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

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The genetic diversity of *Phytophthora* spp. was investigated in potted ornamental and fruit tree species. A metabarcoding approach was used, based on a semi-nested PCR with *Phytophthora* genus-specific primers targeting the ITS1 region of the rDNA. More than 50 ITS1 sequence types 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 some indications of structure amongst populations 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 favouring the introduction, dissemination and evolution of *Phytophthora* species.

Keywords: genus-specific primers, metabarcoding analyses, nurseries, *Phytophthora* spp., potted plants

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 and twig and/or leaf blights in a huge number of plant species; losses in nurseries can be up to 100% within 1 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 that 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

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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 varieties/species, growth of plants in pots) provides an environment favourable 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 between *Phytophthora* spp. 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 phytosanitary 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 used detection methods that are often based on culturing and baiting (Cooke *et al.*, 2007). Several PCR-based methods have been developed for *Phytophthora* species but the majority of diagnostic assays have been specifically designed to detect only a single species. As a consequence, these assays are inappropriate for broader surveys of *Phytophthora* diversity and distribution in ecosystems in which a method 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 metabarcoding 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 a 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 and 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 nine representative 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. Each analysed sample was represented by five subsamples of roots or soils collected from five different plants with general symptoms of decline on the canopy. Bulked samples were maintained in plastic bags at 4°C for no more than 2 days before processing. Root samples were washed with running tap water, dried on blotting paper and cut to obtain small pieces (*c.* 5 cm). Both roots and soils were freeze-dried and stored at −20°C pending molecular analysis.

DNA extraction

DNA extractions were performed in triplicate from all collected soil and root samples. To extract DNA from soil, the method described by Schena *et al.* (2002) was slightly modified. Lyophilized soil (0·5 g) was transferred to 2 mL Eppendorf tubes and suspended in 1·5 mL of extraction buffer (0·12 M Na_2HPO_4, 1·5 M NaCl, 2% CTAB) in the presence of 0·1 g of acid-washed glass beads (425–600 μm diameter; Sigma Aldrich) and two 5 mm stainless steel ball bearings. The extraction mixture was blended at 300 rpm for 10 min in a Mixer Mill MM 200 (Verder Scientific) and centrifuged at 16 000 ***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 3 M sodium acetate, pH 5·2, washed twice with cold 100% and 70% ethanol, dried and resuspended in 100 μL nuclease-free water.

Extraction of DNA from roots was performed using the protocol described by Schena & Ippolito (2003) with minor modifications. Lyophilized 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 μm diameter), 0·1 g PVP (Sigma Aldrich), two 5 mm stainless steel ball bearings and 1·5 mL 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 set to have vibrational frequency of 30 Hz and centrifuged at 16 000 ***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 nuclease-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 by Ruano-Rosa *et al.* (2007).

Evaluation of DNA quantity and quality

Purified and non-purified environmental DNA samples were analysed by electrophoresis in 1·2% agarose gels containing GelRed nucleic acid gel strain (Biotium) in Tris-borate-EDTA buffer and visualized with UV light using a Gel Doc XR system (Bio-Rad). A spectrophotometer (NanoDrop; Thermo Fisher Scientific Inc.) was used to measure absorbance at 260, 280 and 230 nm

Table 1 Results of surveys conducted on soil and root samples collected from potted plantlets in eight different nurseries located in 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. 1)

		Detected *Phytophthora* species	
Sampling locality	Collected 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
	Convolvulus 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*
	Convolvulus 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 pseudodictamnus	ND	ND
	Hebe veronica	ND	ND
	Eremophila nivea	ND	ND
	Arbutus unedo	ND	ND
	Erica canaliculata	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
	Bougainvillea glabra	ND	*P. cinnamomi*-like
Nursery VM, 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
	Euryops pectinatus	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		ND

(continued)

Table 1 (continued)

Sampling locality	Collected species	Detected *Phytophthora* species	
		Roots	Soils
		P. nicotianae, *P. meadii*, *P. cambivora*, *P. citricola*	
	Dahlia campanulata	*P. nicotianae*, *P. citricola*, *P. cambivora*, *P. meadii*-like, *P. niederhauserii*	ND
	Impatiens hawkeri	*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*

NA, not analysed; ND, no *Phytophthora* detected.

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 the 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 *P. kernoviae* DNA were spiked with 1 μL water (control) or either purified or non-purified DNA. Reaction mixtures without *P. kernoviae* DNA were used to confirm the absence of this species in all analysed samples. PCR amplification was performed as described by Schena *et al.* (2006) using a StepOnePlus Real-Time PCR system (Applied Biosystems) and data acquisition and analysis completed using the supplied software according to the manufacturer's instructions. The quantification cycle (C_q) 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). Minor modifications to the method of Scibetta and co-workers concerned the use of a *Taq* DNA 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), 1× *Pfx50* PCR mix, 0·2 μM each primer and 1 μL purified DNA. According to Scibetta and co-workers, 1 μL of the first-round product was used as template for the second 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 a final step of 68°C for 2 min. All PCR was conducted in a Mastercycler Ep Gradient S (Eppendorf).

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

Great precautions were taken to minimize the risk of DNA contamination of PCR amplifications. First and second round PCR amplifications, DNA extractions and electrophoresis were set up in separate areas and using specific sets of materials including gloves, pipettes, filter tips and laboratory coat. Working positions were repeatedly cleaned with 10% NaOCl 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.

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 chemically competent *Escherichia coli* TOP10 (Invitrogen) using a Zero Blunt TOPO PCR Cloning kit (Invitrogen), according to the manufacturer's protocol. For each cloned amplicon, 20 clones were picked and directly used in PCR amplifications (colony PCR) with the second-round PCR primers (ITS6/5.8-1R) as previously described. Amplified products were analysed by electrophoresis and single bands of the expected size were sequenced with both forward and reverse primers by Macrogen Europe (Amsterdam, Netherlands).

Analysis of sequences and identification of ITS1 sequence types

The ChromasPro v. 1.5 software (http://www.technelysium.com.au/) was used to evaluate the quality of sequences and to create consensus sequences. All sequences were aligned using muscle as implemented in mega 5 (Hall, 2013) and analysed and edited manually to check indels and single nucleotide polymorphisms within homologous groups 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 DnaSP v. 5.10.01 (Librado & Rozas, 2009). In order to reduce the risk of errors due to artefacts 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 analysed with the software ElimDupes (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) to delete multiple identical sequences for each species. All *Phytophthora* species with identical ITS sequences were included in the reference database. 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 the *Phytophthora* Database (http://www.phytophthoradb.org/) and GenBank, with priority given to sequences associated to specific publications (Fig. 1). 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 was represented by a single ST or a closely related cluster of ITS1 sequences that were considered to represent a single distinct taxon. The term phylotype was used as a proxy for species in describing the results, because species cannot be defined formally in the absence of living isolates.

To show the relatedness and relative abundance of different STs graphically, networks were generated for each detected *Phytophthora* clade with the statistical parsimony algorithm implemented in TCS v. 1.21 (Clement *et al.*, 2000). Colour and circle size were 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 used 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 colour (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 (C_q) 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 colourless and did not cause any delay in the C_q, indicating that *Taq* DNA polymerase activity was not affected. The quality of purified DNA was also confirmed by an A_{260}:A_{280} ratio of 1·8–2·1 and A_{260}:A_{230} 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^{-1} (soil samples) and 300 and 500 ng μL^{-1} (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 sample) were obtained and representative ITS1 sequences (STs) were deposited in GenBank with accession numbers KJ6011 90–KJ601244 (Annex S1).

Analysis of sequences and species identification

After the exclusion of singletons, 55 unique STs representing known species and subspecies variants, species complexes or representatives of unknown *Phytophthora* taxa were identified. Phylogenetic analysis of these STs against reference sequences (Fig. 1) 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 (e.g. *P. meadii*-like) and 22 (e.g. *P. nicotianae*; Fig. 1). 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. 1). Other phylotypes were associated to *P. citricola* taxon E or III (ST Citr; Table S1) 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, three 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. 1).

Subspecies variation, with indications of host association, was observed for some species (Fig. 2). 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 within the homothallic species such as *P. lateralis* and *P. citricola* taxon E or III (Fig. 2). 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

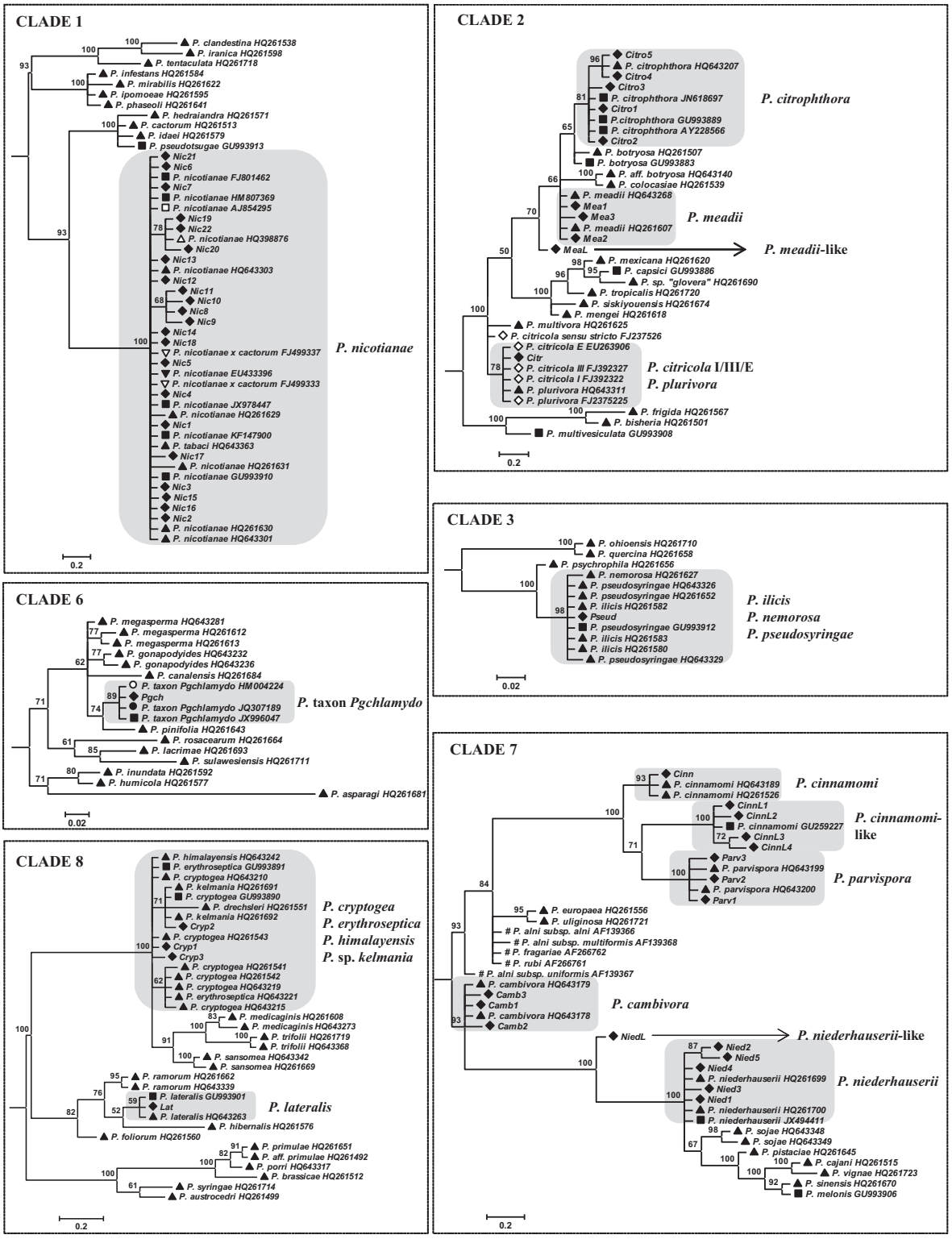

Figure 1 Phylogenetic trees built using unique sequences representative of all detected sequence types, ♦; along with sequences of reference isolates from Aragon-Caballero *et al.* (2008), ▼; Brasier *et al.* (2004), #; Blomquist *et al.* (2012), ●; Camele *et al.* (2005), □; French *et al.* (2011), △; 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.

low abundance as they were detected in only single root or soil samples (Fig. 2).

Dissemination of *Phytophthora* in soil and root samples

Most positive root samples were found to be infected by one (eight samples) or two (six samples) *Phytophthora* phylotypes (Table 1). However, root samples of *Petunia parviflora*, *Lobelia erinus* and *Dahlia campanulata* from the nursery BL were infected by three, four and five different phylotypes, respectively (Table 1; Fig. 2). Similarly, most positive soil samples were found to be infested by one (13 samples) or two (six samples) phylotypes, although five 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. 2). Some STs of *P. nicotianae* were sourced from both soil and roots (13) while others were detected in just soil (five) or root samples (four) (Figs 1 & 2). 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. 2). STs differed at 12 single base pair locations with two homopolymeric runs of nucleotide bases A (0–3 repeats) or T (0–2 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 (Figs 1 & 2).

Four phylotypes clustering in the *Phytophthora* clade 2 were detected (Fig. 1). They comprised five STs of *P. citrophthora* and three STs of *P. meadii*, detected in the citrus orchard and in the nurseries CP and BL (Fig. 2). 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. 2).

Phytophthora phylotypes clustering in clade 7 were detected in six different nurseries and in the citrus orchard (Fig. 2). Among the detected species, *P. cambivora* was represented by three STs and was found in eight different hosts from the citrus orchard and nurseries BL and PG. *Phytophthora niederhauserii* was represented by five STs and was found in eight hosts from nurseries TP, PV, CP, ST and BL. A single ST of *P. cinnamomi* was detected in nursery TP from *Grevillea lanigera* while three STs of *P. parvispora* were found in the citrus orchard soil. Furthermore, four 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 three hosts (*G. lanigera*, *Bougainvillea glabra* and *Cercis siliquastrum*) in nursery TP and ZZ (Table 1; Figs 1 & 2). Similarly, an ST related to *P. niederhauserii* (NiedL) was detected in soil samples of *Cyclamen persicum* collected in nursery BL (Figs 1 & 2).

Two phylotypes clustering within the *Phytophthora* clade 8 were identified as *P. lateralis* or associated to the species complex of *P. cryptogea* (Figs 1 & 2). *Phytophthora 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 with the species complex of *P. cryptogea* was represented by three STs (Cry1, Cry2, Cry3) detected in three 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).

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). A molecular method was used, based on a semi-nested PCR with *Phytophthora* genus-specific primers (Scibetta *et al.*, 2012). This metabarcoding approach provided considerable detail on the diversity of species present in these nurseries and valuable information about the population structure in some taxa.

Among the identified phylotypes, *P. nicotianae* was by far 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 the authors' knowledge, the present study represents the first evidence of *P. nicotianae* on roots and/or in soils of *Armeria maritima*, *Convolvulus 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). The recorded high genetic variation within ITS1 sequences of *P. nicotianae* is also in agreement with the hypothesis 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

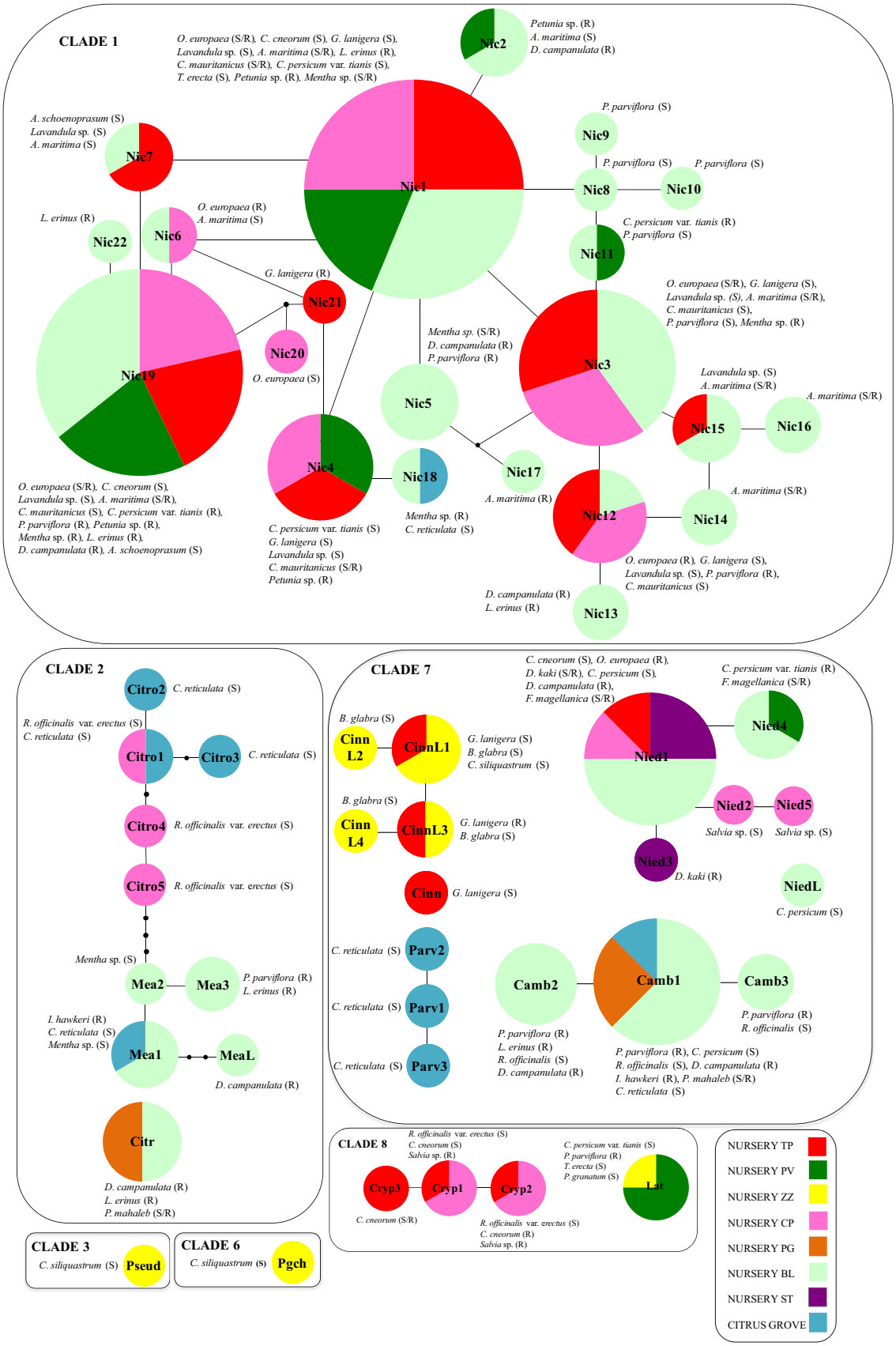
CLADE 1
O. europaea (S/R), C. cneorum (S), G. lanigera (S), Lavandula sp. (S), A. maritima (S/R), L. erinus (R), C. mauritanicus (S/R), C. persicum var. tianis (S), T. erecta (S), Petunia sp. (R), Mentha sp. (S/R)
Nic2
Petunia sp. (R)
A. maritima (S)
D. campanulata (R)
A. schoenoprasum (S)
Lavandula sp. (S)
A. maritima (S)
Nic7
Nic1
P. parviflora (S)
Nic9
P. parviflora (S)
Nic8
P. parviflora (S)
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)
Nic3
Mentha sp. (S/R)
D. campanulata (R)
P. parviflora (R)
Nic20
O. europaea (S)
Nic19
Nic5
Lavandula sp. (S)
A. maritima (S/R)
A. maritima (S/R)
Nic15
Nic16
Nic4
Nic18
Nic17
A. maritima (R)
A. maritima (S/R)
Nic14
Nic12
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)
C. persicum var. tianis (S)
G. lanigera (S)
Lavandula sp. (S)
C. mauritanicus (S/R)
Petunia sp. (R)
Mentha sp. (R)
C. reticulata (S)
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. hawkeri (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)
B. glabra (S)
Cinn L2
CinnL1
G. lanigera (S)
B. glabra (S)
C. siliquastrum (S)
Nied4
Nied1
B. glabra (S)
Cinn L4
CinnL3
G. lanigera (R)
B. glabra (S)
Nied2
Nied5
Salvia sp. (S)
Salvia sp. (S)
Nied3
D. kaki (R)
Cinn
G. lanigera (S)
NiedL
C. persicum (S)
C. reticulata (S)
Parv2
C. reticulata (S)
Parv1
C. reticulata (S)
Parv3
Camb2
Camb1
Camb3
P. parviflora (R)
L. erinus (R)
R. officinalis (S)
D. campanulata (R)
P. parviflora (R)
R. officinalis (S)
P. parviflora (R), C. persicum (S)
R. officinalis (S), D. campanulata (R)
I. hawkeri (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 2 Sequence type (ST) network based on *Phytophthora* ITS1 sequences detected in soil and root samples collected from potted plantlets in eight different nurseries and in a soil sample collected from a citrus grove. The network was constructed using a statistical parsimony algorithm implemented in TCS v. 1.21 (Clement *et al.*, 2000). Different colours 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 three to five letters of the corresponding identified species.

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 the results of the present study, a recent survey on ornamental nurseries in Maryland revealed a high diversity of *Phytophthora* spp. from small samples of recycled potting media, suggesting the potting medium was a source of introduction and spread of variability among *Phytophthora* spp. (Bienapfl & Balci, 2014). 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 the USA (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* in *Citrus* spp. and *Rosmarinus officinalis* soils is not surprising because these species are well-documented hosts of this pathogen (Erwin & Ribeiro, 1996). Similarly, *P. citricola* has already been 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 four 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 the authors' knowledge it has 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 in the potting soil of *C. siliquastrum*, a tree native to the eastern Mediterranean region and widely distributed in western Asia, mainly along the banks of streams. *Phytophthora* taxon *Pgchlamydo* has been widely detected in nurseries in Minnesota and California 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 in soils of *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 on three hosts, including *G. lanigera*, in two different nurseries. BLAST analyses revealed the existence of a sequence (GU259227) identical to the detected ST (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 suggests it is a new species (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 long been considered as a variety of *P. cinnamomi* but based on morphological, physiological and molecular analyses it was recently elevated to a new species (Scanu *et al.*, 2014). Scanu *et al.* (2014) 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 because 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.

Other species found on previously unreported hosts (to the best of the authors' knowledge) include *P. cambivora* and *P. niederhauserii*. Indeed, *P. cambivora* is a well-known forest pathogen and in the USA 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). *Phytophthora niederhauserii* has been previously reported in Sicily (Cacciola *et al.*, 2009a,b; Faedda *et al.*, 2013a) and Valencia (Spain) (Perez-Sierra & Jung, 2013). However, the detection of *P. niederhauserii* from potted plantlets of *Diospyros kaki* in nursery ST was of interest because 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 and

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 it being recommended for listing as an A1 quarantine organism by the European Plant Protection Organism in 2006 (Schlenzig *et al.*, 2011). The detection of a single ST in two Italian nurseries on roots and/or in soils of four different hosts suggests a recent introduction of the pathogen, but also indicates a serious threat because it suggests rapid dissemination is possible once the pathogen is introduced to 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; Leonberger *et al.*, 2013).

In the present study, variability was generally higher in heterothallic species than in homothallic ones. This may be a reflection of outcrossing events that are particularly favoured in the nurseries where many different plant species are grown together, favouring 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). This is in agreement with an analysis of mitochondrial and nuclear markers within a broad population of *P. nicotianae*, which revealed an important role of nurseries in increasing genetic recombination within the species (Mammella *et al.*, 2013). These authors speculated that nursery populations play an important role 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* because 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 used to determine sequences. Although this technique is much less powerful than 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* because 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 artefacts 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 factors support the authenticity of the data. First, the identified STs were represented by at least two sequences given that singleton sequences were excluded as a precaution. As the introduction of identical errors in two independently generated sequences is improbable, it seems unlikely that the last two steps (plasmid replication and colony PCR) introduced errors. 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, because some of the excluded single sequences could actually be true STs.

In conclusion, the results of the present study highlighted a very complex situation in potted ornamental nurseries with a large number of *Phytophthora* taxa detected in all investigated sites. These important pathogens were detected amongst nurseries that vary in terms of the kind of business (smaller family-run or larger 'industrial' premises), origin of the material (produced on-site or purchased from other nurseries), the range and quantity of cultivated species, and irrigation systems used; therefore, this indicates a wide-ranging threat, not specifically related to the investigated sites. The findings confirm the urgent need for new management strategies, based on the enforcement of proactive and preventative approaches to nursery plant production, in order to minimize the risks posed by *Phytophthora* species (Parke & Grünwald, 2012).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Annex S1. List of *Phytophthora* species and ITS1 sequence types (STs) identified in potted nursery roots and soils in Apulia and Calabria (Southern Italy). These were determined using an amplicon metagenomics approach, based on the use of *Phytophthora* genus-specific primers (Scibetta *et al.*, 2012).

Table S1. 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).