*; (6) *P. viticola*; (7)

P. cinnamomi; (8, tied) *P. nicotianae*; (8, tied) *P. ultimum*; and (10) *Albugo candida* (Kamoun *et al.*, 2014).

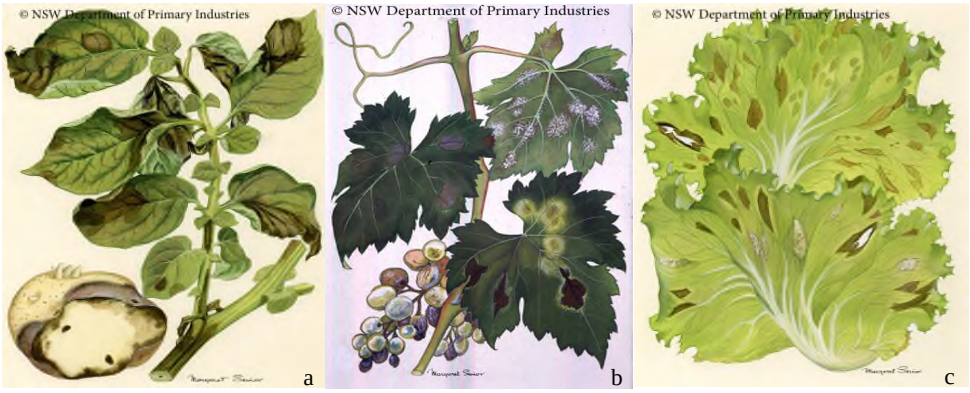

Figure 3. Coloured illustrations of the Late blight *Phytophthora infestans* (a), the Downy mildew of grapes *Plasmopara viticola* (b) and the Downy mildew *Bremia lactucae* (c). [Reproduced from the Division of Science of the NSW Department of Agriculture (1964)].

The genus *Phytophthora*

Phytophthora (the plant destroyer in Greek) is one of the best-studied genus that is mainly, if not entirely, parasitic on various plant hosts in both natural and agricultural settings (Judelson & Blanco, 2005; Blair *et al.*, 2008). Virtually every dicot plant is affected by one or more species of *Phytophthora*, and several monocot species are infected as well.

Since de Bary first established the genus *Phytophthora*, over 140 species have been described, although for sure, this is an underrepresentation of the number of species existing in nature (Kroon *et al.*, 2012). This number of species is increasing yearly due to the availability of more sophisticated tools for species delimitation and for the discovery of novel *Phytophthora* species in natural and agricultural settings.

For a long time identification and classification of species within the genus *Phytophthora* were based on the key developed by Waterhouse (1963). The mycologists in the pre-molecular era used host range, spore morphology, presence or absence of chlamydospores, optimal growth temperature, colony

morphology, surface structure of oospores and other “Waterhouse” criteria to define species boundaries and to position a new species in one of the six Waterhouse groups. The allocation of an isolate to a particular species was arduous work and required trained experts with a good eye and attention to detail. The description of a new species was even more challenging, requiring the researcher to be a skilled mycologist able to distinguish the potential new species from all other species, an artist to draw spore structures by hand and a classicist to phrase the findings in Latin.

Due to their significant environmental and economic importance, several approaches based on molecular analysis have been developed. Technological advances, increased automation and reduced costs of sequencing have contributed to the success of new approaches to the study of microbial diversity via molecular methods (Cooke *et al.*, 2007; Kroon *et al.*, 2012).

Traditional detection techniques of *Phytophthora*

Since *Phytophthora* species are often more difficult to isolate than those of the closely related genus *Pythium* and other unrelated types of fungi, much research has been devoted to the development of supplemental strategies to increase the frequency of selective isolation of *Phytophthora* (Tsao 1970, 1983). However over the last decades, progress has been made in the development of techniques utilizing selective antibiotics and chemicals in the culture media to facilitate the isolation of *Phytophthora* from environmental samples.

Conventional detection methods applicable to *Phytophthora* species generally include: i) direct microscopic examination of diseased material, ii) baiting with plant materials, and iii) isolation of the pathogens from infected plant tissues, water and soil using general or selective agar media. Although widespread, traditional techniques are based on morphological and cultural criteria and require skilled and specialized microbiological expertise, which often takes many years to be acquired. These methods are also very time consuming requiring days or weeks to complete and results are not always

conclusive, e.g. when closely related organisms need to be discriminated (Cooke *et al.*, 2007). Furthermore, traditional methods may not be sensitive enough to assess the occurrence and distribution of the overall diversity in a sample since many species can be excluded during the detection process. It's generally accepted that the absence of *Phytophthora* in a natural ecosystem must be interpreted with caution (Erwin & Ribeiro, 1996). Although, one rather obvious advantage of these methods is that successful isolation of the pathogen yields objective proof of its presence, the limitations of such approaches have led to the development of novel methods for detecting and identify *Phytophthora* species as well as other plant pathogens.

Molecular detection techniques of *Phytophthora*

Despite the large number of studies conducted in the past using traditional detection methods (selective media and baiting techniques), the presence and activity of *Phytophthora* in natural ecosystems was still underestimated. The method of species identification and classification began to change when molecular assays became more readily available. First diagnostic reports of the molecular era were based on the use of hybridization probes. However, in recent years this technique has been replaced by PCR-based approaches because of their greater sensitivity, simplicity and speed. Like phenotypic traits, PCR products started being used as diagnostic tools for the identification of plant pathogens in several ecosystems (Drenth *et al.*, 1993; 1994).

There are several significant advantages of PCR-based detection methods over the traditional methods of diagnosis; i.e., microrganisms do not need to be cultured; the potential to detect a single target molecule in a complex mixture (Lee & Taylor, 1990); their speed and versatility; the possibility to use such automated diagnostic systems for large-scale applications also in field (Tomlinson *et al.*, 2005; Cooke *et al.*, 2007). Despite this, major assays has been developed for the detection of one or few known target plant pathogens. As such, they are inappropriate for broader surveys of microbial

diversity and distribution in natural ecosystems in which a system capable of detecting multiple species or even undescribed species is required.

Recently an innovative molecular approach for the study of *Phytophthora* species in forests and other natural ecosystems has been developed (Scibetta *et al.*, 2012). This method is based on DNA extraction and purification from environmental samples, amplification by nested PCR with a new set of *Phytophthora* genus-specific primers enabling the amplification of the internal transcribed spacer 1 (ITS1) region of the ribosomal DNA (rDNA) and the cloning of the second round PCR product. Database comparisons of the DNA sequence derived from the cloned fragments are used for species identification.

The ITS regions provide attractive targets because they are highly stable, can be easily amplified and sequenced with universal primers, occur in multiple copies, and possess conserved as well as variable sequences (White *et al.*, 1990). However, in recent years, the discovery and ITS sequencing of many new *Phytophthora* species have raised concerns about the specificity of the ITS-based molecular detection methods. This is due to cases where the ITS sequences are not sufficiently variable, making the design of primers to identify and detect closely related taxa very difficult or impossible. Important Phytophthoras such as *P. nemorosa*, *P. ilicis*, *P. psychrophila*, and *P. pseudosyringae* have very similar ITS regions sequences and the design of effective and robust specific primer sets is very challenging (Martin & Tooley, 2003; Schena & Cooke, 2006). Similarly *P. alni*, *P. cambivora*, *P. fragariae*, and *P. europaea* are phylogenetically closely related and challenging to distinguish via ITS sequences (Brasier *et al.*, 2004). The PCR assay used widely for *P. ramorum* detection was recently found to cross-react with *P. foliorum* a newly discovered and closely related species pathogenic on both wounded and intact azalea leaves (Donahoo *et al.*, 2006).

It's safe to say that a large number of well-known diseases caused by various species of *Phytophthora* are not being properly diagnosed in many area of the world. Also many diseases caused

by *Phytophthora* are not reported in some countries. This often leads to an incorrect concept on the distribution of these diseases. These gaps are mainly the result of the lack of an appropriate method to detect *Phytophthora* from several environmental samples. Molecular methods for detection, identification and monitoring of *Phytophthora* species have proved important tools to predict the threats posed by native pathogens and minimize the risk of further invasive *Phytophthora* diseases in order to circumvent some of the weaknesses inherent in the current plant health systems as described by Brasier (2004). Molecular approaches also have great potential in providing *Phytophthora* diversity in a range of terrestrial and water environments.

Defining *Phytophthora*, or perhaps wider *Oomycetes*, diversity at such scales has not yet been attempted but clearly provides an opportunity to increase general understanding of this group of pathogens and their impact on natural and managed vegetation systems. Of course, molecular methods alone cannot provide all the answers and where novel 'molecular species' are discovered it will serve to focus on new isolation attempts and standard approaches to recover and define the key attributes of the taxonomy, ecology and pathology of these species.

Metabarcoding analysis

Metagenomics has revolutionized microbiology by paving the way for a cultivation-independent assessment and exploitation of microbial communities present in complex ecosystems and it is now considered one of the fastest-developing research areas (Simon & Daniel 2011).

Since 1998, when the term "metagenomics" was coined by Handelsman and coworkers, great progress has been made. Initially, metagenomics was used mainly to recover novel biomolecules from environmental microbial assemblages. The development of new high-throughput sequencing techniques, various bioinformatics tools for the analysis and comparison of these data sets and other affordable methods allowing large-scale analysis of microbial communities, resulted in novel

applications, such as comparative community metagenomics, metatranscriptomics, and metaproteomics (Sjöling & Cowan, 2008; Chistoserdova, 2010). These approaches offered significant promise to advance the measurement and prediction of the *in situ* microbial responses, activities, and productivity. In addition, analyses of the thereby-generated comprehensive data sets had an unprecedented potential to shed light on ecosystem functions of microbial communities and evolutionary processes (Simon & Daniel, 2011).

High-throughput sequencing systems have boosted genetics in the last few years. The reduction of costs, wet-lab workflow complexity and the gain of read length has led to an enormous increase in sequencing projects and sequencing data (Voelkerding *et al.*, 2009). For the past 30 years, the Sanger method has been the dominant approach and gold standard for DNA sequencing. Large scale sequencing projects, including whole genome sequencing, have usually required the cloning of DNA fragments into bacterial vectors, amplification and purification of individual templates, followed by Sanger sequencing using fluorescent chain-terminating nucleotide analogues and either slab gel or capillary electrophoresis. Alternative sequencing methods have been described, although no technology has displaced the use of bacterial vectors and Sanger sequencing as the main generators of sequence information (Prober *et al.*, 1987; Brains *et al.*, 1988; Jett *et al.*, 1989; Jacobson *et al.*, 1991; Nyren *et al.*, 1993; Ronaghi *et al.*, 1996; Sanger *et al.*, 1997).

The commercial launch of the first massively parallel pyrosequencing platform in 2005 ushered in the new era of high-throughput genomic analysis. The transition from Sanger sequencing to 454 sequencing opened new horizons in microbial community analysis by making it possible to collect hundreds of thousands of sequences spanning hundreds of samples (Huse *et al.*, 2007). In the relatively short period since 2005, new sequencing approaches have been developed by giving the opportunity to analyze each samples in unprecedented depth in few hours.

Improvements in DNA sequencing technology offered unprecedented cost-effective opportunities to explore microbial diversity by providing for a significantly greater depth of analysis within an individual sample and through the ability to analyze a large number of samples within a single sequencing run, but also present challenges in data analysis due to the large number of sequences generated. Many bioinformatics tools have been generated to automate such species determination from raw sequence data (Caporaso *et al.*, 2010, 2011; Bik *et al.*, 2012).

SCOPE OF THE THESIS

The central themes in this thesis were all related to the development and application of amplicon-metagenomic approaches to evaluate the diversity of soil- and water- borne *Phytophthora* species. These molecular approaches enabled the direct sequencing of the ITS1 barcode-region with genus-specific primers (Scibetta *et al.*, 2012) from several samples, with particular emphasis on ornamental species from nurseries and water samples from waterways. In particular, three sequencing approaches based on Sanger sequencing, 454 Pyrosequencing and MiSeq Illumina have been tested to evaluate their efficacy in terms of specificity and sensitivity with detection in a number of different samples including water, soil and plant roots.

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CHAPTER 2

Molecular analysis of *Phytophthora* diversity in nursery-grown ornamental and fruit plants

ABSTRACT

The genetic diversity of *Phytophthora* spp. was investigated in potted ornamental and fruit tree species using a metagenomic approach based on a semi-nested PCR with *Phytophthora* genus-specific primers targeting the ITS1 region of the rDNA. More than 50 ITS1 sequence types (STs) representing at least 15 distinct *Phytophthora* taxa were detected. Nine had ITS sequences that grouped them in defined taxonomic groups (*P. nicotianae, P. citrophthora, P. meadii, P.* taxon *Pgchlamydo, P. cinnamomi, P. parvispora, P. cambivora, P. niederhauserii,* and *P. lateralis*) whereas three phylotypes were associated to two or more taxa (*P. citricola* taxon E or III; *P. pseudosyringae, P. ilicis or P. nemorosa*; and *P. cryptogea, P. erythroseptica, P. himalayensis or P.* sp. *"kelmania"*) that can be challenging to resolve with ITS1 sequences alone. Three additional phylotypes were considered as representatives of novel *Phytophthora* taxa and defined as *P. meadii*-like, *P. cinnamomi*-like and *P. niederhauserii*-like. Furthermore, the analyses highlighted a very complex assemblage of *Phytophthora* taxa in ornamental nurseries within a limited geographic area and provided insights into the population structure of *P. nicotianae* (the most prevalent taxon) and other taxa. Data revealed new host-pathogen combinations, evidence of new species previously unreported in Italy (*P. lateralis*) or Europe (*P. meadii*) and phylotypes representative of species that remain to be taxonomically defined. Furthermore, the results reinforced the primary role of plant nurseries in favoring the introduction, the dissemination and evolution of *Phytophthora* species by favoring intra- and inter-specific sexual recombination.

INTRODUCTION

The outbreak of new plant disease can have negative economic and environmental consequences and in the worst cases even societal repercussions. Among plant pathogens, the genus *Phytophthora* is one of the most damaging with more than 100 species responsible for devastating diseases in agricultural and natural ecosystems (Brasier, 2009). They cause root rot, stem rot, twig and/or leaf blights in a huge number of plant species and losses in nurseries can be up to 100% within one year (Themann *et al.*, 2002). Many recently described invasive *Phytophthora* species were previously unknown and have been identified only when they have caused severe disease in non-native environments. It has been hypothesized that between 100 and 500 species are still unknown to the scientific community (Brasier, 2009; Kroon *et al.*, 2012). Having adapted and co-evolved with their hosts, many of these pathogens may do little noticeable damage in their native ecosystems and so are less likely to be detected.

New plant diseases can be the result of many factors including adaptation of pathogens to new hosts, incursions of pathogens from other geographic regions and factors such as climate change that trigger an endemic pathogen to cause disease. However, the plant trade is considered the primary cause of new disease outbreaks due to its role in the introduction of invasive alien pathogens (Brasier, 2008). The unprecedented growth of international travel and trade results in huge disturbance to ecosystems with severe socio-economic impact. A specific program for monitoring emerging diseases (ProMED; http://www.promedmail.org) has revealed a 13-fold increase of disease alerts for plant-infecting fungi from 1995 to 2010. In this context the nursery trade, with particular emphasis on the potted ornamentals sector is particularly exposed as a consequence of its globalization, intensive cultivation techniques and the frequent turnover of new varieties and/or species. Ornamental plants have proved to be especially susceptible, probably because they represent artificial ecosystems grown under harsh conditions which expose them to pathogen attack.

The role of the nursery trade in the spreading of *Phytophthora* inoculum has been investigated for *P. ramorum* and *P. nicotianae* but there are many more *Phytophthora* species involved (Goss *et al.*, 2011; Mammella *et al.*, 2011, 2013; Parke *et al.*, 2014). In Italy more than 20 *Phytophthora* species were reported in nurseries of ornamentals and the majority of these were identified on new hosts for the first time (Cacciola *et al.*, 2008). In Germany, Minnesota, California, Virginia and Spain between 10 and 17 different species of *Phytophthora* were detected during surveys carried out in nurseries and garden centres (Themann *et al.*, 2002; Schwingle *et al.*, 2007; Moralejo *et al.*, 2009; Yakabe *et al.*, 2009; Bienapfl & Balci, 2014). It has been suggested that the movement of plant material allows the introduction of pathogens and that conditions typical of nurseries (e.g. warm temperature, high humidity due to frequent irrigation, close and repeated cultivation of many variety/species, growth of plants in pots) provides an environment favorable for growth and sporulation of *Phytophthora* species. In particular, contaminated recycled irrigation water is an important pathway for the dissemination of motile zoospores of *Phytophthora* spp. (Themann *et al.*, 2002).

Nurseries may also play a major role in favouring hybridization due to the presence of multiple plant species with their own pathogens. The contact between related but previously geographically isolated pathogens can accelerate the evolutionary process and generate better adapted or entirely new pathogen species. Relevant examples are represented by *P. alni*, a hybrid between *P. cambivora* and *P. fragariae*-like species (Brasier *et al.*, 2004), and *Phytophthora* × *pelgrandis*, a hybrid between *P. nicotianae* and *P. cactorum* (Faedda *et al.*, 2013b).

To limit the introduction of new invasive pathogens, plants moving in trade are covered by phyto-sanitary certificates. However, certification is commonly based on a simple visual inspection and many *Phytophthora* infections are not detected due to latency and the suppression of symptoms by intensive chemical applications that increase the risk of cryptic pathogen dissemination.

Data on pathogen dissemination are quite limited, frequently contrasting and probably underestimated due to the limited power of commonly utilized detection methods that are often based on culturing and baiting (Cooke *et al.*, 2007). Several PCR-based methods have been also developed for *Phytophthora* species but the majority of diagnostic assays have been specifically designed to detect only a single species and as a consequence they are inappropriate for broader surveys of *Phytophthora* diversity and distribution in ecosystems in which a system capable of detecting multiple species or even undescribed species is required (Cooke *et al.*, 2007; Martin *et al.*, 2012; Sanzani *et al.*, 2013).

The aim of the present study was to evaluate the application of a metagenomics approach based on the use of genus-specific primers to examine the presence and spread of *Phytophthora* species in potted plant nursery roots and soils with particular emphasis on ornamental species (Scibetta *et al.*, 2012). This molecular approach enables the direct sequencing of the ITS1 region and its use as barcode marker for the detection of the overall *Phytophthora* diversity in environmental samples (Scibetta *et al.*, 2012). This culture-free molecular method has the potential to significantly improve the depth of coverage in *Phytophthora* diversity detection (Cooke *et al.*, 2007).

MATERIALS & METHODS

Sampling

A total of 115 soil and root samples were collected from many ornamental and a single fruit tree species during 2012 and 2013 in 9 different nurseries across Apulia and Calabria, Southern Italy (Table 1). The samples were all from potted plants transplanted or sown between 3 months and 3 years before the survey. Plants showing general symptoms of decline on the canopy and/or roots rots were targeted for sampling. All analysed samples were represented by root and soil from five plants and were maintained in plastic bags at 4°C for no more than two days before processing. Roots samples were

washed with running tap water, dried on blotting paper and cut to obtain small pieces (about 5 cm). Both roots and soils were freeze-dried and stored at -20°C pending molecular analysis.

Table 1. Results of surveys conducted on soil and root samples collected from potted plantlets in 8 different nurseries located in Apulia and Calabria (Southern Italy) and on a soil sample collected in a citrus grove in Calabria. Detected *Phytophthora* phylotypes were identified according to their phylogenetic analysis along with reference sequences (Fig. 3).

Sampling locality	**Collected species**	**Detected *Phytophthora* species***	
		Roots	Soils
Nursery TP - Apulia	*Grevillea lanigera*	*P. nicotianae, P. cinnamomi*-like	*P. nicotianae, P.cinnamomi, P. cinnamomi*-like
	Lavandula sp	ND	*P. nicotianae*
	Chamelaucium uncinatum	NA	ND
	Convolvolus cneorum	*P. cryptogea*	*P. niederhauserii, P. cryptogea*
	Armeria maritima	NA	*P. nicotianae*
Nursery CP - Apulia	*Rosmarinus officinalis* var. *erectus*	ND	*P. cryptogea, P. citrophthora*
	Salvia sp.	*P. cryptogea*	*P. niederhauserii*
	Olea europaea	*P. nicotianae, P. niederhauserii*	*P. nicotianae*
	Convolvolus mauritanicus	*P. nicotianae*	*P. nicotianae*
	Rosmarinus officinalis var. *prostratus*	ND	ND
	Pistacia lentiscus	ND	ND
	Cotoneaster salicifolius	ND	ND
	Teucrium brevifolium	ND	ND
	Convolvulus cneorum	ND	ND
	Origanum pseudodictamus	ND	ND
	Hebe veronica	ND	ND
	Eremophila nivea	ND	ND
	Arbutus unedo	ND	ND
	Erica caniculata	ND	ND
	Cytisus sp.	ND	ND
	Russelia equisetiformis	ND	ND
Nursery ST - Calabria	*Diospyros kaki*	*P. niederhauserii*	*P. niederhauserii*
Nursery PV - Apulia	*Cyclamen persicum* var. *halios*	ND	ND
	Cyclamen persicum var. *tianis*	*P. nicotianae, P. niederhauserii*	*P. nicotianae, P. lateralis*
	Tagetes erecta	ND	*P. nicotianae, P. lateralis*
	Tagetes patula	ND	ND
	Petunia parviflora	*P. nicotianae, P. lateralis*	ND
	Petunia sp.	*P. nicotianae*	ND
Nursery ZZ - Apulia	*Cercis siliquastrum*	ND	*P.* taxon *Pgchlamydo, P. cinnamomi*-like, *P. pseudosyringae*
	Punica granatum	ND	*P. lateralis*
	Arbutus unedo	ND	ND

	Rosa sp.	ND	ND
	Grevillea juniperina	ND	ND
	Bouganvillaea glabra	ND	*P. cinnamomi*-like
Nursery DM - Apulia	*Polygala myrtifolia*	ND	ND
	Lantana sellowiana	ND	ND
	Nerium oleander	ND	ND
	Polygala myrtifolia	ND	ND
	Grevillea lanigera	ND	ND
	Lithodora sp.	ND	ND
	Eugenia myrtifolia	ND	ND
	Euriopsis pectinatum	ND	ND
	Coleonema pulchrum	ND	ND
Nursery BL - Apulia	*Thymus sp*	ND	ND
	Mentha sp	*P. nicotianae*	*P. nicotianae, P. meadii*
	Allium schoenoprasum	ND	*P. nicotianae*
	Rosmarinus officinalis	ND	*P. cambivora*
	Armeria maritima	*P. nicotianae*	*P. nicotianae*
	Cyclamen persicum	ND	*P. niederhauserii*-like, *P. niederhauserii*
	Petunia parviflora	*P. nicotianae, P. cambivora, P. meadii*	*P. nicotianae*
	Fuchsia magellanica	*P. niederhauserii*	*P. niederhauserii*
	Lobelia erinus	*P. nicotianae, P. meadii, P. cambivora, P. citricola*	ND
	Dahlia campanulata	*P. nicotianae, P. citricola, P. cambivora, P. meadii*-like, *P. niederhauserii*	ND
	Impatiens nuova guinea	*P. cambivora, P. meadii*	ND
Nursery PG - Apulia	*Quercus ilex*	ND	ND
	Prunus mariana	ND	ND
	Prunus mahaleb	*P. citricola, P. cambivora*	*P. citricola, P. cambivora*
	Crataegus azarolus	ND	ND
Citrus Grove - Calabria	*Citrus reticulata*	NA	*P. nicotianae, P. parvispora, P. cambivora, P. citrophthora, P. meadii*
****N.A. = non-analyzed samples; N.D. = Analyzed samples in which no Phytophthora was detected.***			

DNA extraction

Triplicate DNA extractions were performed from all collected soil and root samples. To extract DNA from soil, the method described by Schena *et al.* (2002) was slightly modified. Lyophilised soil (0.5 g) was transferred in 2 ml Eppendorf tubes and suspended in 1.5 mL of extraction buffer (0.12 M Na2HPO4, 1.5 M NaCl, 2% CTAB) in the presence of 0.1 g of acid-washed glass beads (425-600 um diameter; Sigma Aldrich, USA) and two 5 mm stainless steel ball bearings. The extraction mixture was blended at 300 rpm for 10 min and centrifuged at 16000 g for 10 min at 4°C. The upper phase was extracted with an equal volume of chloroform, precipitated for 1 h at -20°C with two volumes of isopropanol and a tenth of volume of 3M Sodium Acetate, pH 5.2, washed twice with cold 100% and 70% ethanol, dried and re-suspended in 100 µL of RNase free water.

Extraction of DNA from roots was performed using the protocol described by Schena & Ippolito (2003) with minor modifications. Lyophilised tissues were pulverized using mortar and pestle under liquid nitrogen. Approximately 0.5 g of the resulting powder was transferred into 2 ml Eppendorf tubes containing 0.1 g of acid-washed glass beads (425-600 um diameter), 0.1 g of PVP (Sigma Aldrich, USA), two 5 mm stainless steel ball bearings and 1.5 mL of extraction buffer (200 mM Tris-HCl pH 7.7, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The mixture was blended for 5 min using a Mixer Mill MM 400 (Verder Scientific, Italy) set to have vibrational frequency of 1800 and centrifuged at 16000 g for 10 min at 4°C. The upper phase was extracted twice with an equal volume of phenol/chloroform (1:1) and chloroform, respectively. Nucleic acids were precipitated, washed and resuspended in 100 µL of RNase free water as described for soil.

Total DNA from all soil and roots samples was divided into two equal aliquots of 50 µl. Aliquots were stored at -20°C without any additional treatment or after purification through chromatography columns as described Ruano-Rosa *et al.* (2007).

Evaluation of DNA quantity and quality

Purified and non-purified environmental DNA samples were analyzed by electrophoresis in 1.2% agarose gels containing GelRed™ nucleic acid gel strain (Biotium, USA) in TBE buffer and visualized on UV light using a Gel Doc™ XR (BioRad, USA). A spectrophotometer (Nanodrop, Thermo Fisher Scientific Inc.) was used to measure absorbance at 260, 280 and 230 nm and estimate concentration and contamination with protein and humic acid.

Furthermore, to confirm that DNA samples were of sufficient quality to be amplified by PCR, 1 µl of a representative number of DNA samples (purified and non-purified) was analysed by real-time PCR using specific hydrolysis probes method designed to detect *P. kernoviae* (Schena *et al.*, 2006). Primers and probe for *P. kernoviae* were selected because this species was experimentally verified to be absent in all the samples. Amplifications were performed in duplicate and reaction mixtures containing 50 ng of *P. kernoviae* DNA were spiked with 1 µL of water (control) or either purified or non-purified DNA. Reaction mixtures without *P. kernoviae* DNA were utilised to confirm the absence of this species in all analysed samples. PCR amplification was performed as described by Schena *et al.* (2006) using an StepOnePlus™ Real-Time PCR System (Applied Biosystems®, USA) and data acquisition and analysis completed using the supplied software according to the manufacturer's instructions. The quantification cycle (Cq) values for each reaction were calculated automatically by the software by determining the PCR cycle number at which the reporter fluorescence exceeded background.

Amplification of *Phytophthora* spp. ITS1 region from soil and root samples

The ITS1 region of the rDNA of *Phytophthora* spp. was amplified in triplicate from all soil and root samples using a semi-nested assay with the SP primers described by Scibetta *et al.* (2012) (Fig. 1). Minor modifications to the method of Scibetta and co-workers concerned the use of a TaqDNA

polymerase with proofreading activity to reduce the risk of PCR artefacts during PCR amplifications. First and second rounds of amplification were performed in a final volume of 25 µL containing 1 U *Pfx50*™ DNA Polymerase (Invitrogen, USA), 1X *Pfx50*™ PCR Mix, 0.2 µM of primers and 1 µL of purified DNA. According to Scibetta and co-workers 1 µL of the 1st round-product was used as template for the 2nd round PCR. All amplification conditions were slightly modified for the DNA polymerase requirements and consisted of 30 s at 94°C followed by 35 cycles of 94°C for 20 s, 61°C for 25 s and 68°C for 30 s and by a final step of 68°C for 2 min. All PCR was conducted in an Mastercycler Ep Gradient S. (Eppendorf, Germany).

Amplicons from the second round PCR were separated by electrophoresis as described previously and a 100 bp DNA ladder (Invitrogen, USA) used to estimate amplicon size.

Great precautions were taken to minimise the risk of DNA contamination of PCR amplifications. First and second round PCR reactions, DNA extractions and electrophoresis were set up in separate areas and using specific sets of materials including gloves, pipettes, filter tips and lab coat. Working positions were repeatedly cleaned with 10% NaClO to denature potential contaminating nucleic acids. Furthermore, an additional *Phytophthora*-free soil sample and several sterile water samples were processed exactly as collected samples and served as negative controls in all experiments.

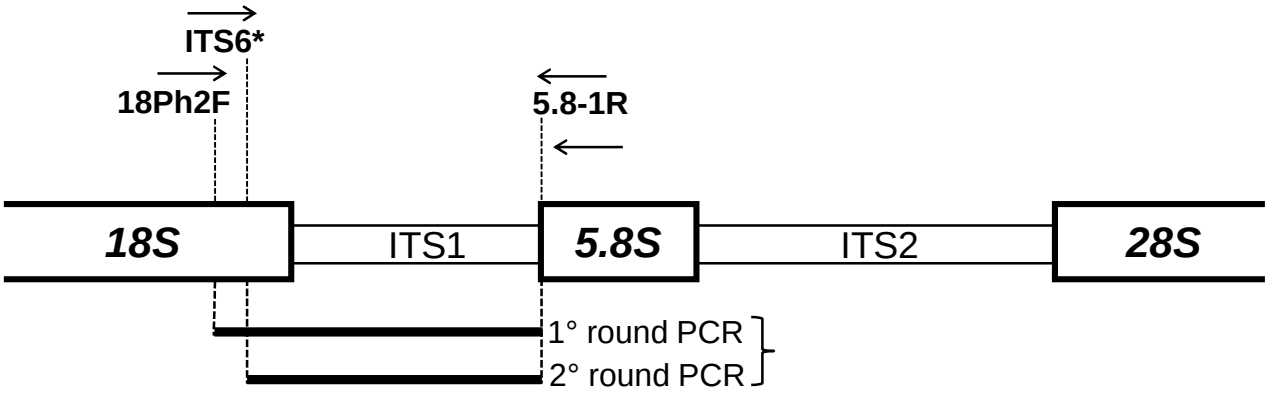


Figure 1. Schematic representation of the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) with location of primers utilized in this study. From Scibetta *et al.*, 2012.

Cloning and sequencing of PCR fragments

Triplicate PCR products of the expected size obtained with the second round PCR from each soil and root sample, were combined in a single sample and cloned into One Shot® TOP10 Chemically Competent *Escherichia coli* (Invitrogen) using a Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, USA), according to the manufacturer's protocol. For each cloned amplicon, 20 clones were picked and directly used in PCR reactions (colony PCR) with the second round PCR primers (ITS6/5.8-1R; 74) as previously described. Amplified products were analyzed by electrophoresis and single bands of the expected size were sequenced with both forward and reverse primers by Macrogen Europe (Amsterdam, The Netherlands).

Analysis of sequences and identification of ITS1 sequence types

The 'ChromasPro version 1.5' software (http://www.technelysium.com.au/) was utilized to evaluate the quality of sequences and to create consensus sequences. All sequences were aligned using the software MUSCLE as implemented in MEGA5 (Hall, 2013) and analyzed and edited manually to check indels and single nucleotide polymorphisms within homologous group of sequences. Prior to analysis, sequences of primers were removed. ITS1 sequence types (STs), defined as the distinct and reproducible ITS1 sequences recovered in this study, were identified in MUSCLE and confirmed using the DnaSP ver. 5.10.01 software (Librado & Rozas, 2009). In order to reduce the risk of errors due to artifacts during PCR and/or plasmid replication, only STs represented by at least two sequences were considered for further analysis.

To identify the species detected, single representative sequences for each ST were subject to phylogenetic analysis along with validated barcode sequences of the genus *Phytophthora* (Robideau *et al.*, 2011). Before analyses the complete panel of *Phytophthora* reference sequences (Robideau *et al.*, 2011) were trimmed to match the sequence lengths determined in this study and analyzed with the

software ElimDupes (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) to delete multiple identical sequences for each species. Identical reference sequences were only included in the panel when they represented different *Phytophthora* species. In cases where no matches were found in the reference sequence from Robideau *et al.* (2011) more closely related sequences were examined using BLAST searches of GenBank with priority given to sequences associated to specific publications (Fig. 2). The complete panel of selected reference sequences and STs were grouped according to their clade (Cooke *et al.*, 2000; Kroon *et al.*, 2012), aligned using ClustalX (Thompson *et al.*, 1997) and introduced to TOPALi for phylogenetic analysis with the MrBayes methods based on Bayesian Tree Estimation (Milne *et al.*, 2008). Bayesian analysis was performed with four runs conducted simultaneously for 500,000 generations with 10% sampling frequency and burn in of 30%. By this process all ITS1 sequences were associated with a phylotype. A phylotype may be represented by single ST or a closely related cluster of ITS1 sequences that are considered to represent a single distinct taxon. We use the term phylotype as a proxy for species in describing the results as species cannot be defined formally in the absence of living isolates.

To graphically show the relatedness and relative abundance of different STs, networks were generated for each detected *Phytophthora* clade with the statistical parsimony algorithm implemented in TCS ver. 1.21 (Clement *et al.*, 2000). Colour and circle size was used to associate STs to the nursery/field of provenance and to the abundance of each ST, respectively. Abundance of STs was determined in terms of number of samples (roots and/or soil) in which each ST was detected.

RESULTS

DNA extraction from soil and root samples

Protocols utilized to extract DNA from root and soil proved to be appropriate for PCR amplifications after the purification step with chromatography columns. Prior to purification, extracted solutions were dark in color (from brown to black) and caused a significant inhibition of PCR reactions. In real-time PCR reactions with *P. kernoviae* DNA a delay of the quantification cycle (Cq) of at least 3 was revealed in reaction mixtures spiked with 1 µl of non-purified soil or root DNA (data not shown). Some DNA extracts completely inhibited PCR reactions. Once purified the DNA samples were clear and did not affect TaqDNA polymerase activity. The quality of purified DNA was also confirmed by a 260/280A ratio of 1.8-2.1 and 260/230A ratio of 1.3-2.0 for both soil and root DNA extracts. The concentration of nucleic acids ranged between 50 and 100 ng/µl (soil samples) and 300 and 500 ng/µl (root samples).

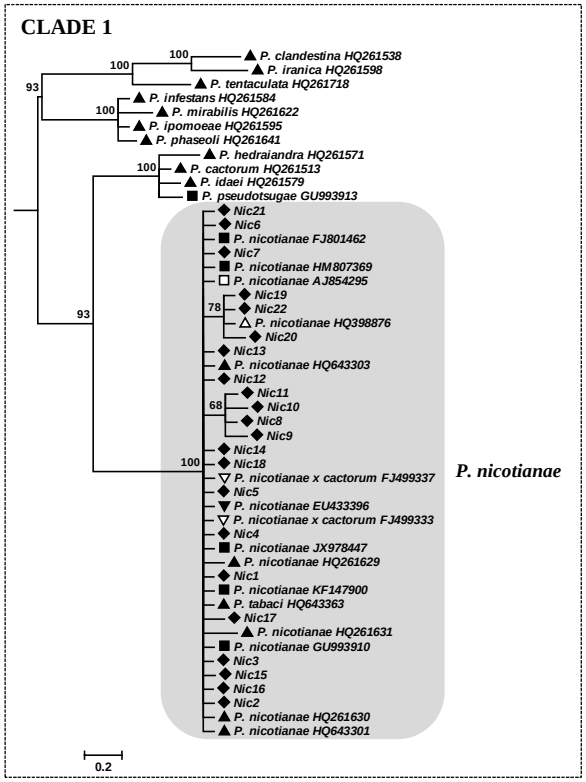
Amplification results

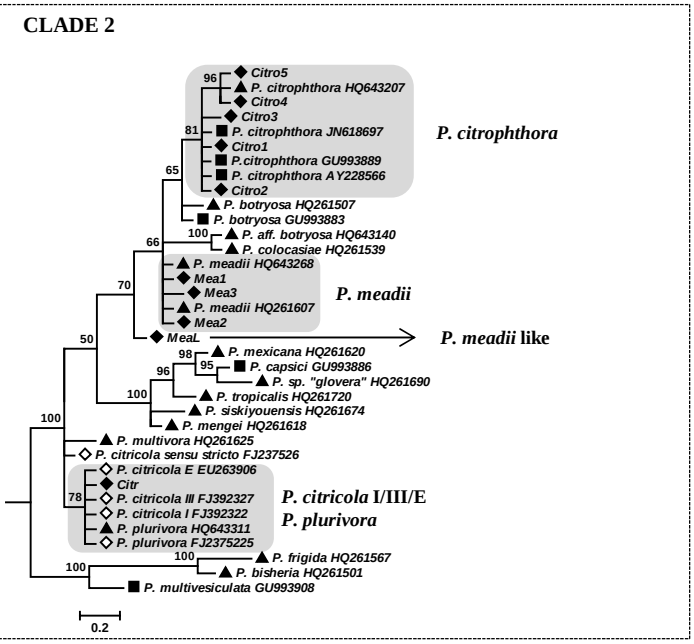
A total of 115 soil and root samples were analysed by the semi-nested assay and 40 of them (17 roots and 23 soils) produced a positive amplification in at least one of the three analysed replications (Table 1). Among these, very few samples produced a positive amplification after the first PCR step, confirming the need for a nested approach to yield reliable levels of sensitivity (Scibetta *et al.*, 2012). For each positive sample, PCR fragments obtained from replicate extractions after semi-nested PCR were combined, cloned and sequenced in both directions. A total of 800 high quality DNA sequences of the ITS1 region (20 clones per each sample) were obtained and representative ITS1 sequences (STs) were deposited in GenBank with accession numbers KJ601190-KJ601244 (Table 2).

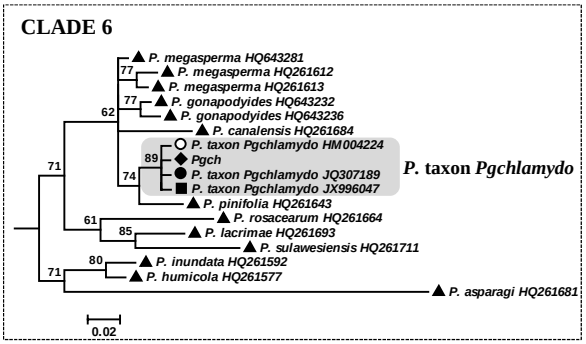
Analysis of sequences and species identification

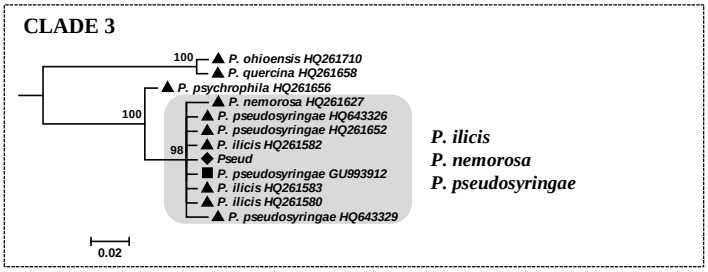
After the exclusion of singletons, 55 unique STs representing known species and sub-species variants, species complexes or representatives of unknown *Phytophthora* taxa were identified. Phylogenetic analysis of these STs against reference sequences (Fig. 2) identified 15 distinct phylotypes in six different ITS clades (Cooke *et al.*, 2000; Kroon *et al.*, 2012). Each phylotype was represented by a number of STs ranging between 1 and 22, as in the case of *P. nicotianae* (Fig. 3). Nine phylotypes were identified to the species level: *P. nicotianae, P. citrophthora, P. meadii, P.* taxon *Pgchlamydo, P. cinnamomi, P. parvispora, P. cambivora, P. niederhauserii,* and *P. lateralis* (Table 1; Fig. 2). Other phylotypes were associated to *P. citricola* taxon E or III (ST Citr; Table 3) or were unresolved within their species complexes: i) *P. pseudosyringae, P. ilicis,* or *P. nemorosa* (ST Pseud); and ii) *P. cryptogea, P. erythroseptica, P. himalayensis* or *P.* sp. *"kelmania"* (STs Cryp1, Cryp2 and Cryp3) because the available genetic variation within the ITS1 region did not enable the reliable differentiation of species (Jung & Burgess, 2009; Robideau *et al.*, 2011). Finally, 3 STs that were markedly different from all reference sequences were defined as *P. meadii*-like (ST MeaL), *P. cinnamomi*-like (STs CinnL1, CinnL2, CinnL3, and CinnL4) and *P. niederhauserii*-like (ST NiedL) phylotypes (Table 1; Fig. 2).

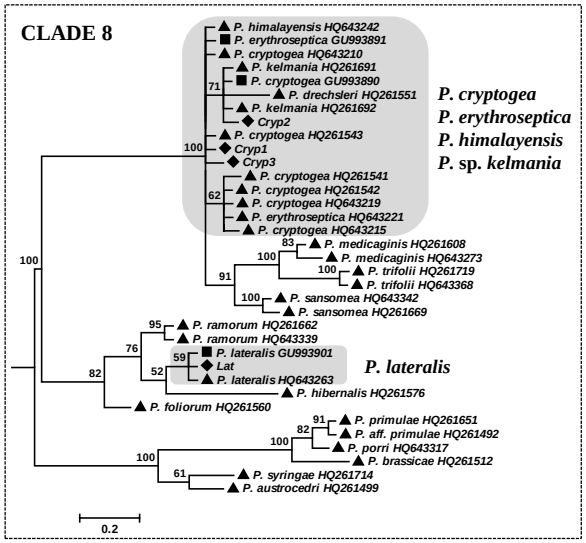
Sub-species variation, with indications of host association, was observed for some species (Fig. 3). In particular, several STs were identified within the heterothallic species *P. nicotianae, P. niederhauserii, P. cambivora, P. citrophthora, P. meadii, P. parvispora,* and the *P. cryptogea* species complex, but not for homothallic species like *P. lateralis* and *P. citricola* taxon E or III (Fig. 3). Four different STs were also identified within the *P. cinnamomi*-like taxon. Single STs only were detected for *P. cinnamomi, P.* taxon *PgChlamydo*, the *P. pseudosyringae* species complex, *P. meadii*-like and *P. niederhauserii*-like but this was probably due to their low abundance since they were detected in only single root or soil samples (Fig. 3).


CLADE 1
P. clandestina HQ261538
P. iranica HQ261598
P. tentaculata HQ261718
P. infestans HQ261584
P. mirabilis HQ261622
P. ipomoeae HQ261595
P. phaseoli HQ261641
P. hedraiandra HQ261571
P. cactorum HQ261513
P. idaei HQ261579
P. pseudotsugae GU993913
Nic21
Nic6
P. nicotianae FJ801462
Nic7
P. nicotianae HM807369
P. nicotianae AJ854295
Nic19
Nic22
P. nicotianae HQ398876
Nic20
Nic13
P. nicotianae HQ643303
Nic12
Nic11
Nic10
Nic8
Nic9
Nic14
Nic18
P. nicotianae x cactorum FJ499337
Nic5
P. nicotianae EU433396
P. nicotianae x cactorum FJ499333
Nic4
P. nicotianae JX978447
P. nicotianae HQ261629
Nic1
P. nicotianae KF147900
P. tabaci HQ643363
Nic17
P. nicotianae HQ261631
P. nicotianae GU993910
Nic3
Nic15
Nic16
Nic2
P. nicotianae HQ261630
P. nicotianae HQ643301
P. nicotianae
0.2


CLADE 2
Citro5
P. citrophthora HQ643207
Citro4
Citro3
P. citrophthora JN618697
Citro1
P.citrophthora GU993889
P. citrophthora AY228566
Citro2
P. citrophthora
P. botryosa HQ261507
P. botryosa GU993883
P. aff. botryosa HQ643140
P. colocasiae HQ261539
P. meadii HQ643268
Mea1
Mea3
P. meadii HQ261607
Mea2
P. meadii
MeaL
P. meadii like
P. mexicana HQ261620
P. capsici GU993886
P. sp. "glovera" HQ261690
P. tropicalis HQ261720
P. siskiyouensis HQ261674
P. mengei HQ261618
P. multivora HQ261625
P. citricola sensu stricto FJ237526
P. citricola E EU263906
Citr
P. citricola III FJ392327
P. citricola I FJ392322
P. plurivora HQ643311
P. plurivora FJ2375225
P. citricola I/III/E
P. plurivora
P. frigida HQ261567
P. bisheria HQ261501
P. multivesiculata GU993908
0.2


CLADE 6
P. megasperma HQ643281
P. megasperma HQ261612
P. megasperma HQ261613
P. gonapodyides HQ643232
P. gonapodyides HQ643236
P. canalensis HQ261684
P. taxon Pgchlamydo HM004224
Pgch
P. taxon Pgchlamydo JQ307189
P. taxon Pgchlamydo JX996047
P. taxon Pgchlamydo
P. pinifolia HQ261643
P. rosacearum HQ261664
P. lacrimae HQ261693
P. sulawesiensis HQ261711
P. inundata HQ261592
P. humicola HQ261577
P. asparagi HQ261681
0.02


CLADE 3
P. ohioensis HQ261710
P. quercina HQ261658
P. psychrophila HQ261656
P. nemorosa HQ261627
P. pseudosyringae HQ643326
P. pseudosyringae HQ261652
P. ilicis HQ261582
Pseud
P. pseudosyringae GU993912
P. ilicis HQ261583
P. ilicis HQ261580
P. pseudosyringae HQ643329
P. ilicis
P. nemorosa
P. pseudosyringae
0.02


CLADE 8
P. himalayensis HQ643242
P. erythroseptica GU993891
P. cryptogea HQ643210
P. kelmania HQ261691
P. cryptogea GU993890
P. drechsleri HQ261551
P. kelmania HQ261692
Cryp2
P. cryptogea HQ261543
Cryp1
Cryp3
P. cryptogea HQ261541
P. cryptogea HQ261542
P. cryptogea HQ643219
P. erythroseptica HQ643221
P. cryptogea HQ643215
P. cryptogea
P. erythroseptica
P. himalayensis
P. sp. kelmania
P. medicaginis HQ261608
P. medicaginis HQ643273
P. trifolii HQ261719
P. trifolii HQ643368
P. sansomea HQ643342
P. sansomea HQ261669
P. ramorum HQ261662
P. ramorum HQ643339
P. lateralis GU993901
Lat
P. lateralis HQ643263
P. lateralis
P. hibernalis HQ261576
P. foliorum HQ261560
P. primulae HQ261651
P. aff. primulae HQ261492
P. porri HQ643317
P. brassicae HQ261512
P. syringae HQ261714
P. austrocedri HQ261499
0.2

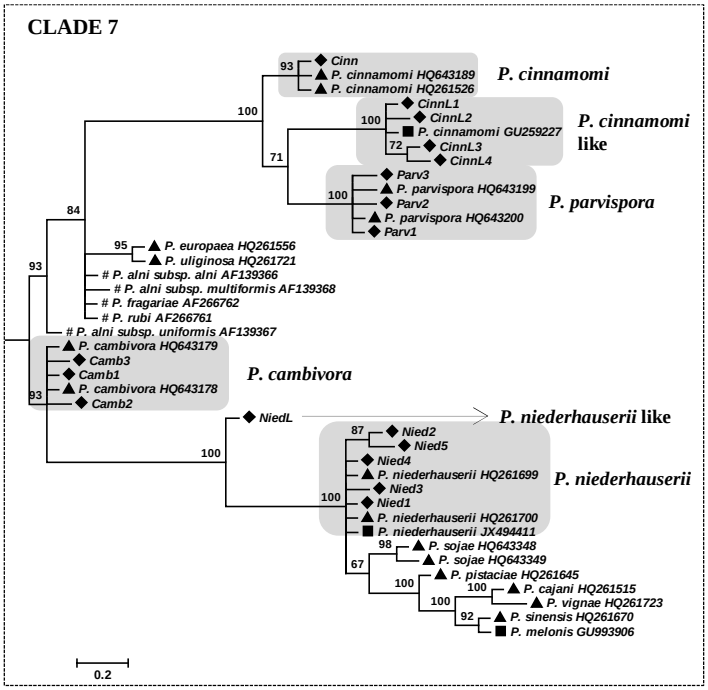

CLADE 7
Cinn
P. cinnamomi HQ643189
P. cinnamomi HQ261526
P. cinnamomi
CinnL1
CinnL2
P. cinnamomi GU259227
CinnL3
CinnL4
P. cinnamomi like
Parv3
P. parvispora HQ643199
Parv2
P. parvispora HQ643200
Parv1
P. parvispora
P. europaea HQ261556
P. uliginosa HQ261721
P. alni subsp. alni AF139366
P. alni subsp. multiformis AF139368
P. fragariae AF266762
P. rubi AF266761
P. alni subsp. uniformis AF139367
P. cambivora HQ643179
Camb3
Camb1
P. cambivora HQ643178
Camb2
P. cambivora
NiedL
P. niederhauserii like
Nied2
Nied5
Nied4
P. niederhauserii HQ261699
Nied3
Nied1
P. niederhauserii HQ261700
P. niederhauserii JX494411
P. niederhauserii
P. sojae HQ643348
P. sojae HQ643349
P. pistaciae HQ261645
P. cajani HQ261515
P. vignae HQ261723
P. sinensis HQ261670
P. melonis GU993906
0.2

Figure 2. Phylogenetic trees built using unique sequences representative of all detected sequence types (◆) along with sequences of reference isolates from Aragon-Caballero *et al.*, 2008 ▼, Brasier *et al.*, 2004 #, Blomquist *et al.*, 2012 ●, Camele *et al.*, 2005 □, French *et al.*, 2011 △, Hurtado-Gonzales *et al.*, 2009 ▽, Jung & Burgess, 2009 , Reeser *et al.*, 2011 ○, Robideau *et al.*, 2011 ▲ and GenBank deposited sequences not associated with specific published articles (■). Separate analyses were conducted for each *Phytophthora* spp. clade. Numbers on nodes represent the statistical support for the Bayesian method.

Dissemination of *Phytophthora* in soil and root samples

Most positive root samples were found to be infected by 1 (8 samples) or 2 (6 samples) *Phytophthora* phylotypes (Table 1). However, root samples of *Petunia parviflora, Lobelia erinus,* and *Dahlia campanulata* from the nursery BL were infected by 3, 4 and 5 different phylotypes, respectively (Table 1; Fig. 3). Similarly, most positive soil samples were found to be infested by 1 (13 samples) or 2 (6 samples) phylotypes, although 5 different *Phytophthora* phylotypes were detected in the soil sample collected in the citrus orchard.

Sequences within the *P. nicotianae* phylotype were detected most abundantly, accounting for 22 out of 55 STs and associated with 15 different hosts from four different nurseries (TP, PV, CP and BL) and the citrus orchard (Table 1; Fig. 3). Some STs of *P. nicotianae* were sourced from both soil and roots (13) while others were detected in just soil (5) or root samples (4) (Fig. 2, 3). Several different *P. nicotianae* STs were associated with a single host from samples collected in the nurseries but a single ST was detected in the soil sample collected in the citrus orchard (Table 1; Fig. 3). STs differed at 12 single base pair locations with two homopolymeric runs of nucleotide bases A (0 to 3 repeats) or T (0 to 2 repeats). Most STs were identical or almost identical to sequences deposited in GenBank. However, four STs (Ni8, Nic9, Nic10 and Nic11) mainly detected in *P. parviflora*, were not present in GenBank and formed a separate bootstrap-supported clade (Fig. 2, 3).

Four phylotypes clustering in the *Phytophthora* clade 2 were detected (Fig. 3). They comprised 5 STs of *P. citrophthora* and 3 STs of *P. meadii*, detected in the citrus orchard and in the nurseries CP and BL (Fig. 3). For both species there was an association between ST and host. Another phylotype

defined as *P. meadii*-like, was represented by a single ST (MeaL) and detected in the nursery BL. Finally, a phylotype represented by a single ST (Citr) matched taxa described as *P. citricola* E or III and was detected in soil samples of *D. campanulata* and *L. erinus* (nursery BL) and in soil and root samples of *Prunus mahaleb* (nursery PG) (Table 1; Fig. 3).

Phytophthora phylotypes clustering in clade 7 were detected in six different nurseries and in the citrus orchard (Fig. 3). Among the detected species, *P. cambivora* was represented by 3 STs and was found in 8 different hosts from the citrus orchard and the nurseries BL and PG. *P. niederhauserii* was represented by 5 STs and was found in 8 hosts from nurseries TP, PV, CP, ST and BL. A single ST of *P. cinnamomi* was detected in nursery TP from *Grevillea lanigera* while 3 STs of *P. parvispora* were found in the soil of the citrus orchard. Furthermore, 4 STs (CinnL1, CinnL2, CinnL3 and CinnL4) defined as *P. cinnamomi*-like, constituted a well-supported group between *P. cinnamomi* and *P. parvispora* and were detected in 3 hosts (*G. lanigera*, *Bouganvillaea glabra* and *Cercis siliquastrum*) in nursery TP and ZZ (Table 1; Fig. 2, 3). Similarly, a ST related to *P. niederhauserii* (NiedL) was detected in soil samples of *Cyclamen persicum* collected in nursery BL (Fig. 2, 3).

Two phylotypes clustering within the *Phytophthora* clade 8 were identified as *P. lateralis* or associated with the species complex of *P. cryptogea* (Fig. 2, 3). *P. lateralis* was represented by a single ST (Lat) detected in the soil of *C. persicum*, *Tagetes erecta* and *Punica granatum* and on the roots *P. parviflora*, in two different nurseries (PV, ZZ). The phylotype associated to the species complex of *P. cryptogea* was represented by 3 STs (Cry1, Cry2, Cry3) detected in 3 different hosts from nurseries TP and CP.

Finally, two single STs detected in nursery ZZ from *C. siliquastrum* were associated with the species complex of *P. pseudosyringae* (clade 3) and to *P.* taxon *Pgchlamydo* (clade 6).

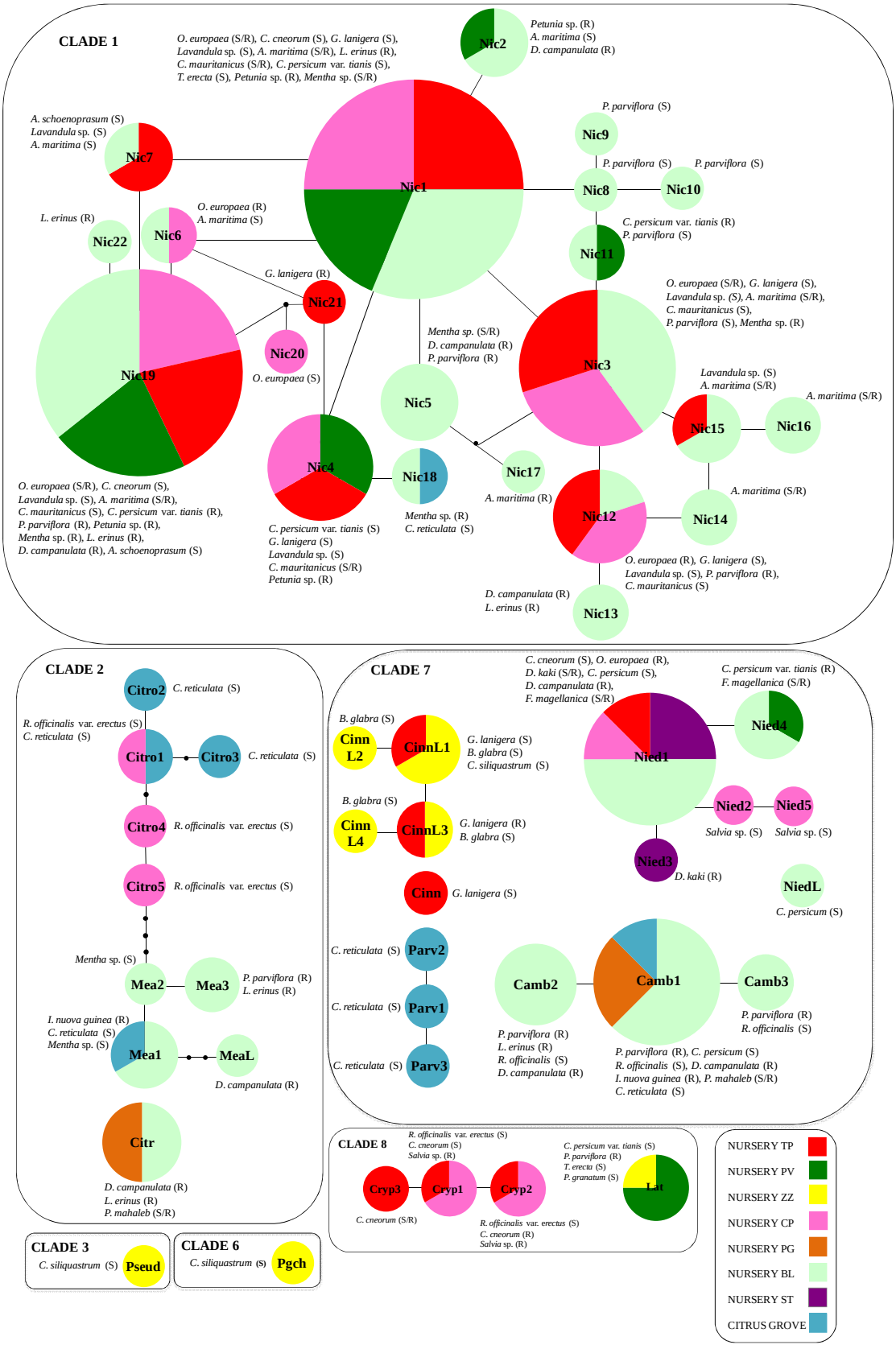

CLADE 1
O. europaea (S/R), C. cneorum (S), G. lanigera (S), Lavandula sp. (S), A. maritima (S/R), L. erinus (R), C. mauritanicus (S/R), C. persicum var. tianis (S), T. erecta (S), Petunia sp. (R), Mentha sp. (S/R)
Nic2
Petunia sp. (R)
A. maritima (S)
D. campanulata (R)
A. schoenoprasum (S)
Lavandula sp. (S)
A. maritima (S)
Nic7
Nic1
P. parviflora (S)
Nic9
P. parviflora (S)
P. parviflora (S)
Nic8
Nic10
L. erinus (R)
Nic22
O. europaea (R)
A. maritima (S)
Nic6
C. persicum var. tianis (R)
P. parviflora (S)
Nic11
G. lanigera (R)
Nic21
O. europaea (S/R), G. lanigera (S), Lavandula sp. (S), A. maritima (S/R), C. mauritanicus (S), P. parviflora (S), Mentha sp. (R)
Nic20
O. europaea (S)
Mentha sp. (S/R)
D. campanulata (R)
P. parviflora (R)
Nic19
Nic3
Nic5
Lavandula sp. (S)
A. maritima (S/R)
A. maritima (S/R)
Nic15
Nic16
Nic4
Nic18
Nic17
A. maritima (R)
A. maritima (S/R)
O. europaea (S/R), C. cneorum (S), Lavandula sp. (S), A. maritima (S/R), C. mauritanicus (S), C. persicum var. tianis (R), P. parviflora (R), Petunia sp. (R), Mentha sp. (R), L. erinus (R), D. campanulata (R), A. schoenoprasum (S)
Mentha sp. (R)
C. reticulata (S)
Nic12
Nic14
C. persicum var. tianis (S)
G. lanigera (S)
Lavandula sp. (S)
C. mauritanicus (S/R)
Petunia sp. (R)
O. europaea (R), G. lanigera (S), Lavandula sp. (S), P. parviflora (R), C. mauritanicus (S)
D. campanulata (R)
L. erinus (R)
Nic13
CLADE 2
Citro2
C. reticulata (S)
R. officinalis var. erectus (S)
C. reticulata (S)
Citro1
Citro3
C. reticulata (S)
Citro4
R. officinalis var. erectus (S)
Citro5
R. officinalis var. erectus (S)
Mentha sp. (S)
Mea2
Mea3
P. parviflora (R)
L. erinus (R)
I. nuova guinea (R)
C. reticulata (S)
Mentha sp. (S)
Mea1
MeaL
D. campanulata (R)
Citr
D. campanulata (R)
L. erinus (R)
P. mahaleb (S/R)
CLADE 7
C. cneorum (S), O. europaea (R), D. kaki (S/R), C. persicum (S), D. campanulata (R), F. magellanica (S/R)
C. persicum var. tianis (R)
F. magellanica (S/R)
Nied4
B. glabra (S)
Cinn L2
CinnL1
G. lanigera (S)
B. glabra (S)
C. siliquastrum (S)
Nied1
Nied2
Nied5
Salvia sp. (S)
Salvia sp. (S)
B. glabra (S)
Cinn L4
CinnL3
G. lanigera (R)
B. glabra (S)
Nied3
D. kaki (R)
NiedL
C. persicum (S)
Cinn
G. lanigera (S)
C. reticulata (S)
Parv2
Camb2
Camb1
Camb3
C. reticulata (S)
Parv1
P. parviflora (R)
R. officinalis (S)
P. parviflora (R)
L. erinus (R)
R. officinalis (S)
D. campanulata (R)
C. reticulata (S)
Parv3
P. parviflora (R), C. persicum (S)
R. officinalis (S), D. campanulata (R)
I. nuova guinea (R), P. mahaleb (S/R)
C. reticulata (S)
CLADE 8
R. officinalis var. erectus (S)
C. cneorum (S)
Salvia sp. (R)
C. persicum var. tianis (S)
P. parviflora (R)
T. erecta (S)
P. granatum (S)
Cryp3
Cryp1
Cryp2
Lat
C. cneorum (S/R)
R. officinalis var. erectus (S)
C. cneorum (R)
Salvia sp. (R)
CLADE 3
C. siliquastrum (S)
Pseud
CLADE 6
C. siliquastrum (S)
Pgch
NURSERY TP
NURSERY PV
NURSERY ZZ
NURSERY CP
NURSERY PG
NURSERY BL
NURSERY ST
CITRUS GROVE

Figure 3. Sequence type **(**ST) network based on *Phytophthora* ITS1 sequences detected in soil and root samples collected from potted plantlets in 8 different nurseries and in a soil sample collected from a citrus grove. The network was constructed using a statistical parsimony algorithm implemented in TCS 1.21 (Clement *et al.*, 2000). Different colors are used to link each ST to sampling locality while the circle size represents the relative frequency of positive samples in which each ST was detected (smallest and largest circles represent 1 and 15 STs, respectively). STs were directly connected without dots when differing by a single change. Every additional putative change was indicated by adding a dot. The name of the host species along with the letters "R" (root samples) and/or "S" soil (soil samples) is reported alongside each ST. Delimitated groups of STs represent different *Phytophthora* spp. clades (Cooke *et al.*, 2000; Kroon *et al.*, 2012). STs were identified according to their phylogenetic collocation (Fig. 1) and named using the first 3-5 letters of the corresponding identified species.

DISCUSSION

In the present study the genetic diversity of *Phytophthora* spp. was investigated in potted ornamental and fruit tree species collected in nurseries located in Apulia and Calabria (Southern Italy) using a molecular method based on a semi-nested PCR with *Phytophthora* genus specific primers (Scibetta *et al.*, 2012). This amplicon-based metagenomic approach provided considerable detail on the diversity of species present in these nurseries and valuable information about the population structure in some taxa. Within the 800 ITS1 clones sequenced in this study we identified nine phylotypes corresponding to defined *Phytophthora* taxa (*P. nicotianae, P. citrophthora, P. meadii, P.* taxon *Pgchlamydo, P. cinnamomi, P. parvispora, P. cambivora, P. niederhauserii,* and *P. lateralis*) and a phylotype associated to *P. citricola* taxon E or III. Although the phylogenetic analysis did not provide an adequate level of resolution for *P. citricola* the accurate analysis of reference sequences showed the consistence of a few polymorphic bases available within the ITS1 region and enabled the reliable identification of the taxa (Table 3; Fig. 1; Jung & Burgess, 2009). On the contrary the identification of phylotypes associated to the *P. cryptogea* and *P. pseudosyringae* species complexes was more challenging due to the remaining taxonomic uncertainties and limited diversity within the ITS1 regions of these groups (Robideau *et al.*, 2011). The ITS1 region is however more variable than the ITS2 region and provides the optimal barcode locus given its sequence length and the risk of PCR chimaera formation when amplifying across the ITS1 and ITS2 regions (Cooke *et al.*, 2000; Robideau *et al.*,

2011; Scibetta *et al.*, 2012). In addition to the identified species or species complex, three phylotypes were considered as representative of unknown *Phytophthora taxa* and defined as *P. meadii*-like, *P. cinnamomi*-like, and *P. niederhauserii*-like.

Among the identified phylotypes, *P. nicotianae* was largely the most abundant. This result was partially expected considering that *P. nicotianae* is responsible for severe foliar and fruit diseases as well as root and crown rots on herbaceous and perennial plant species in more than 250 genera, including horticultural and fruit trees. Different reports have revealed the wide dissemination of *P. nicotianae* in nurseries of potted ornamentals and fruit tree species, however, to the best of our knowledge the present study represents the first evidence of *P. nicotianae* on *Armeria maritima, Convolvolus mauritanicus, T. erecta, Allium schoenoprasum, L. erinus* and *D. campanulata* (Moralejo *et al.*, 2009). According to BLAST analyses many STs detected in the present study were shared with isolates of worldwide origin and from a wide range of hosts including ornamental species. These data support a primary role of the nursery trade as one of the most efficient dissemination pathways of *P. nicotianae* as well as other *Phytophthora* species (Mammella *et al.*, 2011, 2013). Furthermore, in agreement with the presence of both mating types in nurseries (Mammella *et al.*, 2013), the recorded high genetic variation within ITS1 sequences of *P. nicotianae* suggests the existence of multiple introductions throughout the crop cycle, but accurate population analyses would be necessary to confirm this hypothesis and determine the source of genotypes. New introductions could lead to the development of new genotypes or hybrids, which could undermine management practices in areas where these organisms appear to be under control and constitutes a growing threat to local agriculture and natural ecosystems (Brasier, 2008). In agreement with our results, a recent survey on ornamental nurseries in Maryland, revealed a high diversity of *Phytophthora* spp. from small samples of recycled potting media, suggesting the large amount of sources of introduction and spread of variability among *Phytophthora* spp. (Bienapfl & Balci, 2014). In addition, *P. nicotianae* will undoubtedly benefit from

the climatic change. Its host range generally includes those of other species of prime economic importance such as *P. capsici*, *P. infestans* and *P. citrophthora* but generally requires warmer conditions than these other species (Erwin & Ribeiro, 1996). Consequently, the increased risk of rapid spread due to global nursery trade and potential global warming are likely to provide a strong catalyst for the expansion of this species as already reported under Mediterranean climates (Andrés *et al.*, 2003), and Eastern India (Guha Roy *et al.*, 2009).

Ten different STs clustered within the *Phytophthora* clade 2 and were associated with *P. citricola* taxon E or III (Jung & Burgess, 2009), *P. citrophthora* and *P. meadii*. A single ST defined as *P. meadii*-like was genetically distant from the others and probably represents an undescribed taxon. Both *P. citrophthora* and *P. citricola* have been detected in nurseries and have been reported as responsible for serious losses in United States (MacDonald *et al.*, 1994; Donahoo *et al.*, 2006; Garibaldi *et al.*, 2006; Schwingle *et al.*, 2007; Warfield *et al.*, 2008; Yakabe *et al.*, 2009; Leonberger *et al.*, 2013). The detection of *P. citrophthora* on *Citrus* spp. and *Rosmarinus officinalis* is not surprising since these species are well documented hosts of this pathogen (Erwin & Ribeiro, 1996). Similarly, *P. citricola* has been already reported on several flowering plants and *P. mahaleb* (Gadgil, 2005). Much more surprising was the detection of three different STs of a phylotype matching *P. meadii* in soil and/or roots of 4 different ornamental species in the nursery BL and in the citrus orchard. This pathogen is normally distributed in tropical countries and to the best of our knowledge it had not been previously recorded in Italy. Although currently available data does not enable speculation about origin and introduction pathways, the detection of three different STs on different hosts suggests the occurrence of multiple introduction and/or recombination events.

A single ST of *P.* taxon *Pgchlamydo* was detected on *C. siliquastrum*, a tree native of the eastern Mediterranean region and widely distributed in western Asia mainly along the banks of streams. The presence of *P.* taxon *Pgchlamydo* on this plant species is in agreement with its abundance in

streams and the high temperature optima for growth and survival (Jung *et al.*, 2011). *Phytophthora* taxon *Pgchlamydo* has been widely detected in Minnesota and California nurseries and identified as the causal agent of diseases on *Rhododendron, Taxus* spp. and evergreen nursery stock (Schwingle *et al.*, 2007; Yakabe *et al.*, 2009; Blomquist *et al.*, 2012).

A single ST was identified as *P. cinnamomi* and was detected on *Grevillea lanigera*. This pathogen is well established in natural environments and common on economically important plants including *G. lanigera* (Shivas, 1989). Apart from *P. cinnamomi* a closely related phylotype defined as *P. cinnamomi*-like was detected in 3 hosts, including *G. lanigera*, in two different nurseries. BLAST analyses revealed the existence of a sequence (GU259227) identical to the detected STs (CinnL1) for an isolate (P16233) of the World Oomycete Genetic Resource Collection (http://phytophthora.ucr.edu/databasemain.html). Although the sequence of this isolate was deposited as *P. cinnamomi* the phylogenetic analyses conducted in the present study revealed a significant genetic distance that support it as possible new species in line with currently available barcode sequences (Robideau *et al.*, 2011). Interestingly, isolate P16233 was obtained from an ornamental plant (*Rosmarinus* sp.) in a nursery located in California. Another closely related species, *P. parvispora,* was detected in the soil of the citrus orchard with three different STs. This species had been long considered as a variety of *P. cinnamomi* but based on morphological, physiological and molecular analyses it was recently elevated to new species (Scanu *et al.*, 2014). Scanu and co-workers (2014) have reported that almost all findings of *P. parvispora* are linked to the trade of nursery plants and escape from its unknown native environment must have happened only recently since the first record of *P. parvispora* in Europe dates back only 20 years. In this context the detection of *P. parvispora* in the soil of a citrus orchard in Europe represents a new record.

Of relevant importance seem to be also the detection of *P. cambivora* in the soil of the citrus orchard and on seven different ornamental hosts in 2 different nurseries. To the best of our knowledge

none of the hosts identified in the present survey has been reported previously. Indeed, *P. cambivora* is a well known forest pathogen and in the United States is a common root pathogen of commercial stone fruit orchards but has been rarely found in association with nursery crops (Warfield *et al.*, 2008; Yakabe *et al.*, 2009).

A phylotype identified as *P. niederhauserii* was detected in 4 nurseries from 4 different hosts. In recent years, this species has been increasingly reported in greenhouses in association with shrubs and herbaceous ornamentals, but to the best of our knowledge none of the hosts identified in the present study has been previously reported (Abad *et al.*, 2014). In Sicily (Southern Italy) it was isolated from roots of potted sage-leaf rock rose, banksia, mimosa and crimson bottlebrush (Cacciola *et al.*, 2009a, b; Faedda *et al.*, 2013a) while in Valencia province (Spain) it was associated with a severe decline of 2-years-old almond trees in a nursery (Perez-Sierra & Jung, 2013). The detection of *P. niederhauserii* from potted plantlets of *Diospyros kaki* in nursery ST was of interest since it was the only species of *Phytophthora* detected and plantlets showed severe symptoms of dieback. The frequent detection of *P. niederhauserii* phylotypes in the present study and the presence of different STs suggests multiple introductions and spread from nurseries to open field orchards may be a significant threat.

A phylotype clustering within the *Phytophthora* clade 8 was identified as *P. lateralis*. This species has never before been recorded in Italy and until recently was considered to be absent in Europe. It has been rarely detected in nurseries in France and The Netherlands but it was believed to be eradicated (Hansen *et al.*, 1999; Green *et al.*, 2012). Most *P. lateralis* infections in the UK, France and the Netherlands have been on *Chamaecyparis lawsoniana*, however its recent isolation from *Thuja occidentalis* in a nursery led to recommend it for listing as an A1 quarantine organism by the European Plant Protection Organism (EPPO) in 2006 (Schlenzig *et al.*, 2011). The detection of a single ST in two Italian nurseries on four different hosts suggests a recent introduction of the pathogen but also indicates a serious threat since it suggests rapid dissemination is possible once the pathogen is introduced in a

new environment. Another clade 8 phylotype was detected in two nurseries and associated to the species complex of *P. cryptogea* that is a well-documented pathogen in nurseries and greenhouses (MacDonald *et al.*, 1994; Donahoo *et al.*, 2006; Leonberg *et al.*, 2013).

In the present study a total of 55 *Phytophthora* ITS1 STs were detected and considerable within-species variation in ITS1 sequence was observed with between 1 and 22 STs in each of the 15 identified phylotypes. Variability in heterothallic species was generally higher than in the homothallic ones. This may be a reflection of outcrossing events that are particularly favored in the nurseries where many different plant species are grown together, favoring the meeting of different genetically distant isolates. Indeed several different STs of *P. nicotianae* were frequently associated with a single host in samples collected from nurseries, while a single ST of this species was found in the soil of the citrus orchard (Cfr. Fig. 1). In agreement the analysis of mitochondrial and nuclear markers within a broad population of *P. nicotianae* revealed an important role of nurseries in increasing genetic recombination within the species (Mammella *et al.*, 2013). These authors speculated an important role of nursery populations in increasing genetic recombination within the species while isolates from specialized cultivation seem to be mainly the result of asexually propagated clones, adapted to a specific host. In the present study, an exception was represented by *P. cinnamomi* since a single ST (Cinn) was detected in a single host and in a single nursery. However, the high genetic uniformity detected for this heterothallic species may be just the result of its low abundance in the assayed environments suggesting a possible recent introduction of one or both mating types. Furthermore, recent investigations have revealed that hyphal aggregates in plant tissue are a more significant survival strategy for *P. cinnamomi*, even when oospores are present (Jung *et al.*, 2013).

A conventional Sanger sequencing approach was utilized to determine sequences. Although this technique is much less powerful if compared with more recent high-throughput sequencing approaches it has the great advantage of providing very reliable sequences especially if, as done in the present

study, sequences are determined in both directions. This aspect is particularly important for *Phytophthora* since several species are differentiated by a limited number of consistent ITS nucleotide differences (Schena & Cooke, 2006; Robideau *et al.*, 2011; Martin *et al.*, 2012). Considering that artifacts due to DNA polymerase errors could have been theoretically introduced during four different steps (first and second semi-nested PCR, plasmid replication and colony PCR) it is theoretically possible that some of the detected STs are the result of errors. However, several considerations support the authenticity of the data. Firstly, the identified STs were represented by at least two sequences given that singleton sequences were excluded as a precaution. Since the introduction of identical errors in two independently generated sequences is improbable, it seems possible to exclude the existence of errors introduced during the last two steps (plasmid replication and colony PCR). The risk of errors introduced in the first two steps (semi-nested PCR) was greatly reduced by the use of a high-fidelity polymerase (Lindahl *et al.*, 2013). The high-fidelity enzyme also reduced the risk of recombinant (chimeric) amplicons (Lahr & Katz, 2009). The identification of the same STs in different samples (separate extraction and amplifications) provided further evidence for data accuracy. Based on these considerations it is likely that the detected genetic diversity was underestimated rather than overestimated, since some of the excluded single sequences could actually be true STs.

In conclusion results of the present study highlighted a very complex situation in potted ornamental nurseries with large number of *Phytophthora* taxa detected in a limited geographic area. Although some *Phytophthora* species and host pathogen combinations were already well documented, the present study revealed new host-pathogen combinations, new species previously unreported in Italy or Europe and new phylotypes probably representative of still undetermined species showing the high potential value of the molecular diagnostic method used in this study. Although the lack of a culture of these hypothetical new species prevents an accurate evaluation of their role as plant pathogens, this type of molecular analysis offers advance warning of potential threat and enables follow-up targeted

sampling to isolate the organism (Brasier, 2008). Our results reinforce the primarily role of plant nurseries in favoring the introduction and dissemination of *Phytophthora* species and the additional threat of their accelerated evolution via intra- and inter-specific sexual recombination or hybridisation (Brasier, 2008; Faedda *et al.*, 2013b; Leonberger *et al.*, 2013). There is an urgent need for new management strategies based on the enforcement of proactive and preventative approaches to the nursery plant production in order to minimize the risks posed by *Phytophthora* species (Parke & Grünwald, 2012).

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TABLE 2. List of *Phytophthora* species and ITS1 sequence types (STs) identified in potted nursery roots and soils in Apulia and Calabria (Southern Italy), using an amplicon metagenomics approach based on the use of *Phytophthora* genus specific primers (Scibetta *et al*., 2012). Host species in which STs were detected and GenBank accession numbers for sequences are also reported. The name of the host species is followed by the letters "R" and/or "S" to indicate roots and soil samples, respectively. STs were grouped according to the identified species using ITS1 sequence as a barcode gene.

Phytophthora nicotianae

Genotype	SNP sites														Host	Accession number
	7	15	22	39	52	70	75	84	120	121	122	157	164	165		
Nic1	**C**	**-**	**A**	**C**	**G**	**T**	**G**	**T**	**A**	**-**	**-**	**C**	**T**	**-**	*G. lanigera* (S), *Lavandula* sp. (S), *A.* maritima (S/R), *O.* europaea (S/R), *C. mauritanicus* (S/R), *C. cneorum* (S), *C. persicum* var. *tianis* (S), *T.* erecta (S), *Petunia* sp. (R), *Mentha* sp. (S/R), *L. erinus* (R)	**KJ601204**
Nic2	.	.	.	.	.	.	.	.	.	.	.	.	-	.	*Petunia* (R), *A. maritima* (S), *D. campanulata* (R)	**KJ601205**
Nic3	.	.	.	.	.	.	.	.	.	**A**	.	.	.	.	*G. lanigera* (S), *Lavandula* sp. (S), *A. maritima* (S/R), *O. europaea* (S/R), *C. mauritanicus* (S), *Mentha* sp. (R), *P. parviflora* (S)	**KJ601206**
Nic4	.	.	.	.	.	.	.	.	-	.	.	.	.	.	*Lavandula* sp. (S), *G. lanigera* (S), *C. mauritanicus* (S/R), *C. persicum* var. *tianis* (S), *Petunia* sp. (R)	**KJ601207**
Nic5	.	**A**	.	.	.	.	.	.	.	.	.	.	.	.	*Mentha* sp. (S/R), *P. parviflora* (R), *D. campanulata* (R)	**KJ601208**
Nic6	.	.	.	.	.	.	**A**	.	.	.	.	.	.	.	*O. europaea* (R), *A. maritima* (S)	**KJ601209**
Nic7	.	.	.	.	.	.	.	**C**	.	.	.	.	.	.	*Lavandula* sp. (S), *A. maritima* (S), *A. schoenoprasum* (S)	**KJ601210**
Nic8	.	.	.	**T**	.	.	.	.	.	.	.	.	.	.	*P. parviflora* (S)	**KJ601211**
Nic9	.	.	.	**T**	.	.	.	.	.	.	.	**T**	.	.	*P. parviflora* (S)	**KJ601212**
Nic10	.	.	.	**T**	.	**C**	.	.	.	**A**	.	.	.	.	*P. parviflora* (S)	**KJ601213**
Nic11	.	.	.	**T**	.	.	.	.	.	**A**	.	.	.	.	*C. persicum* var. *tianis* (R), *P. parviflora* (S)	**KJ601214**
Nic12	.	.	.	.	.	.	.	.	.	**A**	.	.	.	**T**	*G. lanigera* (S), *Lavandula* sp. (S), *O. europaea* (R), *C. mauritanicus* (S), *P. parviflora* (R)	**KJ601215**
Nic13	.	.	.	.	-	.	.	.	.	**A**	.	.	.	**T**	*D. campanulata* (R), *L. erinus* (R)	**KJ601216**
Nic14	.	.	.	.	.	.	.	.	.	**A**	**A**	.	.	**T**	*A. maritima* (S/R)	**KJ601217**
Nic15	.	.	.	.	.	.	.	.	.	**A**	**A**	.	.	.	*Lavandula* sp. (S), *A. maritima* (S/R)	**KJ601218**
Nic16	.	.	.	.	.	.	.	.	.	**A**	**A**	.	-	.	*A. maritima* (S/R)	**KJ601219**
Nic17	.	**A**	**G**	.	.	.	.	.	.	**A**	.	.	.	.	*A. maritima* (R)	**KJ601220**
Nic18	.	**A**	.	.	.	.	.	.	-	.	.	.	.	.	*Mentha* sp.(R), *C. reticulata* (S)	**KJ601221**

Nic19	.	.	.	.	.	.	**A**	**C**	.	.	.	.	.	.	*Lavandula* sp. (S), *A. maritima* (S/R), *O. europaea* (R), *C. mauritanicus* (S), *C. cneorum* (S), *C. persicum* var. *tianis* (R), *Petunia* sp. (R), *P. parviflora* (R), *Mentha* sp. (R), *L. erinus* (R), *D. campanulata* (R), *A. schoenoprasum* (S)	**KJ601222**
Nic20	**T**	.	.	.	.	.	**A**	**C**	-	.	.	.	.	.	*O. europaea* (S)	**KJ601223**
Nic21	.	.	.	.	.	.	**A**	.	-	.	.	.	.	.	*G. lanigera* (R)	**KJ601224**
Nic22	.	.	.	.	**G**	.	**A**	**C**	.	.	.	.	.	**T**	*L. erinus* (R)	**KJ601225**

Phytophthora citrophthora

Genotype	**SNP sites**					**Host**	**Accession number**
	15	**16**	**71**	**78**	**160**		
Citro1	**A**	**-**	**T**	**C**	**A**	*R. officinalis* var. *erectus* (S), *C. reticulata* (S)	**KJ601190**
Citro2	**.**	**A**	**.**	**.**	**.**	*C. reticulata* (S)	**KJ601191**
Citro3	**.**	**.**	**-**	**T**	**.**	*C. reticulata* (S)	**KJ601192**
Citro4	**-**	**.**	**.**	**.**	**T**	*R. officinalis* var. *erectus* (S)	**KJ601193**
Citro5	**-**	**.**	**-**	**.**	**T**	*R. officinalis* var. *erectus* (S)	**KJ601194**

Phytophthora niederhauserii

Genotype	**SNP sites**				**Host**	**Accession number**
	78	**82**	**149**	**163**		
Nied1	**T**	**A**	**A**	**C**	*O. europaea* (R), *C. cneorum* (S), *D. kaki* (S/R), *F. magellanica* (S/R), *C. persicum* (S), *D. campanulata* (R)	**KJ601226**
Nied2	**.**	**.**	**.**	**T**	*Salvia* sp. (S)	**KJ601227**
Nied3	**C**	**.**	**.**	**.**	*D. kaki* (R)	**KJ601228**
Nied4	**.**	**-**	**.**	**.**	*C. persicum* var. *tianis* (R), *F. magellanica* (S/R)	**KJ601229**
Nied5	**.**	**.**	**G**	**T**	*Salvia* sp.(S)	**KJ601230**

Phytophthora cinnamomi like

Genotype	**SNP sites**			**Host**	**Accession number**
	58	**164**	**166**		
CinnL1	**C**	**G**	**T**	*G. lanigera* (S), *B. glabra* (S), *C. siliquastrum* (S)	**KJ601232**
CinnL2	**G**	**.**	**.**	*B. glabra* (S)	**KJ601233**
CinnL3	**.**	**A**	**.**	*G. lanigera* (R), *B. glabra* (S)	**KJ601234**
CinnL4	**.**	**A**	**C**	*B. glabra* (S)	**KJ601235**

Phytophthora parvispora

Genotype	SNP sites		Host	Accession number
	89	109		
Parv1	**C**	**G**	*C. reticulata* (S)	**KJ601237**
Parv2	**T**	**.**	*C. reticulata* (S)	**KJ601238**
Parv3	**C**	**A**	*C. reticulata* (S)	**KJ601239**

Phytophthora cryptogea/ erythroseptica/ himalayensis/* sp. *"kelmania"

Genotype	SNP sites		Host	Accession number
	3	75		
Cryp1	**A**	**T**	*C. cneorum* (S), *Salvia* sp. (R), *R. officinalis* var. *erectus* (S)	**KJ601200**
Cryp2	**.**	**C**	*C. cneorum* (R), *Salvia* sp. (R), *R. officinalis* var. *erectus* (S)	**KJ601201**
Cryp3	**G**	**.**	*C. cneorum* (S/R)	**KJ601202**

Phytophthora cambivora

Genotype	SNP sites		Host	Accession number
	35	60		
Camb1	**A**	**C**	*R. officinalis* (S), *P. parviflora* (R), *C. persicum* (S), *I. nuova guinea* (R), *D. campanulata* (R), *C. reticulata* (S), *P. mahaleb* (S/R)	**KJ601240**
Camb2	**G**	**.**	*R. officinalis* (S), *P. parviflora* (R), *D. campanulata* (R), *L. erinus* (R)	**KJ601241**
Camb3	**.**	**T**	*R. officinalis* (S), *P. parviflora* (R)	**KJ601242**

Phytophthora meadii

Genotype	SNP sites		Host	Accession number
	99	128		
Mea1	**T**	**-**	*Mentha* sp. (S), *I. nuova guinea* (R), *C. reticulata* (S)	**KJ601195**
Mea2	**.**	**T**	*Mentha* sp. (S)	**KJ601196**
Mea3	**A**	**-**	*P. parviflora* (R), *L. erinus* (R)	**KJ601197**

Phytophthora lateralis

Genotype	Host	Accession number
Lat	*C. persicum* var. *tianis* (S), *P. parviflora* (R), *T. erecta* (S), *P. granatum* (S)	**KJ601203**

Phytophthora citricola* I- III- E/ *plurivora

Genotype	Host	Accession number

Citr	*D. campanulata* (R), *L. erinus* (R), *P. mahaleb* (S/R)	**KJ601199**

Phytophthora cinnamomi

Genotype	Host	Accession number
Cinn	*G. lanigera* (S)	**KJ601236**

***Phytophthora meadii* like**

Genotype	Host	Accession number
MeaL	*D. campanulata* (R)	**KJ601198**

***Phytophthora niederhauserii* like**

Genotype	Host	Accession number
NiedL	*C. persicum* (S)	**KJ601231**

Phytophthora pseudosyringae/ ilicis/ nemorosa

Genotype	Host	Accession number
Pseud	*C. siliquastrum* (S)	**KJ601243**

Phytophthora* taxon *Pgchlamido

Genotype	Host	Accession number
Pgch	*C. siliquastrum* (S)	**KJ601244**

TABLE 3. Comparison of the ITS1 region of the sequence type Citr identified in the present study and reference sequences of *P. plurivora* and *P. citricola* taxon I, III and E (Jung *et al.*, 2011). The alignment shows the consistence of two polymorphic bases enabling the reliable identification of the ST as *P. citricola* taxon III or E.

Genotype	SNP sites*		Accession numbers
	15	66	
Citr	**-**	**-**	KJ601199
***P. citricola* III**	**.**	**.**	FJ392327, EF032477, DQ648146
***P. citricola* E**	**.**	**.**	EU263906, AF266788, EU000081
***P. citricola* I**	**A**	**.**	FJ392322, FJ392321, EU000125, FJ665234, FJ665235
P. plurivora	**.**	**T**	FJ2375223, FJ237523, FJ237524, FJ392324, FJ392325, FJ665225, FJ665226, FJ665227, FJ665228, FJ665229, FJ665230, FJ665231, FJ665232, FJ665233, AF266789, AJ007370, AY879291, DQ486661

*Deleted "-" or identical "." bases as compare to other sequences

CHAPTER 3

Development and validation of 454 pyrosequencing approach to detect *Phytophthora* diversity in ornamental nurseries

ABSTRACT

The diversity of *Phytophthora* communities associated to soil and root samples from potted ornamental and fruit tree species (Cfr. Chapter 2) was investigated by combining genus specific primers and a high-throughput 454 pyrosequencing approch. All sequences were accurately analyzed with appropriate bioinformatic tools and used as barcode for species identification utilizing a validated ITS database. Results were compared with those obtained with the cloning/Sanger sequencing approach (Cfr. Chapter 2). The 454 pyrosequencing confirmed results from cloning/Sanger sequencing and provided a higher levels of accuracy enabling the detection of four additional species (*P. cactorum*, *P. megasperma*, *P. palmivora* and *P. ramorum*) and a general higher level of diversity (number of detected sequence types - STs) within analyzed samples. Moreover, several putative new species undetected with the cloning/Sagner approch were revealed confirming a very high level of sensitivity that enable the detection of rare and/or low abundant phytlotypes. This study represents the first application of pyrosequencing to study *Phytophthora* diversity in nurseries.

INTRODUCTION

Phytophthora is considered one of the most destructive plant pathogens affecting thousands of cultivated and wild plants worldwide with more than 100 species (Brasier, 2009). It has been hypothesized that there might be between 100 and 600 species still unknown to the scientific community (Brasier, 2009; Kroon *et al.*, 2012). Plant trade is considered the primary reason of new disease outbreaks and causes a huge distribution of pathogens beyond their natural endemic ranges with severe socio-economic impact (Brasier, 2008). The increase of international trade of rooted plants as a

consequence of globalization, as well as new production technologies and the continual introduction of new varieties and/or species, exposes nurseries, with particular emphasis on the potted ornamentals sector, to new plant diseases and creates opportunities for pathogens to exploit. Invasive pathogens have been most frequently found on ornamental plants, probably because they represent artificial ecosystems grown under harsh conditions (e.g. warm temperature, high humidity due to frequent irrigation, use of contaminated recycled water, growth of plants in pots) which provide an environment favorable to pathogen attack (Themann *et al.*, 2002).

Many authors speculated about the role of long distance migration via the nursery trade in the spreading of *Phytophthora* inoculum and how the movement of plant material allows the introduction of this pathogen. This phenomenon has been widely investigated for *P. ramorum* and *P. nicotianae* but there are more *Phytophthora* species involved (Goss *et al.*, 2011; Mammella *et al.*, 2011, 2013). In Italy more than 20 *Phytophthora* species were reported in nurseries of ornamentals and the majority of these were identified on new hosts for the first time (Cacciola *et al.*, 2008). In Germany, Minnesota, California, Virginia and Spain between 10 and 17 different species of *Phytophthora* were detected during surveys carried out in nurseries and garden centres (Themann *et al.*, 2002; Schwingle *et al.*, 2007; Moralejo *et al.*, 2009; Yakabe *et al.*, 2009).

The presence of multiple plant species in nurseries and the contact between related but previously geographically isolated pathogens can also play a role in favouring hybridization and generate better-adapted or entirely new pathogen species, e.g. *P. alni* and *Phytophthora* × *pelgrandis* are two hybrids between *P. cambivora* and *P. fragariae*-like (Brasier *et al.*, 2004) and *P. nicotianae* and *P. cactorum* (Faedda *et al.*, 2013) respectively.

The detection of *Phytophthora* spp. in plants (including those currently unknown to science) remains a challenge due to the low efficiency of biological methods (Cooke *et al.*, 2007) and several invasive and previously unknown species have been identified only when they have caused severe

disease in non-native environments. Furthermore, many of these pathogens may do little noticeable damage in their native ecosystems and so are less likely to be detected, having adapted and co-evolved with their hosts. Therefore, an accurate description of the *Phytophthora* community is of an ecological and epidemiological relevance.

Several molecular assay based on PCR have been developed for *Phytophthora* species, even if most of them are inappropriate for broader surveys of its diversity and distribution in ecosystems and detects only one or few species (Cooke *et al.*, 2007; Martin *et al.*, 2012; Sanzani *et al.*, 2013). The development of molecular techniques based on sequencing of specific regions of microorganisms, provides a means to assess the diversity of microbial communities without requiring their cultivation in laboratory and offers a considerable reduction in the cost, complexity and time required to analyze large amount of samples. High-resolution techniques can adequately assist in the task of deep investigation of microbial distribution in environmental samples for epidemiological studies when describing target communities of plant pathogens by providing much greater sequencing depth (Orgiazzi *et al.*, 2013). Furthermore, the large number of reads produced in a single sequencing run provides unprecedented sampling depth, leading to the detection of low abundant and rare phylotypes (Sogin *et al.*, 2006).

Recently 454 pyrosequencing was used to detect *Phytophthora* communities in environmental samples using Oomycete specific primers but the identification of putative species was not as accurate as needed to differentiate closely related *taxa* (Vettraino *et al.*, 2012; Vannini *et al.*, 2013). The aim of the present study was the development and validation of a high-throughput sequencing assay based 454 pyrosequencing (Acosta-Martínez *et al.*, 2008) in combination with genus-specific primers (Scibetta *et al.*, 2012). In order to evaluate the reliability of the method, the same samples (roots and soils from ornamental nurseries) analyzed in Chapter 2 with a cloning/Sanger sequencing were reanalyzed and result were compared.

MATERIALS & METHODS

Sampling and DNA extraction and amplification

Soil and root samples from many ornamental potted plants species were collected during 2013 and 2014 in 8 different nurseries across Apulia and Calabria, Southern Italy (Cfr. Chapter 2). Triplicate DNA extractions were performed from all collected soil and root samples. Purified DNA samples from each nursery were merged and amplified in triplicates using the *Phytophthora* genus specific primers as described in chapter two (Scibetta *et al.*, 2012).

However, SP primers used in the 2nd round-amplification (Scibetta *et al.*, 2012) were modified to obtain fusion primers as recommended for the tag-encoded 454 GS-FLX amplicon pyrosequencing method (Acosta-Martinez *et al.*, 2008). Specific pyrosequencing 25-bp adaptors (Primer A, CGTATCGCCTCCCTCGCGCCATCAG and Primer B, CTATGCGCCTTGCCAGCCCGCTCAG) were ligated to forward and reverse oligos, respectively. Furthermore, eight different Multiplex Identifiers (MIDs) sequences were utilized to associate amplicons to the different sampled nurseries. Soil and root sample from the same nursery were amplified with the same MID as described in Table 1.

In the 1st round of amplifications 1 µL of purified DNA was amplified in a total volume of 10 µL containing 1X AccuPrime™ PCR Buffer II, 0.4 µM of primers and 1 U of AccuPrime™ *Taq* High Fidelity. The same reaction mixture was used in the second round amplification with 1 µL of the 1st round-product as template and 0.2 µM of fusion primers containing different MIDs according to the samples. All amplification conditions consisted of 1 min at 94°C followed by 30 and 25 cycles (1st round and 2nd round amplifications respectively) of 94°C for 30 s, 61°C for 25 s and 68°C for 30 s and by a final step of 68°C for 2 min. All PCRs were conducted in a Mastercycler Ep Gradient S (Eppendorf, Germany).

Amplicons from the 2nd round PCR were purified using the PCRExtract Mini Kit (5PRIME, USA) and quantified as described in Chapter 2. All purified PCR products were pooled in equal volume and sequenced using a 1/8 of a PicoTiter Plate with the 454 GS FLX+ System (Macrogen, Seoul, Korea).

Analysis of data and identification of sequence types (STs)

Raw images obtained from 454 pyrosequencing were processed by Genome Sequencer FLX software provided by 454 platform and translated into raw data. The complete dataset was processed with the bioinformatics pipeline QIIME Version 1.8 (Caporaso *et al.*, 2010). Sequences were subjected to demultiplex, quality trimming and chimera detection. All cleaned sequences were pooled, trimmed to remove sequences of primers, and analyzed with the software ElimDupes (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) to delete multiple identical sequences and identify ITS1 sequence types (STs), defined as the distinct and reproducible ITS1 sequences recovered in this study. Singletons (sequence reads occurring only once across the complete panel of analyzed samples) were removed from the dataset.

To identify the species detected, single representative sequences for each ST were subject to phylogenetic analysis along with validated barcode sequences of the genus *Phytophthora* (Robideau *et al.*, 2011). Before analyses the complete panel of *Phytophthora* reference sequences (Robideau *et al.*, 2011) were trimmed to match the sequence lengths determined in this study and analyzed with the software ElimDupes (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) to delete multiple identical sequences for each species. Identical reference sequences were only included in the panel when they represented different *Phytophthora* species. In cases where no matches were found in the reference sequence from Robideau *et al.* (2011) more closely related sequences were examined using BLAST searches of GenBank with priority given to sequences associated to specific publications

(Fig. 1-5). The complete panel of selected reference sequences and STs detected using the 454 approch were grouped according to their clade (Cooke *et al.*, 2000; Kroon *et al.*, 2012), aligned using ClustalX (Thompson *et al.*, 1997) and introduced to TOPALi for phylogenetic analysis with the PhyML method based on maximum-likelihood principle (Guindon & Gascuel, 2003). By this process, all ITS1 sequences were associated with a phylotype. A phylotype may be represented by single ST or a closely related cluster of ITS1 sequences that are considered to represent a single distinct taxon. We use the term phylotype as a proxy for species in describing the results as species cannot be defined formally in the absence of living isolates.

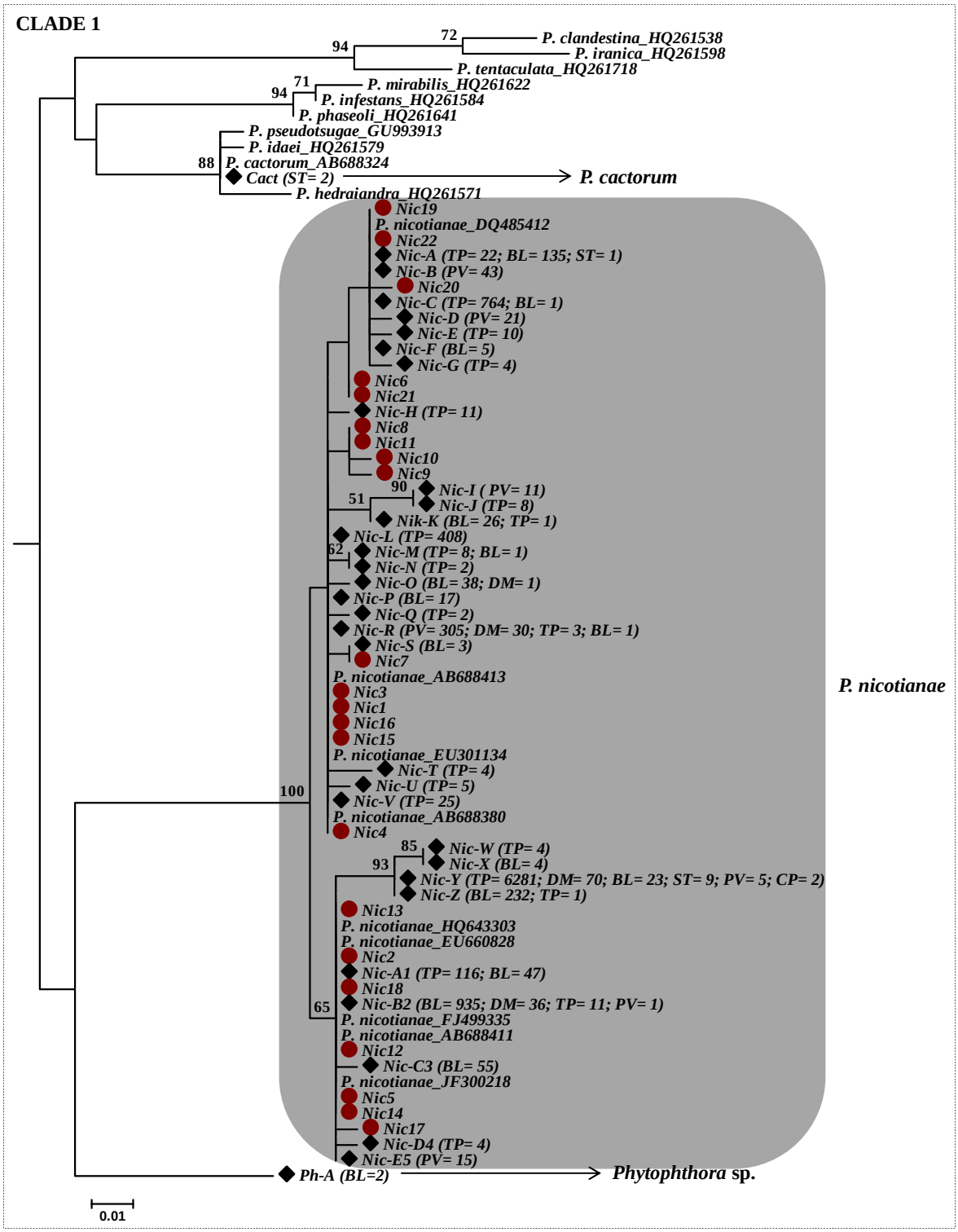

Figure 1. Phylogenetic tree built using unique sequence types (STs) clustering in clade 1. Sequences types detected with the 454 pyrosequencing approach (♦) were analyzed along with STs detected with the cloning/Sanger sequencing approch (●; Cfr. Chapter 2) and sequences of selected reference sequences from GenBank (Robideau *et al.*, 2011). Numbers in parentes indicate the number of sequences represented by each detected ST and the nurseries in which they have beed detected. Numbers on Numbers represent the posterior probabilities for the maximum likelihood method.

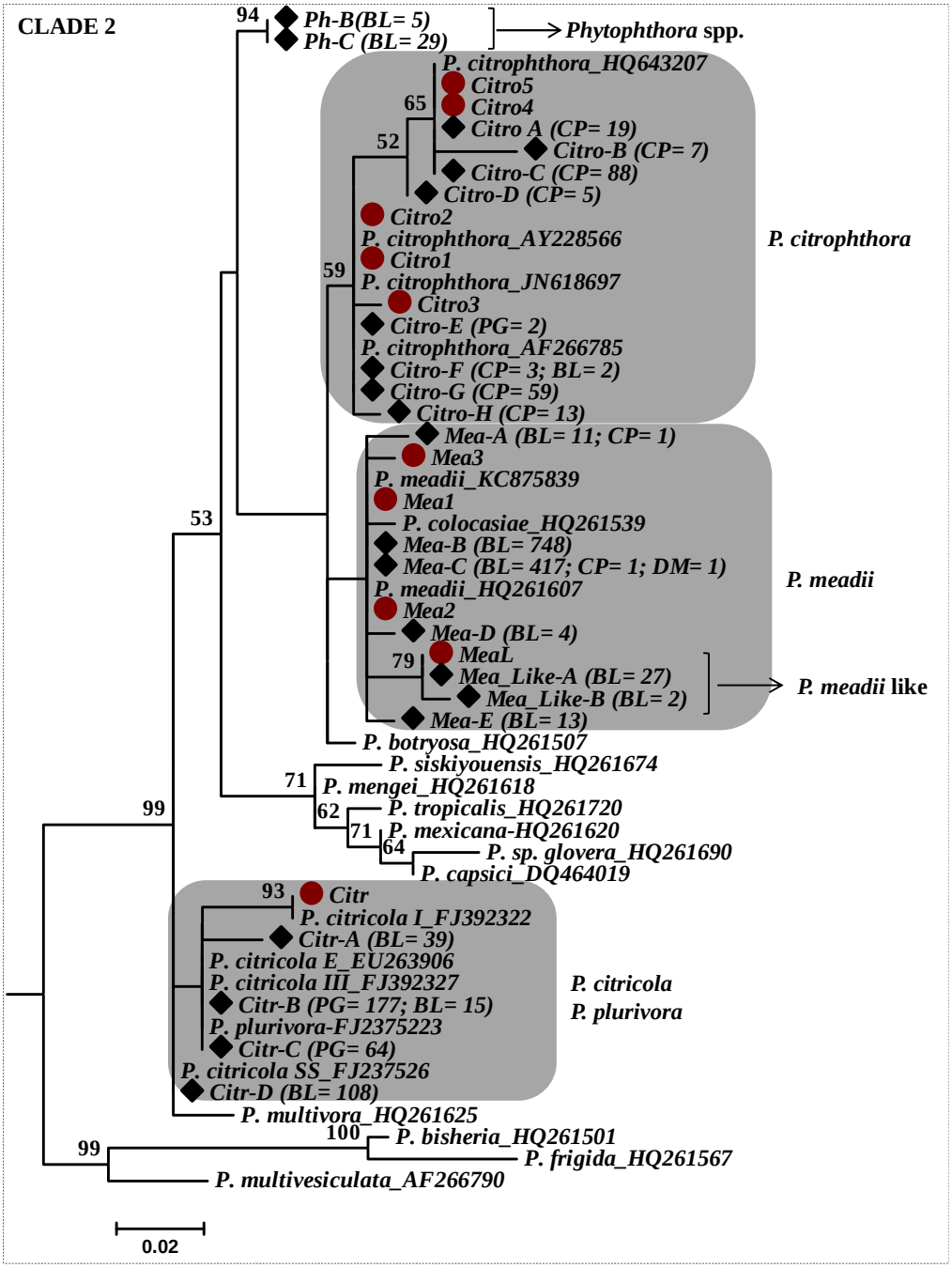

Figure 2. Phylogenetic tree built using unique sequence types (STs) clustering in clade 2. Sequences types detected with the 454 pyrosequencing approach (♦) were analyzed along with STs detected with the cloning/Sanger sequencing approch (●; Cfr. Chapter 2) and sequences of selected reference sequences from GenBank (Robideau *et al.*, 2011). Numbers in parentes indicate the number of sequences represented by each detected ST and the nurseries in which they have beed detected. Numbers on Numbers represent the posterior probabilities for the maximum likelihood method.

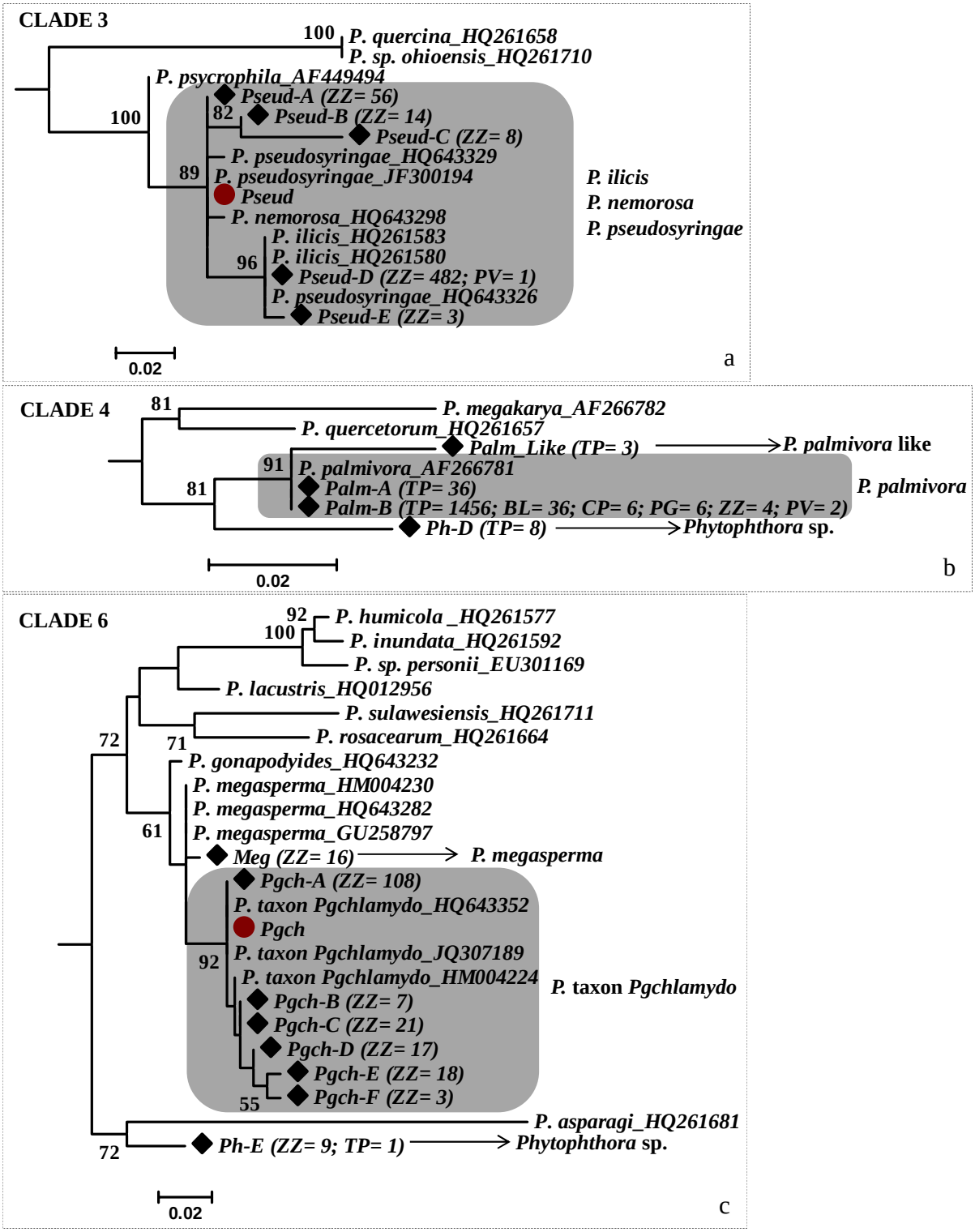

Figure 3. Phylogenetic tree built using unique sequence types (STs) clustering in clades 3 (a), 4 (b) and 6 (c). Sequences types detected with the 454 pyrosequencing approach (♦) were analyzed along with STs detected with the cloning/Sanger sequencing approch (●; Cfr. Chapter 2) and sequences of selected reference sequences from GenBank (Robideau *et al.*, 2011). Numbers in parentes indicate the number of sequences represented by each detected ST and the nurseries in which they have beed detected. Numbers on Numbers represent the posterior probabilities for the maximum likelihood method.

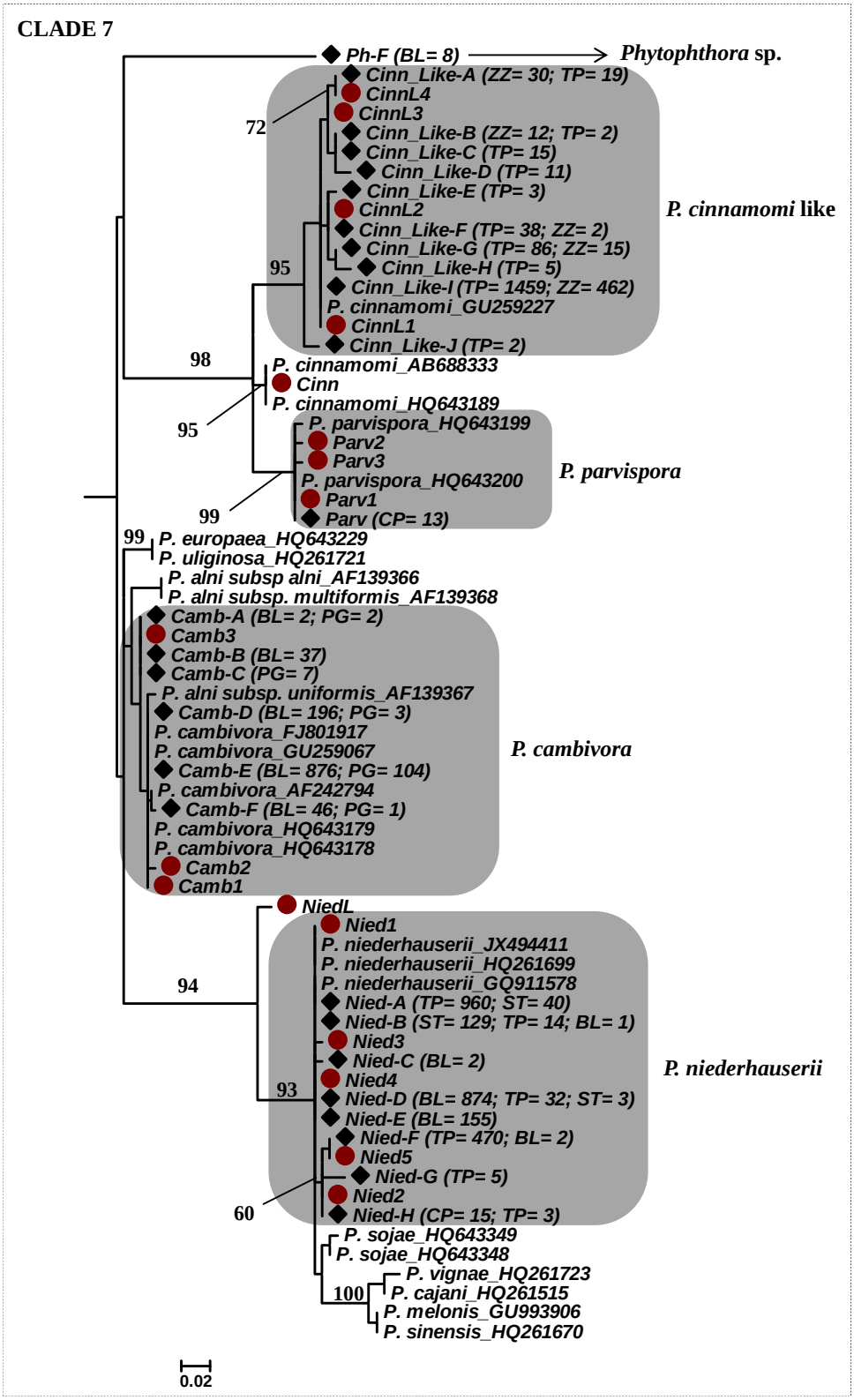

Figure 4. Phylogenetic tree built using unique sequence types (STs) clustering in clade 7. Sequences types detected with the 454 pyrosequencing approach (♦) were analyzed along with STs detected with the cloning/Sanger sequencing approch (●; Cfr. Chapter 2) and sequences of selected reference sequences from GenBank (Robideau *et al.*, 2011). Numbers in parentes indicate the number of sequences represented by each detected ST and the nurseries in which they have beed detected. Numbers on Numbers represent the posterior probabilities for the maximum likelihood method.

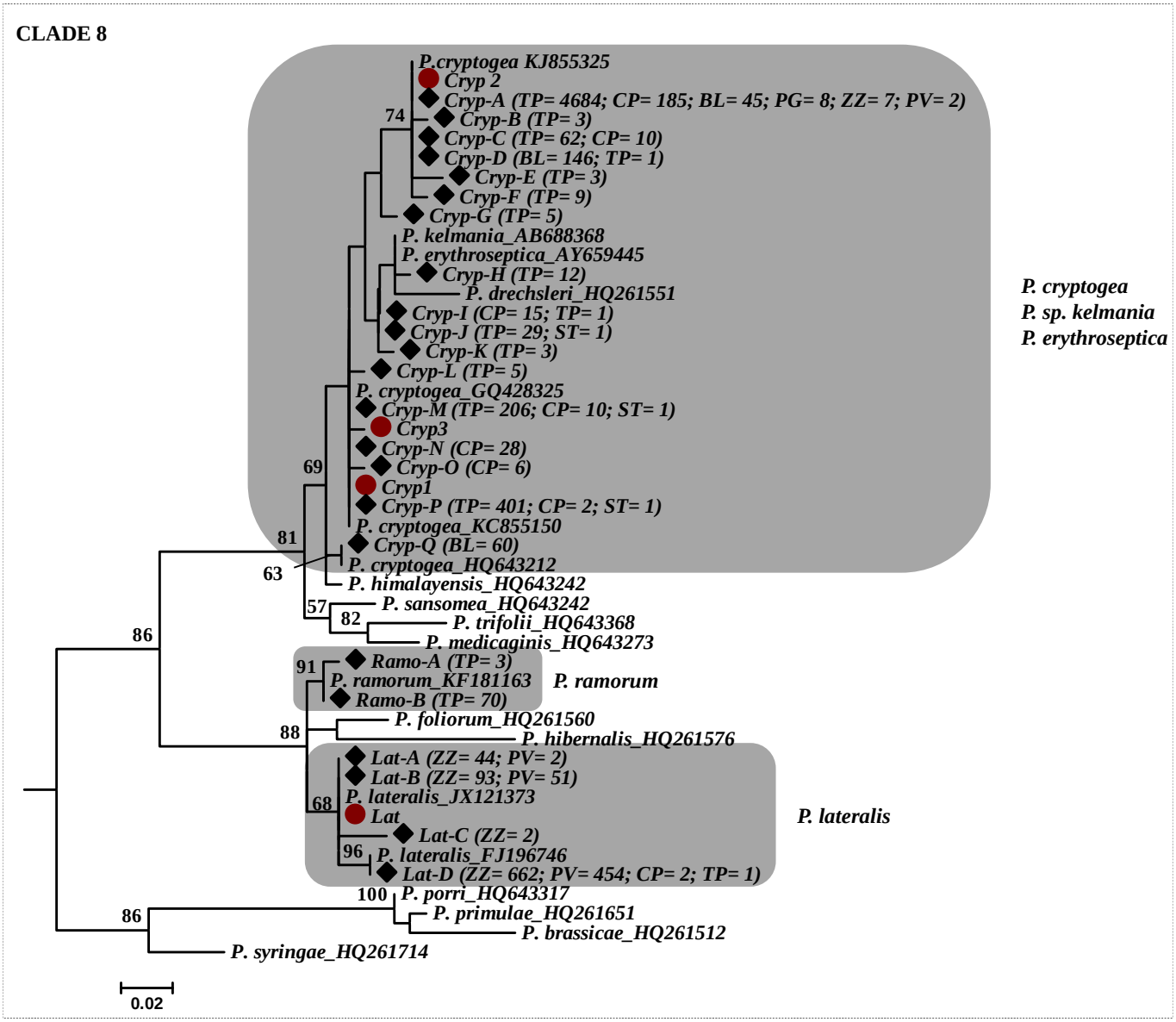

Figure 5. Phylogenetic tree built using unique sequence types (STs) clustering in clade 8. Sequences types detected with the 454 pyrosequencing approch (♦) were analyzed along with STs detected with the cloning/Sanger sequencing approch (●; Cfr. Chapter 2) and sequences of selected reference sequences from GenBank (Robideau *et al.*, 2011). Numbers in parentes indicate the number of sequences represented by each detected ST and the nurseries in which they have beed detected. Numbers on Numbers represent the posterior probabilities for the maximum likelihood method.

To graphically show the relative abundance of different putative *Phytophthora* species, identified according to their filogenetic collocation (Figures 1-5), specific histograms were generated for each sampled nursery according to results of 454 pyrosequencing and cloning/Sanger sequencing (Fig. 6).

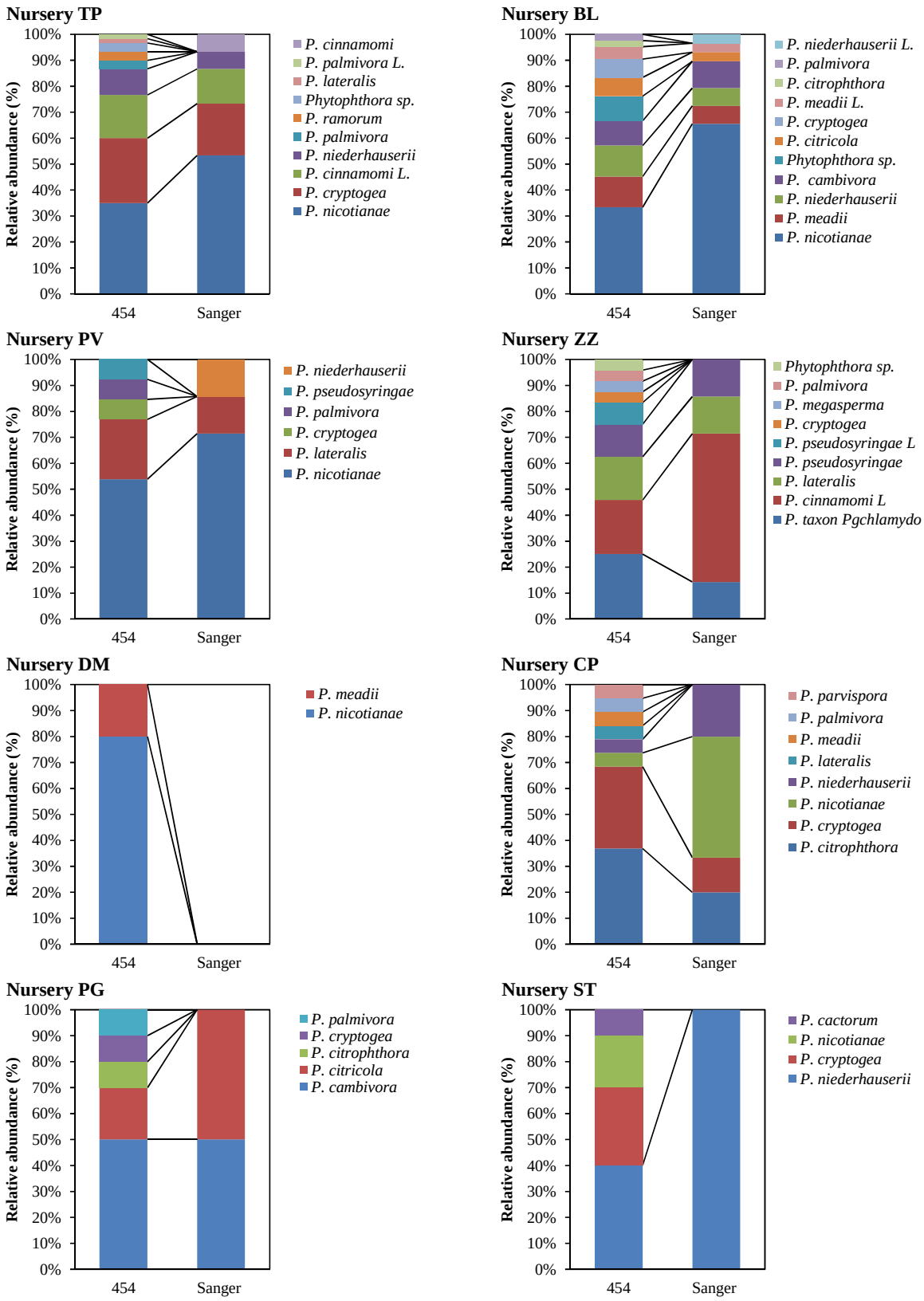

Figure 6. Histograms showing the relative abundance of different putative *Phytophthora* species in the investigated nurseries according to 454 pyrosequencing and cloning/Sanger sequencing approaches. The lettel "L" after the name of some detected phylotypes means "like".

RESULTS

Sequencing results

A total of 76.612 sequence reads were obtained after data processing with 454 Genome Sequencer system with an average length of 270 bp. After quality trimming, denoising and chimera removal 28.067 high quality ITS1 sequences were recorded. The number of reads per sample are reported in Table 1 and ranged from 139 (nursery DM) to 18199 (nursery TP).

Table 1. List of Multiplex Identifier (MID) sequences utilized during 2nd round-amplification to amplify samples from 8 different nurseries located in Apulia and Calabria (Southern Italy; Cfr. Chapter 2). After purification, libraries were mixed and sent for pyrosequencing. The final number of sequences after quality trimming, denoising and chimera removal is also reported.

Sampling Locality	MID Sequences (5'-3')	Sequence Count
Nursery TP (Apulia)	MID 28 (ACTACTATGT)	18.199
Nursery CP (Apulia)	MID 30 (AGACTATACT)	495
Nursery PV (Apulia)	MID 32 (AGTACGCTAT)	924
Nursery ZZ (Apulia)	MID 33 (ATAGAGTACT)	2.100
Nursery DM (Apulia)	MID 37 (TACACACACT)	139
Nursery BL (Apulia)	MID 38 (TACACGTGAT)	5.632
Nursery PG (Apulia)	MID 39 (TACAGATCGT)	375
Nursery ST (Calabria)	MID 29 (ACTGTACAGT)	203

Identification of putative *Phytophthora* species

After the exclusion of singletons, 120 representative STs were identified in the complete panel of analyzed samples (nurseries). According to phylogenetic analyses all STs clustered within the genus *Phytophthora* and were assigned to 24 *Phytophthora* phylotypes distributed in 7 different clades (Fig. 1-5).

Twelve phylotypes were identified to the species level: *P. cactorum, P. nicotianae, P. citrophthtora, P. meadii, P. palmivora, P. megasperma, P.* taxon *Pgchlamydo, P. parvispora, P. cambivora, P. niederhauserii, P. lateralis* and *P. ramorum* (Fig. 1-5). Three phylotypes were

unresolved within their species complexes because the available genetic variation within the ITS1 region did not enable the reliable differentiation of species (Jung & Burgess, 2009; Robideau *et al.*, 2011). They comprised: i) *P. citricola* or *P. plurivora* (from Citr-A to Citr-D; Fig. 2); ii) *P. pseudosyringae, P. ilicis,* or *P. nemorosa* (Pseud-A, B, C, D, E; Fig. 3); and iii) *P. cryptogea, P. erythroseptica,* or *P.* sp. *"kelmania"* (from Cryp-A to Cryp-Q; Fig. 5). Three STs were defined as *Phytophthora* sp.-like because represented well defined clusters phylogenetically related to accepted *Phytophthora* species: i) *P. meadii*-like (STs Mea_Like-A and Mea_Like-B; Fig. 2), ii) *P. palmivora*-like (ST Palm_Like; Fig. 3); and iii) *P. cinnamomi*-like (STs form Cinn_Like-A to Cinn_Like-J; Fig. 4). Finally, 6 STs were defined as *Phytophthora* spp. phylotypes because they clearly clustered within the genus but were very distant from all currently defined species (*Phytophthora* sp. A to F; Fig. 1-5). These putative new species were associate to clade 1 (1 ST), clade 2 (2 STs), clade 4 (1 ST), Clade 6 (1ST), and clade 7 (1ST).

Relative abundance of detected phylotypes

According to the phylogenetic analyses, STs and phylotypes detected with the 454 pyrosequencing approach largely confirmed those previously detected using the cloning/Sanger sequencing approach (Cfr. Chapter 2). Exceptions were represented by a ST associated to *P. cinnamomi* in nursery TP and a ST formerly defined as *P. niederhauserii* like in nursery BL wich were not detected with the pyrosequencing (Fig. 6). Furthermore, *P. niederhauserii* was detected in nursery PV by cloning/Sanger sequencing but not by 454 pyrosequencing.

Apart from above few exeptions, the 454 approach enabled a much more accurate investigation of the genetic diversity with the detection of 29 additional phylotype/nursery combinations as compared to the cloning/Sanger approach (Fig. 6). In nursery DM two species (*P. nicotianae* and *P.*

meadii) were detected with 454 pyrosequencing while no Phytophthoras had been detected with the cloning/Sanger approch.

Among new phylotype/nursery combinations there were previously undetected phylotypes which were associated to the species *P. cactorum* in nursery ST (Fig. 1; Fig. 6), *P. palmivora* in nurseries TP, BL, CP, PG, ZZ and PV (Fig. 3; Fig. 6), *P. megasperma* in nursery ZZ (Fig. 3; Fig. 6), and *P. ramorum* in nursery TP (Fig. 5; Fig. 6). The 454 approach enabled the detection of 6 putative new *Phytophthora* species in clade 1 (ST Ph-A; Fig. 1), clade 2 (STs Ph-B and Ph-C; Fig. 2), clade 4 (ST Ph-D; Fig. 3), clade 6 (ST Ph-E; Fig. 3) and clade 7 (ST Ph-F; Fig. 4). Furthermore, a new phylotype represented by a single ST (Palm_Like) was defined as *P. palmivora* like because of a phylogenetic relatedness to this species (Fig. 3). In general, newly detected phylotypes with the 454 pyrosequencing represented a small portion of the detected populations in each nursery suggesting their presence as rare or low abundant phylotypes (Fig. 6).

Results of 454 pyrosequencing approach confirmed *P. nicotianae* as the most abundant species in ornamental nurseries in Southern Italy (Cfr. Chapter 2). This species accounted for 31 out of 120 STs and was detected in 6 out of 8 nurseries; in 4 of them (nurseires TP, BL, PV, and DM) was the prevalent species (Fig. 6). In other nurseries, prevalent species were *P. niederhauserii* and *P. cryptogea* (nursery ST), *P. cambivora* (nursery PG), *P. citrophthora* and *P. cryptogea* (nursery CP) and *P. taxon PgChlamydo, P. cinnamomi like and P. lateralis* (nursery ZZ).

DISCUSSION

Pyrosequencing is one of the leading technologies supplanting Sanger sequencing for metagenomics (Margulies *et al.*, 2005). In the present study, a protocol based on pyrosequencing was tested to detect the genetic diversity of *Phytophthora* spp. in soil and root samples of potted ornamental and fruit tree species collected in nurseries located in Apulia and Calabria (Southern Italy). In order to

evaluate the reliability of the method, it was utilized to analize the same samples already investigated with a conventional cloning and Sanger sequencing approach (Cfr. Chapter 2). Both methods were based on *Phytophthora* specific primers targeting the ITS1 region of rDNA (Scibetta *et al*., 2012).

With two sole exceptions (a ST associated to *P. cinnamomi* and a ST formerly defined as *P. niederhauserii* like) the 454 pyrosequencing confirmed the detection of all phylotypes revealed using the cloning/Sanger sequencing approach. On the other hand, the 454 approach enabled a much more accurate investigation of the genetic diversity with the detection of 29 additional nursery/phylotype combinations wich included four previously undetetced species (*P. cactorum*, *P. palmivora, P. megasperma*, and *P. ramorum*) and 6 putative new *Phytophthora* clustering with species of clade 1, 2, 4, 6 and 7. Furthermore, a new phylotype defined as *P. palmivora* like because of its phylogenetic relatedness to this species, was detected. These data represented an indirect confirmation of the reliability both molecular methods since identical or very similar STs were detected with the two methods and, as expected, confirmed the higher resolution of the pyrosequencing approach. Indeed all new detected phylotypes with the 454 pyrosequencing represented a small portion of the detected populations in each nursery suggesting their presence as rare or low abundant phylotypes. Therefore, it is likely that they were not detected with the cloning/Sanger sequencing approach just because of a lower resolution of the technique related to the limited number of sequenced clones (20). As regard to the few cases of phylotypes deteced only with the cloning/Sanger sequencing approach, it was likely just the result of the chance in consideration of the low abundance of target sequences.

With both methods, major drawbacks were related to difficulties in discriminating phylogenetically related species that might have sequences identical or differing only by a few nucleotide positions (Kiss, 2012). Most currently utilized bioinformatics tools and genetic databases enable good identification of microorganisms up to the level of the genus but they become less reliable when used to identify species. For this reason results from pyrosequencing were demultiplexed, and

analysed for quality trimming and chimera detection with the bioinformatics pipeline QIIME Version 1.8 (Caporaso *et al.*, 2010), a useful tool to handle thousands of sequences from high-throughput sequencing techniques. After this steps all single representative STs were subjected to a traditional phylogenetic analysis based on the use of common phylogenetic tools along with selected reference sequences in GenBank to guarantee a more detailed identification of single phylotypes.

Among the identified phylotypes, *P. nicotianae* was largely the most abundant. This result confirmed data from Sanger sequencing and supported the wide dissemination of *P. nicotianae* in nurseries of potted ornamentals and fruit tree species (Mammella *et al.*, 2011, 2013). A new ITS1 sequence was detected in soil and root samples from fruit trees collected in nursery ST and was associated to *P. cactorum*. This pathogen occurs worldwide and is capable of infecting more than 200 species in 160 genera including many economically important crops such as apple, pear, rhododendron, azalea, and strawberry (Erwin & Ribeiro, 1996).

Seventeen different STs grouped within the *Phytophthora* clade 2 and were associated with *P. citrophthora*, *P. meadii* and *P. citricola*/*plurivora* complex. These data confirmed results from Sanger sequencing (Fig. 2) even though the phylogenetic analysis did not provide an adequate level of resolution for *P. citricola*/*plurivora* complex (Jung & Burgess, 2009). Moreover, pyrosequencing revealed the presence of *P. citrophthora* and *P. meadii* in more sampled sites and confirmed the presence of a *P. meadii*-like taxon in nursery BL.

A phylotype was associated to the *P. pseudosyringae* complex was mainly detected in nursery ZZ and in a single case in nursery PV. The identification of this phylotype at the species level was not possible due to the limited diversity within ITS1 regions of several related species including *P. ilicis* and *P. nemorosa*.

Phytophthora palmivora was detected in 6 nurseries (TP, BL, CP, PG, ZZ, PV) confirming the ubiquity of this pathogen responsible for many different diseases on a wide range of plants including

ornamental species (Erwin & Ribeiro, 1996; Hansen *et al.*, 2012). A single ST defined as *P. palmivora*-like and detected in nursery TP is likely to represent a still unknown species.

Phylogenetic analysis of pyrosequencing data confirmed the presence of *P. taxon Pgchlamydo* in nursery ZZ and revealed the presence of a new ST not detected with cloning and associated to *P. megasperma*. The detection of this species in nursery ZZ is not surprising since among the sampled plants there was a species (*Cercis siliquastrum*) belonging to the family of *Fabaceae*, a well known group of hosts of this pathogen (Erwin & Ribeiro, 1996).

A phylotype defined as *P. cinnamomi*-like was detected in soil and root samples collected in nurseries TP and ZZ. This group of STs clustered with the ITS1 sequences revealed by Sanger sequencing in the same nurseries. Another closely related species, *P. parvispora*, was detected in nursery CP confirming its presence in Italy (Scanu *et al.*, 2014).

An important result came from the analysis of two STs recorded in nursery TP and identify as *P. ramorum*, a species that was not detected with cloning/Sanger sequencing approch. *P ramorum* is a relatively recently described species of *Phytophthora* (Werres *et al.*, 2001) causing high mortality of oak trees in California, where the disease is known as 'sudden oak death' (Rizzo *et al.*, 2002). In Europe, it is included in the A2 list of the European Plant Protection Organism (EPPO). The pathogen has also been found causing 'ramorum dieback' and 'ramorum leaf blight' on a range of native plants and species of conifer in California and on a range of ornamental plants in Europe. Furthermore, it represents a growing threat from natural ecosystem in UK (http://www.forestry.gov.uk/forestry/INFD-8XLE56). Another critical species within clade 8 (*P. lateralis*) was detected in four nurseries (ZZ, PV, TP and CP) with the 454 pyrosequencing approach while it was only detected in two nurseries (PV and ZZ) by Sanger sequencing. *Phytophthora lateralis* is currently considered quarantine organism locally present in the EPPO regions (A2 list). According to our data there is an urgent need for appropriate management strategies to minimize the risks posed by these two *Phytophthora* species.

A rather obvious limitation of metagenomic sequencing studies is the absence of living microrganisms cultures and the resulting impossibility of determining their actual role in the assayed environment. In the present study 5 different phylotypes clustering within the genus *Phytophthora* were found to be very distant as compared to all currently known species (Ph-A to F) and as a consequence they were considered as representative of putative new *Phytophthora* species. Although, our data indicate that they were present as low abundant or rare phylotypes and although available data does not enable specific speculations about their importance and role, it is undoubted that their presence in nurseries represent a potential new risk for agricultural and natural environments. The complementation of metagenomic investigations with conventional isolation tecniques to obtain cultures as well as others "meta-" data (metatranscriptomic, metaproteomic, and metabolomics) may be useful to obtain a more complete view of microbial communities under given conditions (Zhang *et al.*, 2010; Knief *et al.*, 2011; Segata *et al.*, 2013).

Data of the present study indicated that the use of genus specific primers combined with 454 pyrosequencing are valuable tools to investigate *Phytophthora* diversity in different environments and pathosystems. Compared to the cloning/Sanger approach it enabled much more detailed investigations and reduced time and costs of the analyses. On the other hand, particular attention was needed in the analisys of raw sequence data to avoid errors. In comparison to Sanger sequencing, pyrosequencing technology is known for higher error rates which mainly come from homopolymeric miscounts (Margulies *et al.*, 2005; Huse *et al.*, 2007; Kunin *et al.*, 2010; Gilles *et al.*, 2011; Shao *et al.*, 2013; Knief, 2014). The longer the homopolymeric region, the higher the probability of an indel error and the lower the quality scores of the bases toward the end of this region (Luo *et al.*, 2012; Skums *et al.*, 2012; Niklas *et al.*, 2013). However, the use of a high-fidelity polymerase to reduce the risk of errors and of chimeric amplicons and the analysis of raw data with a quality filter based on the removal of reads with one or more unresolved bases or with errors in the barcode or primer sequence greatly increased the

reliability of selected sequences (Sogin *et al.*, 2006). The exclusion of singletons from downstream analyses further increased the reliability of final results. Indeed, important indirect confirmations of the reliability of the method can be achieved from the following considerations: i) many identical STs were identified in different nurseries i.e. as a consequence of separate extraction and amplifications, ii) most STs detected by 454 pyrosequencing were identical to STs identified by using the cloning/Sanger sequencing approach, iii) most STs detetced only with 454 pyrosequencing were identical to GenBank deposited sequences, and iv) STs associated to new putative *Phytophthora* species were too different from all other detected sequences to hypothesize that they were simple sequencing errors. The large number of *Phytophthora taxa* detected in the present study in a limited geographic area confirms a primarily role of nurseries in favoring the diffusion and the evolution of *Phytophthora* species by favoring both intra- and inter-specific sexual recombination (Brasier, 2008; Faedda *et al.*, 2013; Leonberg *et al.*, 2013).

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CHAPTER 4

Development and validation of a metagenomic approach based on MiSeq Illumina sequencing for the study of *Phytophthora* diversity in water samples

ABSTRACT

A metagenomic approach based on *Phytophthora* spp. specific primers and Illumina MiSeq was utilized to investigate *Phytophthora* diversity in natural Scottish streams and rivers. The performance of the method was preliminarily evaluated using mixtures of DNA or PCR amplicons from 10 selected *Phytophthora* species and reveled that the concentration of the libraries utilized for sequencing has a great impact on the performance of the method. The use of a more concentrated library (8pM) significantly increased the number of reads and enabled the detection of all species included in the panel. The great majority of detected sequences was identical to accepted sequence types (STs), however a swarm of sequences characterized by a low frequency and differentiated by one or few nucleotides as compared to STs, was also revealed. The analysis of *Phytophthora* communities in water samples from 4 different localities yielded up to 896 K paired-end reads clustering in 3373 MOTUs associated to several *Phytophthora* and other *Oomycete* species. Significant differences were revealed in the population of the analyzed sites. New *Phytophthora* phylotypes for which no similarity was found in reference databases were also detected and were likely to represent unknown organisms. Major difficulties related to the discrimination of phylogenetically related species and the risk of ambiguous interpretation of data need to be always taken into account. However, according to our results the proposed method has a huge potential to evaluate *Phytophthora* diversity with an unprecedented level of accuracy.

INTRODUCTION

The influence of plant pathogens on biodiversity is not always immediately obvious and, as a consequence, tends to be overlooked. Among plant pathogens, *Phytophthora* represents an essential component of biodiversity for its ecological, evolutionary and socio-economic significance and for its role in devastating diseases in crop and forest systems (Brasier, 2009).

More than 20 species of *Phytophthora*, including *P. ramorum*, the sudden oak death pathogen, have been isolated from irrigation reservoirs and natural waterways (Hong *et al.*, 2005; 2008; Reeser *et al.* 2007; Werres *et al.*, 2007; Tjosvold *et al.*, 2008), and a number of previously unknown taxa also have been documented in aquatic environments (Brasier *et al.*, 2003; Hong *et al.*, 2008). These pathogens pose a threat to agricultural sustainability and natural ecosystems, as agriculture increasingly depends on recycled water for irrigation in light of rapidly spreading global water scarcity (Hong *et al.*, 2001; 2005). Recycling irrigation systems provide an efficient means of pathogen dissemination from a single point of infection to an entire farm and from a single farm to other farms sharing the same water resources (Hong *et al.*, 2005; 2008). Nevertheless, plant health legislation has been enforced worldwide to prevent the spread of these pathogens and avoid negative ecological impact, there is a surprising lack of information on the aquatic ecology of *Phytophthora* species.

Specific tools are required for direct detection of *Phytophthora* in organic substrates, plant hosts and soil or water samples in a relatively rapid assay and from samples of a meaningful size (Martin & English, 1997). For long time, baiting and culture-based detection assays have been the only available techniques to assess the diversity of *Phytophthora* in natural communities (Jung *et al.*, 2000; Vettraino *et al.*, 2005; Davidson *et al.*, 2005; Jung, 2009). Although still valuable, conventional detection methods have been a constraint on the characterization and analysis of the whole *Phytophthora* diversity (Cooke *et al.*, 2007).

Extraction and identification of DNA from environmental samples has proven noteworthy in detecting and monitoring not only common species, but also those that are endangered, invasive, or elusive (Bohmann *et al.*, 2014). In the last two decades, DNA sequence analysis has greatly contributed to general understanding of the diversity and phylogenetic relationships in the *Phytophthora* genus. The internal transcribed spacer (ITS) regions of the rRNA genes was proved a valuable target for the design of many PCR detection assays that have complemented or replaced isolation and baiting techniques in many host/pathogen combinations (Cooke *et al.*, 2000a). Such molecular assays, however, are geared to the detection of one, or very few, specific known target species (Schena *et al.*, 2008), and, therefore unsuitable in cases where multiple or, as yet undescribed, *Phytophthora* spp. are present. This latter point is recognized as a major weakness of protocols used in international plant health legislation (Brasier, 2008).

Metagenomic DNA sequencing approaches based on PCR amplification of single target regions such as rDNA have been widely used to investigate "*in situ*" microbial communities in a range of terrestrial and marine habitats (Lim *et al.*, 2010), demonstrating the power of such methodology and confirming that rDNA sequence diversity is a valid measure of the occurrence and distribution of phylogenetic types in natural communities. Advances in DNA sequencing and bioinformatics now allow massive and accurate biodiversity assessments of microscopic eukaryotes from environmental samples and the use of high throughput sequencing techniques is dramatically increasing the power of this approach (Lim *et al.*, 2010). These new sequencing techniques allow in depth analysis through a variety of sequencing methodologies that are not possible with standard Sanger sequencing. Common features to second generation sequencing approaches are the addition of tags to amplicons to track which amplicons come from what sample, the generation of millions of sequences at once which increases the reliability and scope of analysis, and the ability to generate sequence data in a much more cost-effective manner.

The aim of the present study was the development, validation and application of a high throughput metabarcoding method for studying *Phytophthora* diversity. The method is based on the molecular analysis of the ITS1 region of *Phytophthora* spp. from water samples from Scottish natural and semi-natural habitats and their sequencing with the high-throughput MiSeq Illumina system. MiSeq platforms use bridge amplification to produce up to 1.6 billion sequences per flow cell with a maximum length for paired-end reads up to 300 bp. The evaluation of a suitable protocol for Illumina sequencing was critical to enable detection of the overall diversity and avoid suboptimal sequencing conditions.

MATERIALS & METHODS

Development of artificial systems (positive controls)

The accuracy and sensitivity of the MiSeq Illumina system was preliminarily evaluated using artificially developed systems containing DNA from pure cultures or their PCR products. Ten *Phytophthora* isolates sourced from the culture collection of The James Hutton Institute (Dundee, Scotland) were selected to represent different *Phytophthora* clades (Cooke *et al.*, 2000b) and different sizes of ITS1 amplicons (Scibetta *et al.*, 2012). Furthermore, prevalence was given to species unreported in Scotland (Table 1).

DNA extracted from the 10 isolates was mixed and diluted in order to have a final concentration of 5ng/µl of each *Phytophthora* species. One µl of total DNA was then subjected to semi-nested PCR using the *Phytophthora* spp. specific primers (Scibetta *et al.*, 2012) properly modified to enable analyses with the MiSeq Illumina system (See below).

Alternatively, separate semi-nested PCR amplicons obtained from each isolate were mixed in equal proportions, diluted 20 times and analyzed using the MiSeq Illumina system.

Natural water samplings

Water samples were collected from four different Scottish streams located in Invergowrie, Dundee (IGB), Glensaugh Research Station and the Cairngorms National Park (GSB and ECN, respectively) in Angus, and Sourhope Research Station in "Scottish Borders" (SRB). Samples were collected every two weeks from December 2011 to March 2014. Water (10 L) was filtered directly in the field using a clean knapsack sprayer to pressurize an in-line polypropylene filter holder (XX4304700, Millipore, UK) into which a mixed cellulose esters filter (RW1904700, Millipore, UK) was fitted (diameter 47 mm and porosity 1.2 µm). The cellulose filters were stored in sterile tubes and maintained on ice after sampling. Samples were immediately returned to the laboratory in plastic containers and stored at -20 °C until DNA extraction.

DNA extractions

To extract DNA from pure *Phytophthora* isolates lyophilized mycelia were ground in the presence of silica beads (Ø 0.1) and 23 µl of NaOH in 1.5 ml Eppendorf tube. Tubes were spin at 13000 rpm for 5 min and 10 µl of the upper phase resuspended in 90 µl of sterile distilled water. After extraction, DNA samples were analyzed by electrophoresis in 1.5% agarose gel with SYBR Safe™ DNA gel stain (Invitrogen, UK). Nanodrop (Thermo Fisher Scientific Inc.) was used to measure absorbance at 260, 280 and 230 nm and estimate concentration and contamination with protein and humic acid.

DNA from water samples was extracted using a slightly modified version of the method described by Scibetta *et al.* (2012). Wet filters were halved and one half cut into about 5 mm squares, placed in 2 ml Eppendorf tube and freeze-dried for at least 1 day. Each sub-sample was blended in a Mini-BeadBeater-8 (Bio Spec Products Inc., US) for three 1 min periods at 3000 rpm with 1.5 ml of SDS lysis buffer (200 mM Tris–HCl [pH 8], 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and 0.6 g of a mix of silica beads (Ø 0.1 and 1 mm). Tubes were centrifuged at 13,000 rpm for 15 min and the upper

phase was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and 100% chloroform (−20 °C), respectively. DNA was precipitated by adding an equal volume of isopropanol and a tenth of volume of 3M Sodium Acetate (pH 5.2), washed twice with cold 100% and 70% ethanol, dried and re-suspended in 50 µL of HPLC water.

Total DNA was purified through single chromatography columns (Micro Bio-Spin columns, Bio-Rad, Hercules, CA) filled with 500 µl of Sepharose 2B (Sigma-Aldrich, UK) (Miller, 2001). Before use, the columns were conditioned by two sequential additions of 150 µl of HPLC water, each followed by a 3 min centrifugation at 1500 rpm. All DNA samples (50 µl) were added to the top of the columns and centrifuged for 3 min at 1500 rpm. Purified eluates were collected in a sterile 1.5 ml tube and stored at −20 °C before molecular analysis.

Purified environmental DNA samples were analyzed by electrophoresis in 1.5% agarose gel with SYBR Safe™ DNA gel stain (Invitrogen, UK). Nanodrop (Thermo Fisher Scientific Inc.) was used to measure absorbance at 260, 280 and 230 nm in order to estimate DNA concentration and its level of contamination with proteins and humic acids. Furthermore, to confirm that all DNA samples were of sufficient quality to be amplified by PCR, 1 µl of each undiluted DNA sample was amplified using the universal primers ITS6–ITS4 (White *et al.*, 1990). Extractions were repeated using the second half of the filters in case of poor/low quality DNA.

PCR reactions

One µl of a mixture of DNA from 10 selected *Phytophthora* species or from water samples was amplified by semi-nested PCR using the SP *Phytophthora* spp. specific primers described by Scibetta *et al.* (2012). In both cases, SP primers were modified to fit the MiSeq Illumina protocol requirements. In particular, overhang adapter sequences were appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters. According to the manufacturer protocols the Illumina

overhang adapter sequences added to forward and reverse SP primers were 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3', respectively.

All PCR reactions were carried out in the same conditions using a Primus 96plus Thermalcycler (MWG-Biotech), but first and second rounds of amplifications (nested PCR) were performed in a total volume of 15 µl and 25 µl, respectively. PCR reactions contained 1x KAPA HiFi HotStart Ready Mix (Kapa Biosystems, US), 0.3 µM of primers and 1 µl of DNA (1 µl of the 1st round-product was added in the 2nd round mix). Amplification conditions for *Phytophthora* spp. specific primers consisted of 1 cycle of 95°C for 3 min, 30 cycles (1st round) or 25 cycles (2nd round) of 98°C for 20 s, 61°C for 25 s, 72°C for 40 s (1st round) and 25 s (2nd round) and a final cycle of 72°C for 1 min. After nested-PCR, the Agencourt AMPure XP beads (Beckman Coulter, US) were used to purify amplicons according to the protocol described by the manufacturer.

For the universal primers ITS6–ITS4, 1 µl of DNA was added in a PCR mix of 1X Green GoTaq® Flexi Buffer (Promega, US) 0.4 mM PCR Nucleotide Mix, 1U GoTaq® G2 Flexi DNA Polymerase and 0.4 µM of forward and reverse primers. Amplification conditions consisted of 1 cycle of 95° C, 30 cycles of 95° C for 20 s, 55° C for 25 s, 72° C for 50 s and a final sycle of 72° C for 5 min. Amplicons were separated by electrophoresis in 2% agarose gels containing SYBR Safe™ DNA gel stain (Invitrogen, US), in TBE buffer and visualized on UV light. The size of each band was estimated using a 100 bp DNA ladder (Promega, US).

Many precautions were taken to avoid DNA contamination of the PCR reactions (Van Pelt-Verkuil *et al.*, 2008). In nested PCR, first and second round PCR reactions were set up in separate laminar flow hoods located in separate laboratories. Both laminar flow hoods were repeatedly cleaned with 0.2 M NaOH and 70% EtOH. A special set of pipettes was maintained for DNA amplifications

and filter tips (Axygen Scientific, UC, USA) were used at all times. Prior to use, the HPLC water was UV-treated to denature any contaminating nucleic acids.

Sample preparation for MiSeq Illumina sequencing

After PCR reactions a subsequent limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters and obtain unique libraries. PCR amplifications were performed in a final volume of 50 µl containing 1x KAPA HiFi HotStart Ready Mix (Kapa Biosystems, US), 5 µl of Nextera Index primers A and B and 5 µl of nested-PCR product. Amplification conditions consisted of 1 cycle of 95° C for 3 min, 8 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final cycle of 72°C for 5 min. After PCR, Agencourt AMPure XP beads (Beckman Coulter, US) were used to purify the amplicons.

To verify the size of the final library, 1 µl of a 1:50 dilution of each PCR product was run using the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Using *Phytophthora* specific primers modified for Illumina sequencing, the expected size of the final library was about 350 bp. All libraries were quantified by the Qubit® 2.0 Fluorometer (Invitrogen, UK), diluted to 4 nM and pooled. Before sequencing a qPCR was performed for an accurate quantification of the final library. qPCR was performed in a final volume of 20 µl prepared with 1x KAPA SYBR FAST qPCR Master Mix containing Primer Premix (Kapa Biosystems, US) and 4 µl of 1:1000, 1:2000, 1:4000, 1:8000-diluted library or Illumina DNA standards (Kapa Biosystems, US).

In preparation for cluster generation and sequencing, pooled libraries were denatured with 0.2 N NaOH, diluted with hybridization buffer, and then denatured before MiSeq sequencing. To find optimal sequencing conditions, two different runs were performed at a final concentration of the denatured DNA of 4 and 8 pM. Each run included 10% of PhiX (Illumina, US) as an internal control during sequencing.

For sequencing, the fast-turnaround MiSeq - Illumina system was used with the MiSeq Reagent Kit v2 in the 500-cycles (2x250) format to allow the longest read lengths. It provided up to 525 cycles of sequencing, sufficient for up to a 251-cycle paired-end run plus two eight-cycle index reads. The expected result was approximately 10,000 reads per sample for a total of 500 Mbp in less than 48 hours. The estimated MiSeq run output was, recognized as sufficient for this metabarcoding survey.

Analysis of sequences

Demultiplexed raw data from Miseq were aligned using Pandaseq Assembler software (Masella *et al.*, 2012). Results were processed with the bioinformatics pipeline QIIME Version 1.8 (Caporaso *et al.*, 2010). Chimeras and forward and reverse oligos were removed. The open-reference OTU picking algorithm with a similarity threshold of 99% and the singletons removal option enabled, was utilized for assigning sequences to molecular operational taxonomic units (MOTUs). The BLAST method was used to assign taxonomy from a non-redundant selected and manually checked database derived from GenBank, for a total of 2812 ITS1 sequences from all *Oomycetes*. For statistical analysis, a workflow script for computing β-diversity distance matrices and generating PCoA (Principle coordinates Analysis) plots to assess differences between microbial communities was used.

Table 1. *Phytophthora* species included in this study and used as control for the protocol. Isolates were sourced from the culture collection of The James Hutton Institute (Dundee, Scotland).

Species	Clade	ITS1 size
P. boehmeriae	10	172
P. capsici	2	140
P. cryptogea	8	174
P. idaei	1	184
P. fallax	9	172
P. katsurae	5	182

P. megasperma	6	193
P. palmivora	4	179
P. plurivora	2	152
P. rubi	7	199

RESULTS

Evaluation of MiSeq Illumina sequencing runs with positive controls

Results from positive controls (DNA and PCR mix) revealed a considerable higher numbers of sequences in the concentrated library (8 pM) as compared to standard concentration (4 pM) recomended by Illumina guidelines. Using a mixuture of DNA amplicons, 770 and 3916 reads were obtained with 4 and 8 pM, respectively (Fig. 1). Similarly, using a mixture of DNA, 719 and 4014 reads were obtained with 4 and 8 pM, respectively (Fig. 1). In general, all *Phytophthora* included in the panel were detected using an amplicon mixture at both 4 and 8 pM. On the contrary using a DNA mix and the lower library concentration (4 pM) two species (*P. boehmeriae* and *P. palmivora*) were not detected and another species (*P. fallax*) was associated to a very low number of reads (Fig. 1). The detection coverage greatly increased at 8 pM since all Phytophthoras were detetced although few reads were associated to *P. boehmeriae*, *P. palmivora* and *P. fallax.*

The analyses of sequences revealed that the great majority was identical or highly similar (*P. fallax*, *P. idaei*, *P. megasperma* and *P. rubii*) to sequence types (STs) of species included in the panel (obtained in both directions by Sanger sequencing). However, for all detected species (10) a swarm of sequences characterized by a low frequency and differentiated by one or few nucleotides as compared to STs, was also revealed.

This swarm of sequences greatly complicated the analysis of data with the Qiime pipeline and made the identification of closely related species, challenging.

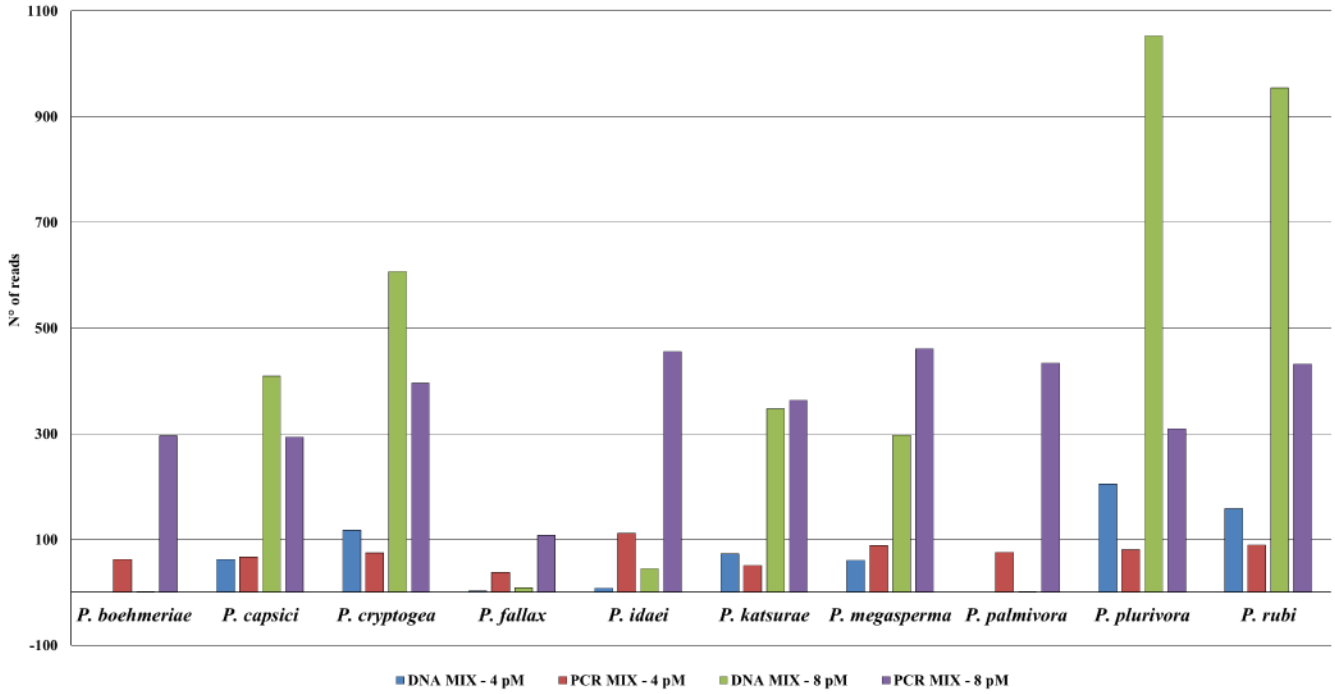

Figure 1. Comparison between sequencing outputs from control reactions (DNA and PCR mix) at 4 and 8 pM – concentrated library.

Sampling and DNA extraction from natural water samples

The filtration strategy utilized for water samples was useful to capture environmental-DNA (eDNA) as it allowed the concentration of eDNA fragments from large volumes of water. Protocols utilized to extract DNA from wet filters yielded nucleic acids appropriate for PCR amplifications after the purification step with chromatography columns. Conventional PCR with ITS6-4 universal primers revealed that once purified DNA samples did not affect TaqDNA polymerase activity (data not shown). In a few cases the presence of residues of soil on filters negatively affected DNA extractions that were repeated.

Amplification results

A total of 149 DNA samples from wet filters collected during the two-year long survey were analysed by the semi-nested assay. 101 of them (6 from ECN, 48 from IGB, 14 from SRB and 31 from GSB) produced a positive amplification in at least one of the two analysed filter halves. Among these, no samples produced a positive amplification after the first PCR step, confirming the need for a nested approach to yield reliable levels of sensitivity (Scibetta *et al.*, 2012). The six positive PCR fragments from ECN samples were pooled to form a single sample before sequencing. As a consequence, a total of 94 unique libraries from environmental samples (1, 48, 14 and 31 from ECN, IGB, SRB and GSB, respectively) and 2 libraries from positive controls (see above) were pooled before Illumina sequencing.

Sequencing results

After sequencing of 4pM and 8 pM-concentrated libraries, 131.478 and 896.206 sequence reads respectively, were obtained. The number of reads per sample ranged from 3 to 7017 after sequencing of the 4 pM-concentrated library and from 59 to 46.369 after sequencing of the 8 pM-concentrated library. Other technical variants are showed in table 2.

Table 2. Sequencing performances with 4 and 8 pM-concentrated libraries.

	4 PM – SEQUENCING RUN	**8 PM – SEQUENCING RUN**
CLUSTERS DENSITY* (K/mm²)	163	744
CLUSTERS PASS FILTER† (%)	86	95
READS PASS FILTER§ (M)	0,217	1,08
READS > Q30 †† (%)	95	78
READS (K)	131	896

*Number of clonal clusters generated from each DNA amplicon on a flow cell before sequencing. Install specifications based on Illumina PhiX control library at supported cluster densities suggested between 467-583 k/mm2 clusters passing filter for v2 chemistry Performance parameters may vary based on sample type, sample quality, and clusters passing filter.
† § The "filter" is a quality control for those clusters, which are capable of producing good sequencing, reads. It is influenced by overall cluster density and relative positions of clusters on the flow cell.
†† A quality score (Q-score) is a prediction of the probability of an error in base calling. The percentage of bases > Q30 is averaged across the entire run. The minimum Q30 score for a 2X250 bp run is >75% "passing filter".
K= thousands, M = millions.

Detection of *Phytophthora* species in natural samples

In agreement with control samples the use of a concentrated library (8 pM) to analyze water samples yielded better results in terms of both number of detected sequences per each sample and number of detected species/phylotypes. A total of 3373 MOTUs were associated to *Phytophthora* spp. and other *Oomycetes* (Fig. 2). Comparison among samples showed a clear site-to-site variation with less diversity in GSB and more in IGB (Fig. 3). In general, the majority of sequences were associated to the genus *Phytophthora* species with the exception of the SRB site, in which 62,2% of sequences corresponded to the genera *Peronospora, Hyaloperonospora, Plasmopara* and *Bremia* (Fig. 3). However, in sites ECN, GSB and IGB, *Phytophthora* species represented 63,6%, 83,8% and 69,8% of total recovered sequences (Fig. 3). These data confirmed results from cloning (data not shown) in which the majority of sequences recovered was assigned to *Phytophthora* spp. while a low percentage was assigned to downy mildew genera (*Peronospora*, *Hyaloperonospora*, *Pseudoperonospora*, *Plasmopora* and *Bremia*). An insignificant proportion of sequence reads belonged to other genera in the oomycetes.

Among *Phytophthora* species, *P. infestans* was most abundant, especially in GSB, where an 81,6% of sequences was assigned to this phylotype (Fig. 3). Interestingly a very low concentration of quarantine pests was detected (Fig. 2-3).

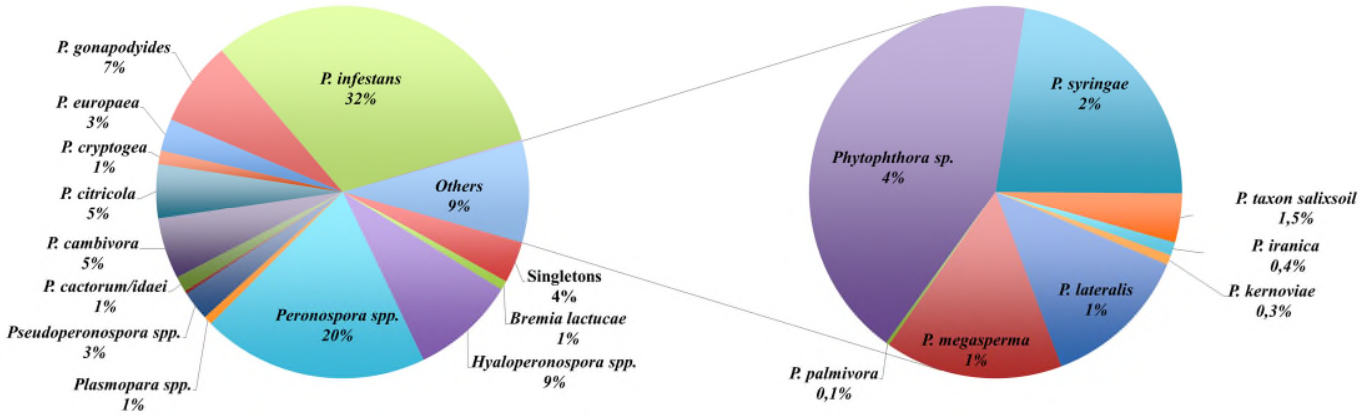

Figure 2. Taxonomic summary describing microbial diversity in natural water samples collected in Scotland using a 8 pM-concentrated library and MiSeq sequencing. Single sequences (singletons) were excluded from the taxonomic analysis. A tiny proportion of reads was assigned to *Pythium*, *Saprolegnia* and *Phytopythium*, revealing a high specificity of primers used.

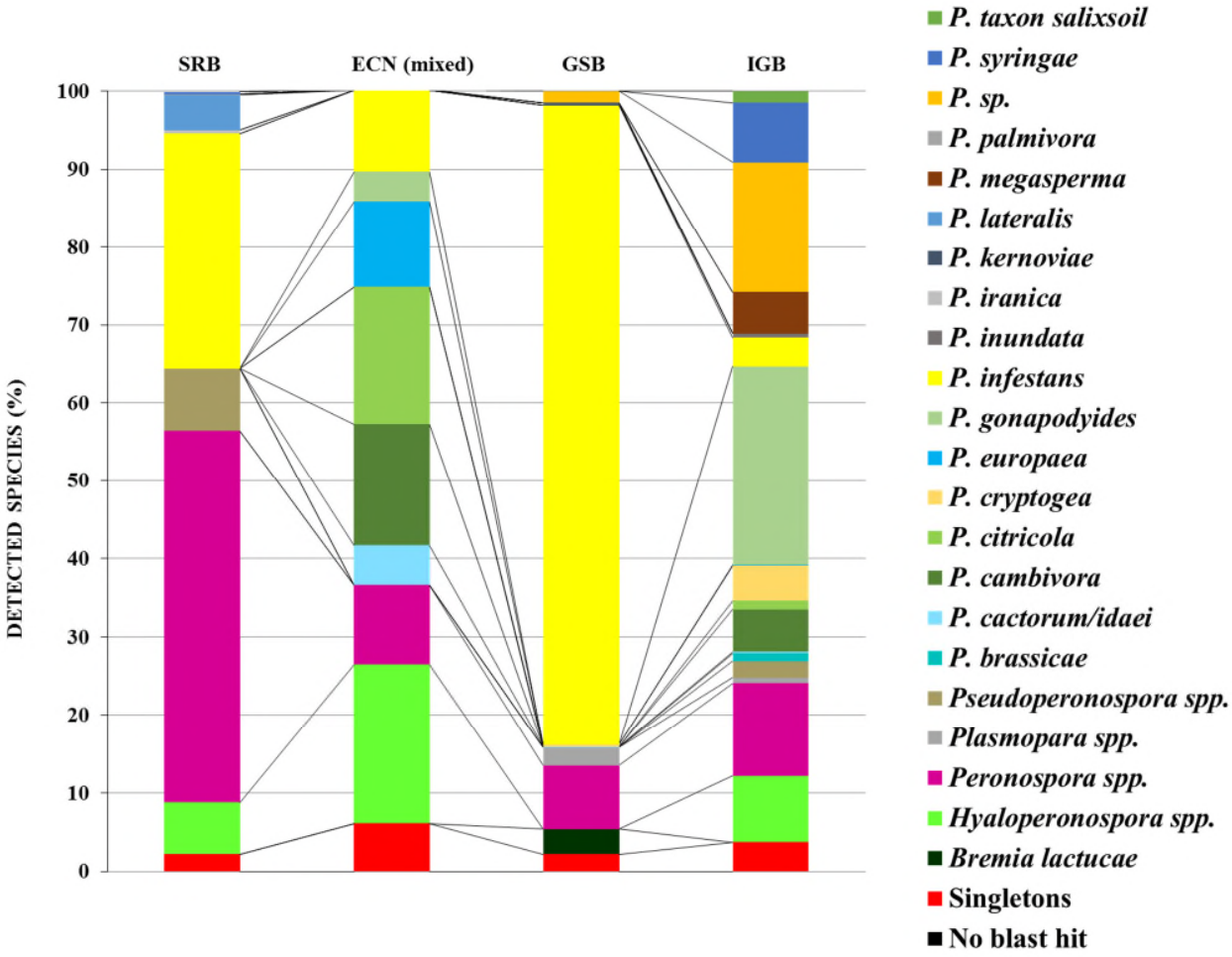

Figure 3. Comparison of microbial diversity in water samples collected during a two years-long survey in four different natural ecosystems in Scoltand. Samples from ECN were mixed before sequencing. Results revealed a clear site-to-site variation with less diversity at GSB Glensaugh (mostly *P. infestans*) and most diversity at Invergowrie (IGB). Results referred to 8 pM-concentrated library sequencing.

The analysis of β-diversity distance matrices revealed evident differences in populations associated to the investigated environments. For example IGB samples were clearly far from SRB and GSB samples which shares a higher number of MOTUS (Fig. 4). The constancy of *Phytophthora* species in IGB sites makes samples cluster closer if compared to other sites in which there is evidence of modifications of the ecosystem.

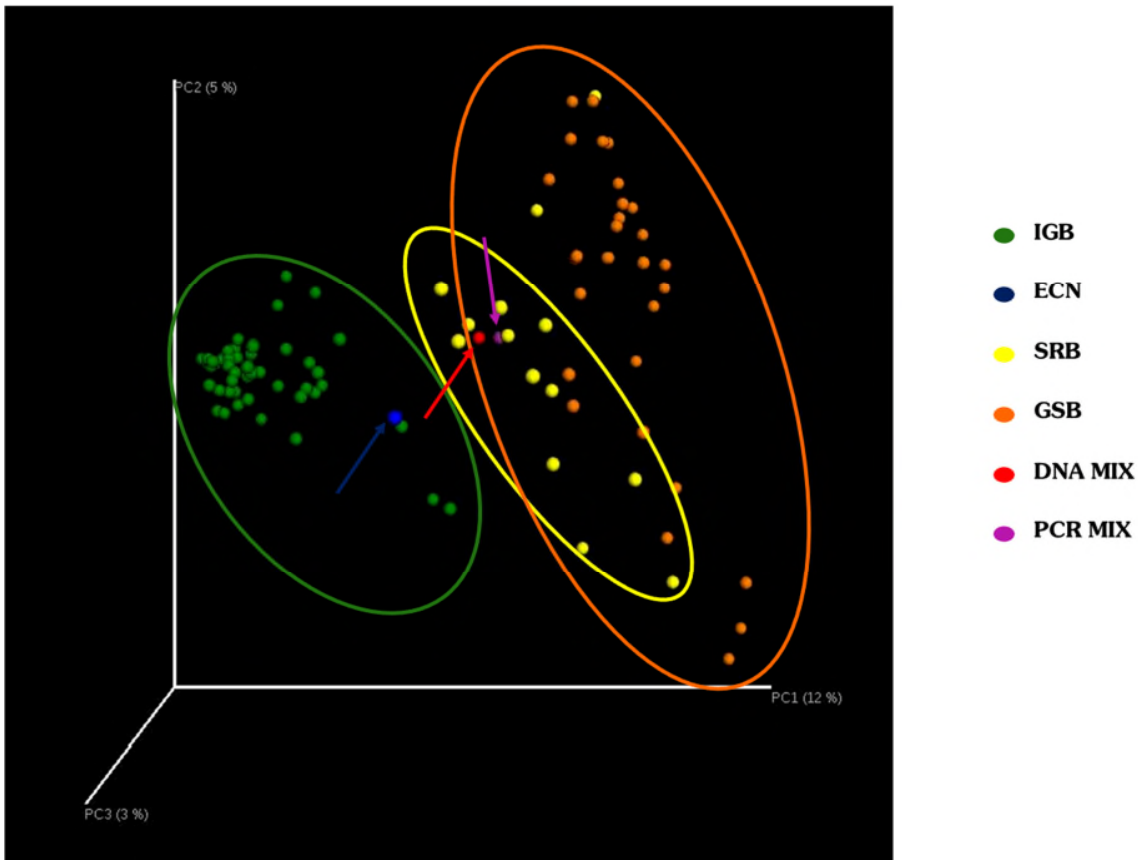

Figure 4. Principal coordinates analysis (PCoA) showing β diversity index, a mathematical expressions that combine species richness and evenness (the homogeneity of abundances) in more samples. In the present study the species that characterize each natural community differed in relative abundance, with a few species quite common and most species much less abundant. Red and purple spots associated to positive controls grouped very closely because of the presence of identical species in each sample.

DISCUSSION

In the present study the application of a metagenomic approach based on *Phytophthora* spp. specific primers and Illumina MiSeq provides an unprecedented sequencing depth to investigate *Phytophthora* diversity in natural Scottish streams and rivers. Illumina MiSeq is one of the leading technologies supplanting Sanger sequencing for metabarcoding analysis. The most popular metabarcoding application is the sequencing of rDNA amplicons to profile the phylogenetic diversity within microbial communities. However, this sequencing platform has to date been primarily used for Bacterial metabarcoding, and little information is currently available about its application to characterize fungal and *Oomycete* (including *Phytophthora*) populations. To our knowledge, this study is the first report evaluating the application of Illumina sequencing of the ITS1 region of the rDNA to evaluate *Phytophthora* diversity.

The analysis of the sequencing performance conducted on artificial systems based on mixtures of DNA or PCR amplicons from 10 selected *Phytophthora* species revealed that the choice of an optimal concentration of the final library (8 pM instead of 4 pM) has a great impact on the performance of the sequencing and on the quality of final results (Table 2). In particular, this choice strongly affected the cluster creation step, considered one of the most critical, with remarkable effects on the clusters and the consequent number of reads. Indeed a low concentration of the final library used for sequencing decreased the level of diversity since, in the case DNA mixtures, two species (*P. boehmeriae* and *P. palmivora*) included in the panel, were not detetced using a 4 pM library. On the other hand, the use of 8pM libraries enabled the detection of all species but with large differences in the number of reads, although an equal quantity of DNA from each species was mixed. These results reveal important challenging aspects during the amplification step, which appear to favour the amplification of some species as compared to others. The size of the ITS1 product was hypothesized to influence the PCR efficiency and therefore the final read number in this study no relationship between product size

and read number (Table 1, Fig.1) was observed. Probably, the quality of extracted DNA from the different isolates played a major role in determining results, but other factors such as copy number within each species cannot be excluded. In particular, since degenerate primers are used to amplify the target region (Scibetta *et al.*, 2012), it cannot be completely excluded that some targets may be favoured over others in complex mixtures, containing DNA from different species. As a confirmation, a much better performance of the methods was obtained when a mixuture of amplicons, amplified in separate reactions, was sequenced. In general our data clearly indicate that quantitative analyses based on the relative abundance of different reads in natural samples need to be interpreted with great caution. Furthermore, a critical aspect remains the use of sensitive and standardized DNA extraction methods.

Another challenging aspect revealed by the analyses of controls is related to the swarm of detected sequences showing high similarity but not identity as compared to accepted sequence types (STs). These sequences are likely to be the results of errors introduced duing the MiSeq sequencing but it cannot be exluded that at least some of them reflect a true variation amongst ITS tandem repeats. Indeed two variant forms of the *P. cryptogea* ITS ST were observed at frequencies of 260 and 160 reads each and these corresponded to a dimorphic base in the Sanger sequence reads of the same isolate (data not shown). Most variant sequences were however detected with a very low frequency as compared to STs, and a threshold may be applied to exclude low abundant reads from the analysis. However, the identification of a correct threshold to enable the deletion of artifacts and at the same time the detection of all true genetic variation is a very complicated task (Vettraino *et al.*, 2012).

Although many aspects need to be addressed and optimized, the analysis of *Phytophthora* communities in natural Scottish water samples demonstrated the feasibility of the proposed method to study the *Phytophthora* microbial diversity with an unprecedented level of detail. Up to 896 K paired-end reads (less then 250 nucleotides in length) were obtained and clustered in 3373 MOTUs associated to several *Phytophthora* species and other *Oomycete* species. The detection of other *Oomycetes* along

with *Phytophthora* spp. was partially expected because is has been alredy reported that primers used in the present study may also amplify the target region from other downy mildew species (Scibetta *et al.*, 2012).

Detected reads where analized with the QIIME toolkit which enables standard analyses on a huge sequence sets, including quality filtering of reads, efficient molecular operational taxonomic units (MOTUs) picking, taxonomy assignment and computation of β diversity measure (Caporaso *et al.*, 2010). The use of this versatile bioinformatics tool enabled many computational challenges to be overcome including the OTU picking step which was performed using the "open-reference OTU picking" option. This workflow clustered all reads against a reference sequence collection and rather than discard sequences that failed to match the reference, these "failures" were clustered *de novo* in a serial process. As a consequence analyses were not restricted to already-known MOTUs and all detected MOTUs (excluding singletons) were combined into a single filtered table (Rideout *et al.*, 2014). The main advantage of this workflow was the possibility to identify new phylotypes for which no similarity was found in reference database and that at list partially are likely to represented unknown organisms. Among identified MOTUs 3169 out of 3373 were assigned by BLAST to the genus *Phytophthora* spp., even if the identification of the species was frequently challenging. This aspect represents a major obstacle in the development of species-specific metabarcoding protocols and further work on bioinformatics pipelines to identify and characterize the 'unknown' species is required.

Indeed most currently utilized bioinformatics tools and genetic databases enable a good identification of microorganisms up to the level of genus but they become less reliable when used to identify species. This is aspect is particularly important for *Oomycete* plant pathogens since related species with very similar ITS sequences may have very different pathogenetic behavior. Although the ITS1 sequence analysis has been widely used for identification of true fungi and oomycetes due to the relatively high number of informative and discriminant bases (Cooke *et al.*, 2000b; Blair *et al.*, 2008),

many species are poorly resolved with ITS (Martin & Tooley, 2003; Jung & Burgess, 2009) and this problem may result in some misclassifications of the *Phytophthora* community. The generation of false MOTUs because of the presence of closely related species in a sample represents an important bottleneck of all high-throughput sequences analysis (Vettraino *et al.*, 2012). A single reliable, non-redundant and regularly updated reference database is required to limit errors in assigning taxonomy and the optimization of the taxonomic threshold to reliably detect OTUs against "noise" still remains a big challenge (Vettraino *et al.*, 2012; Bik *et al.*, 2014).

In the present study, difficulties in discriminating phylogenetically related species with identical sequences or differing only by a few nucleotide positions, were preliminary noticed during the analysis of the artificial systems, based on a mixture of PCR products or DNA of 10 different *Phytophthora* isolates. In particular, the *in silico* taxonomic analysis assigned three groups of MOTUs corresponding to *P. rubi*, *P. idaei* and *P. plurivora* incorrectly to the species *P. cambivora*, *P. cactorum* and *P. citricola*, respectively. This result was anticipated for *P. plurivora* and *P. citricola* that were found to be very similar genetically (Jung & Burgess, 2009) as well for *P. cactorum* and *P. idaei* that differ by only a few bases across the whole ITS region (Cooke *et al.*, 1996; Cooke *et al.*, 2000b). The correct identification of such closely related species can be made via careful comparisons against high quality reference sequences but the need for such manual curation should be avoided by improved bioinformatic tools and reference databases. The variability in data quality in current databases is a major impediment. Furthermore, as stated above, the generation of errors during sequencing or amplification steps can certainly increase the generation of false MOTUs, although the the use of a high-fidelity polymerase, greatly increases reliability of the analyses.

In conclusion, a protocol based on the high-throughput MiSeq Illumina sequencing was tested on artificial control reactions of DNA or amplicons from pure cultures and used to study *Phytophthora* diversity in natural water samples from four apparently healthy Scottish sites. The study was successful

and demonstrates that the method has a huge potential to evaluate *Phytophthora* diversity with an unprecedented level of accuracy. However, many technical and bioinformatic aspects need to be addressed and the risk of ambiguous results need to be always taken into account. In this context, the future development and validation of alternative barcode markers characterized by a higher interspecific variation may be required.

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CHAPTER 5

CONCLUSION

Tasks related to epidemiology, quarantine, eradication, and biosecurity need rapid, accurate and reliable means by which plant pathogens can be adequately detected, identified and quantified. Accurate technologies are required to detect the organisms directly in their living habitat (for example from organic substrates, plant host, soil or water) in a relatively rapid assay and from samples of a meaningful size. This is particularly important in the case of soil-borne/root pathogens such as most *Phytophthora* species since detection is often challenging due to the structural, physical and biological complexity of the rhizosphere and soil environment.

The main objective of the present project was the development and application of appropriate tools based on metabarcoding of the PCR-amplified rDNA ITS regions to evaluate *Phytophthora* diversity in soil and root sample from nurseries and in natural water samples from rivers and streams. Three different methods based on Cloning/Sanger sequencing, 454 pyrosequencing and Illumina MiSeq were evaluated. All methods provided the use of *Phytophthora* spp. specific primers and proved to be appropriate to investigate *Phytophthora* diversity with an unprecedented level of accuracy as compared with traditional baiting and/or cultural methods. The cloning/Sanger sequencing approach produced reliable results due to the trustworthiness of Sanger sequencing but time and costs of the analyses related to this sequencing strategy and to the required cloning step represents an important issue. The 454 pyrosequencing may represent a good compromise between reliability of the analyses and reduced costs and time to afford large scale investigations. Furthermore, this technique enabled much more detailed investigations with the detection or rare and/or low abundant phylotypes including putative new species still unknown to the scientific community. As regard to the MiSeq approach it represents the most powerful investigated approach and it has a huge potential to evaluate *Phytophthora* diversity with an unprecedented level of accuracy. However, many aspects need to be addressed and the risk of ambiguous results need to be always taken into account. A major challenge with all methods is the

discrimination of phylogenetically related species that might have sequences identical or differing only by a few nucleotide positions. In this context the performing of downstream standard phylogenetic analyses may greatly help in the accurate evaluation and interpretation of data but the huge amount of sequences produced with second generation sequencing strategies represents a big challenge. All methods have great scope for further implementations with new approaches. New projects are now focusing on the use of primers ensuring a higher coverage in order to include the genus *Pythium* spp. and other relevant Oomycetes plant pathogens and/or to identify alternative barcode genes providing higher levels of resolution to enable the discrimination of closely related species. This latter aspect is particularly relevant for *Oomycete* plant pathogens since related species with very similar ITS sequences can be characterized by a completely different pathogenetic behavior.

In general, the methods developed and validated in the present study represent valuable tools to study the biology, population genetic, center of origin and diffusion pathways of *Phytophthora* species as well as to update the quarantine lists of international plant protection organizations. The accurate and cost-effective detection of all *Phytophthora* taxa including those still unknown to the scientific community represents another important aspect for a number of applications from pathogen surveys to biosecurity statutory testing. Metagenomic analyses may also effectively integrate more conventional detection methods such as baiting and culturing methods by indicating the presence and localization of new phylotypes and facilitate their isolation and characterization. The achievement of the above-mentioned objectives will be essential to raise public and industry awareness and will have important practical implications in reducing the threat related to the introduction and diffusion of invasive *Phytophthoras*, including those still unknown to science that represent a hidden enemy against which there are few current effective management tools.

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