NILDE 1418009

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Techniques

Metabarcoding Analysis of *Phytophthora* Diversity Using Genus-Specific Primers and 454 Pyrosequencing

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Accepted for publication 8 November 2015.

ABSTRACT

Prigigallo, M. I., Abdelfattah, A., Cacciola, S. O., Faedda, R., Sanzani, S. M., Cooke, D. E. L., and Schena, L. 2016. Metabarcoding analysis of *Phytophthora* diversity using genus-specific primers and 454 pyrosequencing. Phytopathology 106:305-313.

A metabarcoding method based on genus-specific primers and 454 pyrosequencing was utilized to investigate the genetic diversity of *Phytophthora* spp. in soil and root samples of potted plants, from eight nurseries. Pyrosequencing enabled the detection of 25 *Phytophthora* phylotypes distributed in seven different clades and provided a much higher resolution than a corresponding cloning/Sanger sequencing approach. Eleven of these phylotypes, including *P. cactorum*, *P. citricola* s.str., *P. palmivora*, *P. palmivora*-like, *P. megasperma* or *P. gonapodyides*, *P. ramorum*, and five putative new *Phytophthora* species phylogenetically related to clades 1, 2, 4, 6, and 7 were detected only with the 454 pyrosequencing approach. We also found an additional 18 novel records of a phylotype in a particular nursery that were not detected with cloning/Sanger sequencing. Several aspects confirmed the reliability of the method: (i) many identical sequence types were identified independently in different nurseries, (ii) most sequence types identified with 454 pyrosequencing were identical to those from the cloning/Sanger sequencing approach and/or perfectly matched GenBank deposited sequences, and (iii) the divergence noted between sequence types of putative new *Phytophthora* species and all other detected sequences was sufficient to rule out sequencing errors. The proposed method represents a powerful tool to study *Phytophthora* diversity providing that particular attention is paid to the analysis of 454 pyrosequencing raw read sequences and to the identification of sequence types.

Additional keywords: metagenomic analyses, ornamental nurseries.

The increasing international trade in rooted plants and the continual introduction of new varieties and/or species exposes nurseries, with particular emphasis on the potted ornamentals, to new host–pathogen combinations and creates new disease threats. Invasive pathogens have been frequently found on ornamental plants and their trade is considered a primary driver of new disease outbreaks since it causes large-scale distribution of pathogens beyond their natural endemic ranges with severe socio-economic impact (Brasier 2008; Themann et al. 2002).

Among plant pathogens, the approximately 140 species of the genus *Phytophthora* are particularly destructive, affecting thousands of cultivated and wild plants worldwide (Brasier 2009; Kroon et al. 2012; Martin et al. 2012). The role of long-distance migration via the nursery trade in the spread of *Phytophthora* inoculum has been accurately investigated focusing on major species such as *P. ramorum* and *P. nicotianae* (Goss et al. 2011; Kamoun et al. 2015; Mammella et al. 2011, 2013). However, many more species have been reported in nurseries of potted plants in Europe and the United States (Cacciola et al. 2008; Moralejo et al. 2009; Parke et al. 2014; Schwingle et al. 2007; Themann et al. 2002; 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 favoring hybridization and generate better-adapted or entirely new pathogen species (Faedda et al. 2013). Furthermore, the existence of many species of *Phytophthora* still unknown to the scientific community has been postulated (Brasier 2009; Kroon et al. 2012). Having adapted to and coevolved with their hosts, many of these pathogens may not cause noticeable damage in their native ecosystems but represent a serious threat when introduced into a new environment through the nursery trade (Roy et al. 2014).

The detection of *Phytophthora* spp. (including those currently unknown to the scientific community) remains a challenge due to the limits of conventional culturing and bating methods (Cooke et al. 2007). As a consequence, several invasive and previously unknown species have been identified only when they have caused severe disease in nonnative environments. Several polymerase chain reaction (PCR)-based molecular assays have been developed for *Phytophthora* species, but most are inappropriate for broader surveys of its diversity and distribution in ecosystems as they detect only one or a few species (Bilodeau et al. 2014; Cooke et al. 2007; Martin et al. 2012; Sanzani et al. 2014; Schena et al. 2013).

The massive sequencing of PCR amplicons with metabarcoding approaches represents a powerful culture-independent technique to investigate the genetic diversity in microbial communities (Abdelfattah et al. 2015; Lindahl et al. 2013). A number of studies have focused on the design of appropriate universal primers for the amplification of specific barcode genes from all species of major taxonomic groups of fungi and/or oomycetes (Toju et al. 2012). These universal primers represent a valuable tool for ecological studies since they enable the broad characterization of microbial diversity. In particular, oomycete-specific primers proved valuable to study the ecology of this important class of microorganisms using next generation sequencing (Sapkota and Nicolaisen 2015; Vannini et al. 2013; Vettraino et al. 2012). However, primers specific to very broad taxonomic groupings may complicate analyses focusing on specific genera since the target organisms may represent a minority of the

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http://dx.doi.org/10.1094/PHYTO-07-15-0167-R
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complete soil microbiome. Indeed, Sapkota and Nicolaisen (2015) detected few sequences of *Phytophthora* spp. compared with other dominant oomycete genera (*Pythium*, *Saprolegnia*, *Pythiogeton*, and *Aphanomyces*). In this context, the use of more selective primers can greatly improve analyses as demonstrated for the genera *Phytophthora*, *Trichoderma*, and *Colletotrichum* (Friedl and Druzhinina 2012; Mosca et al. 2014; Scibetta et al. 2012).

In a recent study, *Phytophthora* diversity was analyzed in potted ornamental plant nurseries using genus specific primers combined with the Sanger sequencing of cloned amplicons (Prigigallo et al. 2015). The analyses highlighted a complex assemblage of *Phytophthora* species with new host–pathogen combinations, evidence of species previously unreported in the investigated area, and phylotypes representative of species that remain to be taxonomically defined. The use of a conventional cloning Sanger/sequencing (CSS) approach guaranteed a very high level of reliability for detected sequences and this was considered particularly relevant since several *Phytophthora* species are differentiated by a limited number of consistent internal transcribed spacer (ITS) nucleotide differences (Martin et al. 2012; Robideau et al. 2011; Schena and Cooke 2006). However, the CSS approach is much less powerful and more costly than high-throughput sequencing approaches such as 454 pyrosequencing (454P). The large number of reads produced in a single sequencing run with the latter approach provides a far greater sampling depth, thus increasing the detection of low abundance phylotypes. On the other hand, high-throughput sequencing technologies have higher error rates than Sanger sequencing (Knief 2014). This aspect may represent a significant issue in the analysis of *Phytophthora* species since some genetically related species with very similar ITS sequences can have quite different pathogenic behaviors (Martin et al. 2012).

The aim of the present study was to investigate the power and reliability of a metabarcoding approach based on genus-specific primers and 454P to determine *Phytophthora* diversity in environmental samples. To this aim, a number of soil and root samples from potted ornamental nurseries, already analyzed using the CSS approach (Prigigallo et al. 2015), were investigated and results obtained with the two methods were critically compared.

MATERIALS AND METHODS

Sampling, DNA extraction, and PCR amplification. Samples of soil and roots were collected from many ornamental potted plants species during 2013 and 2014 in eight different nurseries across Apulia and Calabria, Southern Italy (Table 1). Each sample comprised five mixed subsamples of roots or soils collected from individual plants of the same species showing general symptoms of decline. Soil and roots from different plant species and nurseries were kept separated and triplicate DNA extractions were performed from each sample as previously described (Prigigallo et al. 2015). After extractions, DNA samples from each nursery (soil and roots) were pooled and the ITS1 region was amplified in triplicate using a semi-nested approach with *Phytophthora* genus specific primers (Scibetta et al. 2012) using the same procedure described by Prigigallo et al. (2015). However, primers used in the present study in the second round amplification were modified to obtain fusion primers according to 454 GS-FLX amplicon pyrosequencing (http://www.454.com/). In particular, eight different multiplex identifier (MID) sequences were selected, paying attention to potential secondary structures with primers and utilized to associate amplicons to the different sampled nurseries (Table 1).

The triplicate amplicons obtained from each sample after the second round PCR were pooled and purified and quantified as previously described (Prigigallo et al. 2015). Finally, purified PCR products from the eight different samples were pooled in equal volume and sequenced using a 1/8 of a PicoTiter Plate with the 454 GS FLX+ System (Macrogen, Seoul, Korea).

Identification of *Phytophthora* phylotypes and sequence types (STs). The complete data set of 454P sequences was processed with the bioinformatics pipeline QIIME Version 1.8 (Caporaso et al. 2010). De-multiplexing and quality filtering analyses were performed using a minimum quality score of 25, a minimum/maximum length ratio of 100/1,000, and a maximum number of homopolymer bases of 10. Additionally, the sliding window test of quality scores (–w) was enabled with a value of 75 to discard sequences with bad windows according to the "–g" command. Sequences were analyzed to reduce erroneous reads (denoised) using the denoise wrapper script (Reeder and Knight 2010) and imeric sequences were identified and filtered using USEARCh 6.1 (Edgar 2010). Cleaned sequences had a length ranging from 150 to 202 bp and were analyzed with the software ElimDupes (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) to identify identical sequences and determine ITS1 STs, defined as the distinct and reproducible ITS1 sequences recovered in this study (Malacrinò et al. 2015; Prigigallo et al.

TABLE 1. List of analyzed samples from eight different nurseries located in Apulia and Calabria (Southern Italy) along with multiplex identifier (MID) sequences associated to each sample and corresponding number of sequences detected by 454 pyrosequencing

Samples	Collected species (roots and soils)[a]	MID	Sequences[b]
Nursery TP Apulia	*Grevillea lanigera*, *Lavandula* sp., *Chamelaucium uncinatum* (S), *Convolvulus cneorum*, *Armeria maritima* (S)	MID 28	18,199
Nursery CP Apulia	*Rosmarinus officinalis* var. *erectus*, *Salvia* sp., *Olea europaea*, *Convolvulus mauritanicus*, *Rosmarinus officinalis* var. *prostratus*, *Pistacia lentiscus*, *Cotoneaster salicifolius*, *Teucrium brevifolium*, *Convolvulus cneorum*, *Origanum pseudodictamnus*, *Hebe veronica*, *Eremophila nivea*, *Arbutus unedo*, *Erica canaliculata*, *Cytisus* sp., *Russelia equisetiformis*	MID 30	495
Nursery ST Calabria	*Diospyros kaki*	MID 32	924
Nursery PV Apulia	*Cyclamen persicum* var. *halios*, *Cyclamen persicum* var. *tianis*, *Tagetes erecta*, *Tagetes patula*, *Petunia parviflora*, *Petunia* sp.	MID 33	2,100
Nursery ZZ Apulia	*Cercis siliquastrum*, *Punica granatum*, *Arbutus unedo*, *Rosa* sp., *Grevillea juniperina*, *Bougainvillea glabra*	MID 37	139
Nursery VM Apulia	*Polygala myrtifolia*, *Lantana sellowiana*, *Nerium oleander*, *Grevillea lanigera*, *Lithodora* sp., *Eugenia myrtifolia*, *Euryops pectinatus*, *Coleonema pulchrum*	MID 38	5,632
Nursery BL Apulia	*Thymus* sp., *Mentha* sp., *Allium schoenoprasum*, *Rosmarinus officinalis*, *Armeria maritima*, *Cyclamen persicum*, *Petunia parviflora*, *Fuchsia magellanica*, *Lobelia erinus*, *Dahlia campanulata*, *Impatiens hawkeri*	MID 39	375
Nursery PG Apulia	*Quercus ilex*, *Prunus mariana*, *Prunus mahaleb*, *Crataegus azarolus*	MID 29	203

[a] The presence of (S) after the species name indicates that only soil samples were analyzed.
[b] Number of detected sequences after quality trimming, denoising, and chimera removal.

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2015). Singletons (sequence reads occurring only once across the complete panel of analyzed samples) were removed from the data set.

To associate detected sequences to specific *Phytophthora* taxa, STs were subject to phylogenetic analysis along with validated barcode sequences of the genus *Phytophthora* (Robideau et al. 2011). Before analysis, sequences from a complete *Phytophthora* reference panel (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 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 and *Phytophthora* Database (http://www.phytophthoradb.org/) with priority given to sequences associated to specific publications (Figs. 1 and 2).

The complete panel of selected reference sequences, STs detected in the present study and STs detected using the CSS approach (Prigigallo et al. 2015), were grouped according to their clade (Cooke et al. 2000; Kroon et al. 2012), aligned using ClustalX and introduced to TOPALi for phylogenetic analysis with the PhyML method based on maximum-likelihood methods (Guindon and Gascuel 2003). By this process, all STs were associated with a phylotype and compared with those previously obtained with the CSS approach (Prigigallo et al. 2015). 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. The term phylotype was used as a proxy for species in describing the results as species cannot be defined formally in the absence of living isolates.

RESULTS

Sequencing results. A total of 76,612 sequence reads with an average length of 270 bp were obtained by 454P. Data were deposited in the SRA database (NCBI) with the accession number SRX884122 (http://www.ncbi.nlm.nih.gov/sra/SRX884122). After quality trimming, denoising, and chimera removal, 28,067 high quality ITS1 sequences were recorded. The number of reads per sample ranged from 139 to 18,199 (Table 1).

Identification of *Phytophthora* phylotypes. After the exclusion of singletons, 120 representative STs were identified in the complete panel of analyzed samples (nurseries) and deposited in GenBank with accession numbers KP691717 to KP691836 (Figs. 1 and 2). According to the phylogenetic analyses, all STs clustered within the genus *Phytophthora* and were assigned to 25 *Phytophthora* phylotypes distributed in seven different clades (Figs. 1 and 2).

Twelve phylotypes were identified to the species level: *P. cactorum* and *P. nicotianae* in clade 1; *P. citricola* s.str., *P. citrophthora*, and *P. meadii* in clade 2; *P. palmivora* in clade 4; *P.* taxon *Pgchlamydo* in clade 6; *P. parvispora*, *P. cambivora*, and *P. niederhauserii* in clade 7; and *P. lateralis* and *P. ramorum* in clade 8 (Figs. 1 and 2). Four 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 and Burgess 2009; Robideau et al. 2011). They comprised the following: (i) *P. citricola* I, *P. citricola* III, *P. citricola* E, or *P. plurivora* in clade 2; (ii) *P. pseudosyringae*, *P. ilicis*, or *P. nemorosa* in clade 3; (iii) *P. gonapodyides* or *P. megasperma* in clade 6; and (iv) *P. cryptogea*, *P. erythroseptica*, *Phytophthora* sp. "*kelmania*", or *P. himalayensis* in clade 8 (Figs. 1 and 2). A further five STs were found to be closely related to accepted *Phytophthora* species and defined as *P. meadii*-like and *P. pseudosyringae*-like A and B (Fig. 1), *P. palmivora*-like, and *P. cinnamomi*-like (Fig. 2). Finally, five STs representative of five phylotypes clustered within the genus but were phylogenetically distant from all currently defined species and were indicated as *Phytophthora* spp. These putative new species were associated with clade 1 (Ph-A), clade 2 (Ph-B and Ph-C), clade 4 (Ph-D), clade 6 (Ph-E), and clade 7 (Ph-F). These phylotypes were 6 (Ph-B), 7 (Ph-C), 11 (Ph-D), 16 (Ph-E), 22 (Ph-A), and 25 (Ph-F) bp different from their closest related taxon (Figs. 1 and 2).

Analysis of phylotypes from 454P and Sanger sequencing. The 454P approach enabled a much more accurate investigation of the *Phytophthora* genetic diversity with the detection of a significantly higher number of STs (120) and phylotypes (25) compared with those previously detected using the CSS approach (55 and 15, respectively). The higher number of STs resulted in the identification of a conspicuous number of previously undetected phylotypes (Fig. 3). They included *P. cactorum* in nursery ST and *P. citricola* s.str. in nursery BL (Figs. 1 and 3), *P. palmivora* in nurseries TP, BL, CP, PG, ZZ, and PV, *P. palmivora*-like in nursery TP, *P. megasperma* or *P. gonapodyides* in nursery ZZ, and *P. ramorum* in nursery TP (Figs. 2 and 3). Other phylotypes detected exclusively with the 454P represented five putative new *Phytophthora* species phylogenetically related to clade 1 (Ph-A), clade 2 (Ph-B and Ph-C), clade 4 (Ph-D), clade 6 (Ph-E), and clade 7 (Ph-F) species. Furthermore, 18 phylotypes already detected with the CSS approach were also detected in other nurseries (new phylotype/nursery combinations) with the 454P (Fig. 3). In contrast, only two phylotypes (*P. cinnamomi* and *P. niederhauserii*-like) and a phylotype/nursery combination (*P. niederhauserii* in nursery PV) formerly detected with the CSS approach were not confirmed with the 454P (Fig. 3).

With the exception of *P. palmivora*, phylotypes detected only with the 454P were represented by a relatively low number of reads suggesting the species were not abundant in the investigated samples (Fig. 3). In terms of relative abundance, the 454P largely confirmed CSS results (Fig. 3). *P. nicotianae* was the most abundant species accounting for 31 out of 120 STs and was detected in six of the eight nurseries. In four nurseries (TP, BL, PV, and DM) it was the most prevalent species (Fig. 3). In other nurseries, the dominant 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

In the present study, a metabarcoding method based on genus-specific primers (Scibetta et al. 2012) and 454P was utilized to investigate the genetic diversity of *Phytophthora* spp. in soil and root samples of potted ornamentals and fruit tree species collected in nurseries located in Apulia and Calabria (Southern Italy). In order to evaluate the method, the results were compared with those obtained in a previous study with a validated CSS approach (Prigigallo et al. 2015). Indeed, many conventional and real-time PCR detection methods based on specific primers and probes have been developed to detect *Phytophthora* species; however, such molecular assays are geared to the detection of one, or very few, specific known target species, and, therefore unsuitable in cases where multiple, or as yet undescribed, *Phytophthora* spp. are present (Cooke et al. 2007; Sanzani et al. 2014). The 454P enabled the detection of 25 *Phytophthora* phylotypes distributed in seven different clades and confirmed almost all phylotypes (15) previously identified with the CSS approach with two exceptions that were likely the result of chance considering the low quantity of soil and roots utilized for DNA extractions. On the other hand, the 454P enabled a much more detailed view of the species diversity with the detection of 11 previously undetected phylotypes including *P. cactorum*, *P. citricola* s.str., *P. palmivora*, *P. palmivora*-like, *P. megasperma* or *P. gonapodyides*, *P. ramorum*, and five putative new *Phytophthora* species phylogenetically related to species in clades 1, 2, 4, 6, and 7. Furthermore, the 454P made possible the identification of 18 new phylotype/nursery combinations compared

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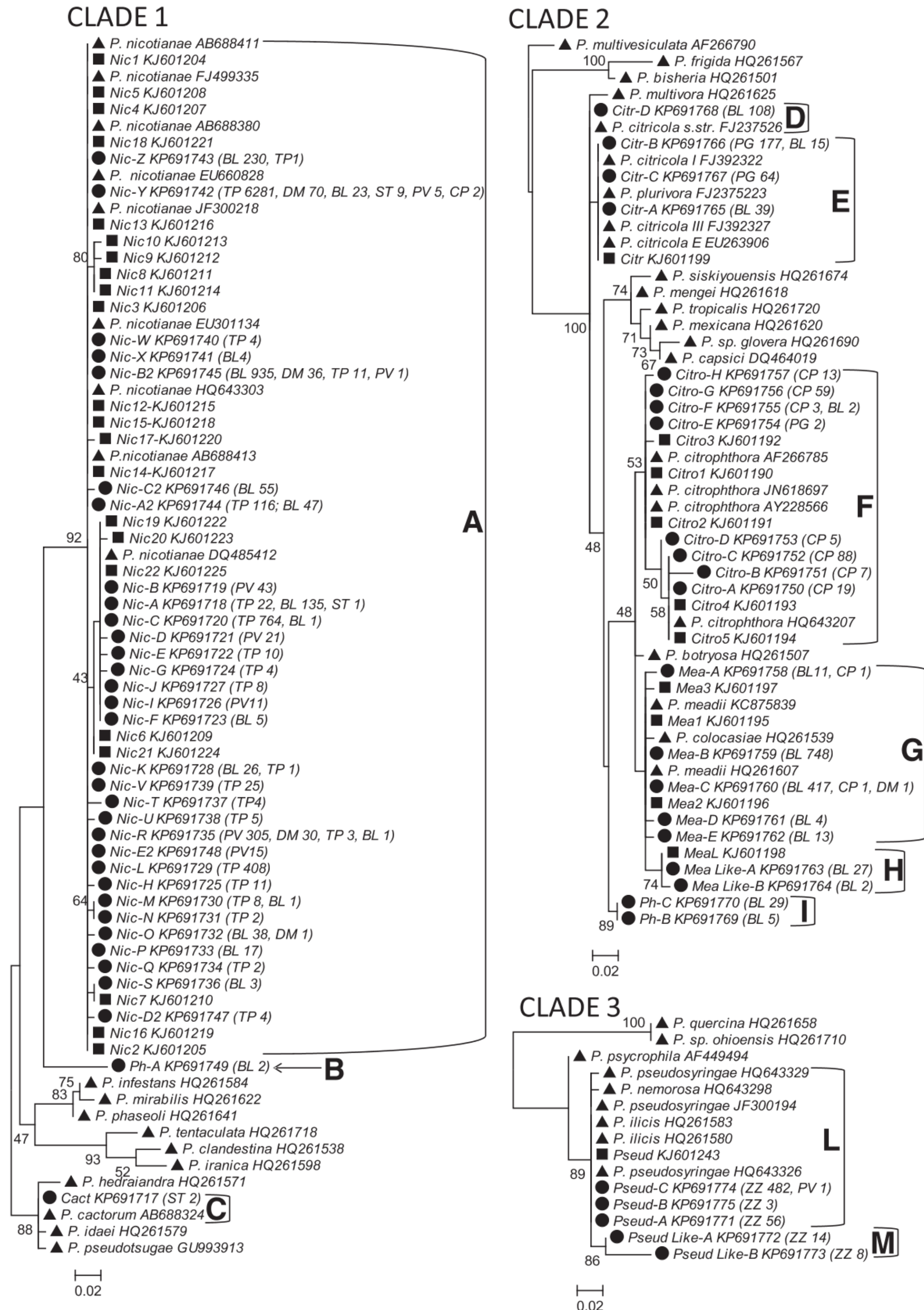

Fig. 1. Phylogenetic trees built for *Phytophthora* clades 1, 2, and 3, using sequence types (STs) detected with 454 pyrosequencing (●) and cloning/Sanger sequencing (■), along with reference sequences (▲) representative of the genetic diversity within each clade (Robideau et al. 2011). Accession numbers are reported for all sequences. The number of identical sequences represented by each ST and the nurseries in which they were detected (Table 1) are reported in parentheses. Numbers on nodes represent the posterior probabilities for the maximum likelihood method. According to this analysis, STs obtained with the 454 pyrosequencing approach were associated with *P. nicotianae* (A), *P. cactorum* (C), *P. citricola* s.str. (D), *P. citricola* I, *P. citricola* III, *P. citricola* E or *P. plurivora* (E), *P. citrophthora* (F), *P. meadii* (G), and *P. pseudosyringae*, *P. nemorosa*, or *P. ilicis* (L). Other STs were defined as *P. meadii*-like (H), *P. pseudosyringae*-like (M), and *Phytophthora* spp. (B and I).

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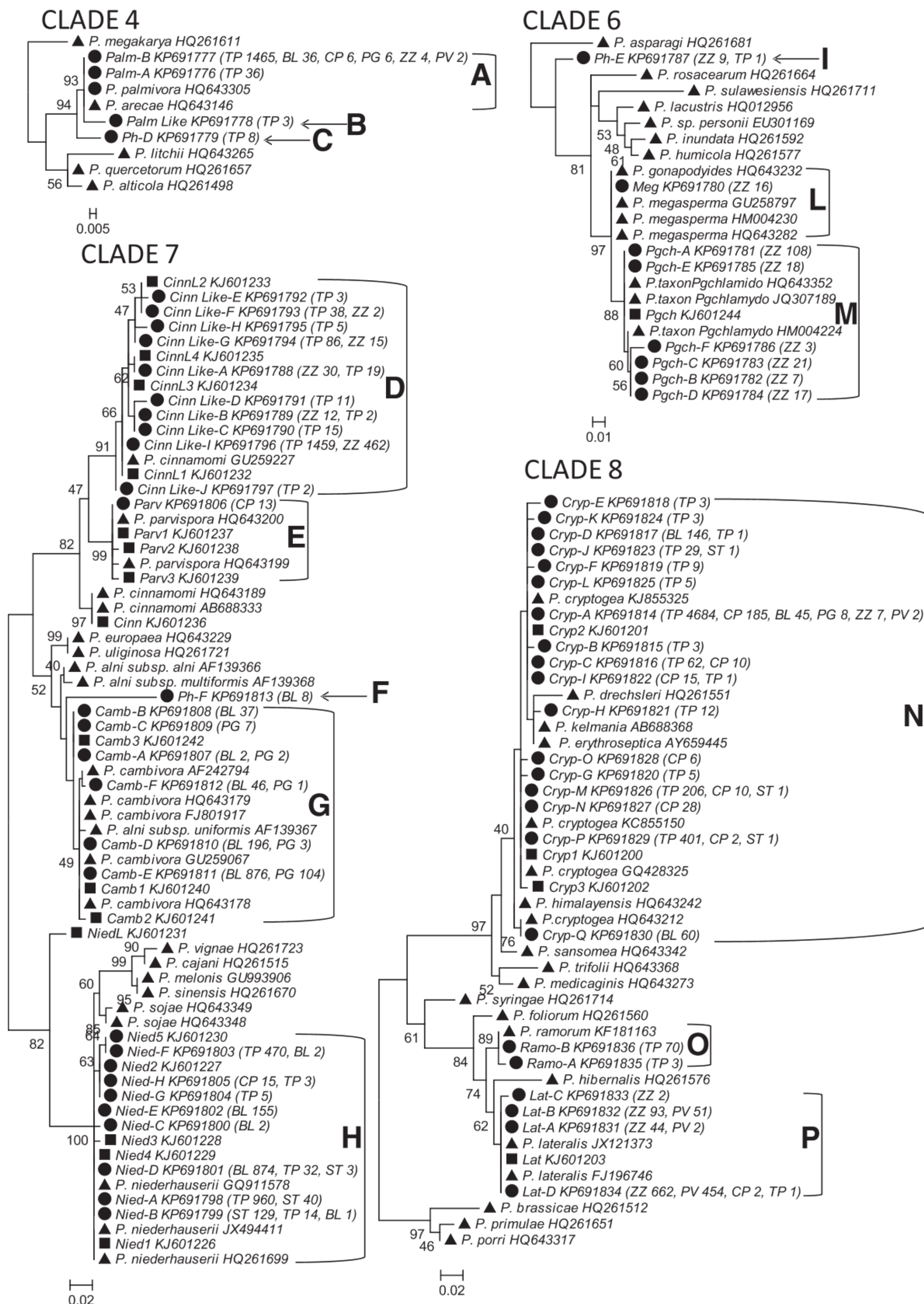

Fig. 2. Phylogenetic trees built for *Phytophthora* clades 4, 6, 7, and 8, using sequence types (STs) detected with 454 pyrosequencing (●) and cloning/Sanger sequencing (■), along with reference sequences (▲) representative of the genetic diversity within each clade (Robideau et al. 2011). Accession numbers are reported for all sequences. The number of identical sequences represented by each ST and the nurseries in which they were detected (Table 1) are reported in parentheses. Numbers on nodes represent the posterior probabilities for the maximum likelihood method. According to this analysis, STs obtained with the 454 pyrosequencing approach were associated with *P. palmivora* (A); *P. parvispora* (E); *P. cambivora* (G); *P. niederhauserii* (H); *P. megasperma* or *gonapodyides* (L); *P.* taxon *PgChlamydo* (M); *P. cryptogea*, *P. drechsleri*, *P. kelmania*, *P. erythroseptica*, or *P. himalayensis* (N); *P. ramorum* (O); and *P. lateralis* (P). Other STs were defined as *P. palmivora*-like (B), *P. cinnamomi*-like (D), and *Phytophthora* spp. (C, F, and I).

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with the CSS approach. Interestingly, most phylotypes and phylotype/nursery combinations previously undetected with the CSS approach were represented by a small number of sequences, suggesting their presence as rare phylotypes in the tested samples. Therefore, these STs were probably not detected with the CSS approach, because of the lower resolution due to the limited number of clones (20) that were sequenced per sample.

A major issue in taxa identification in all metabarcoding approaches is related to difficulties in discriminating phylogenetically related species. The unreliable annotations of sequences in public DNA repositories and the fact that many deposited ITS sequences fail to reflect recent taxonomic advances further complicate analyses (Nilsson et al. 2006). As a consequence, currently utilized bioinformatics tools and genetic databases for

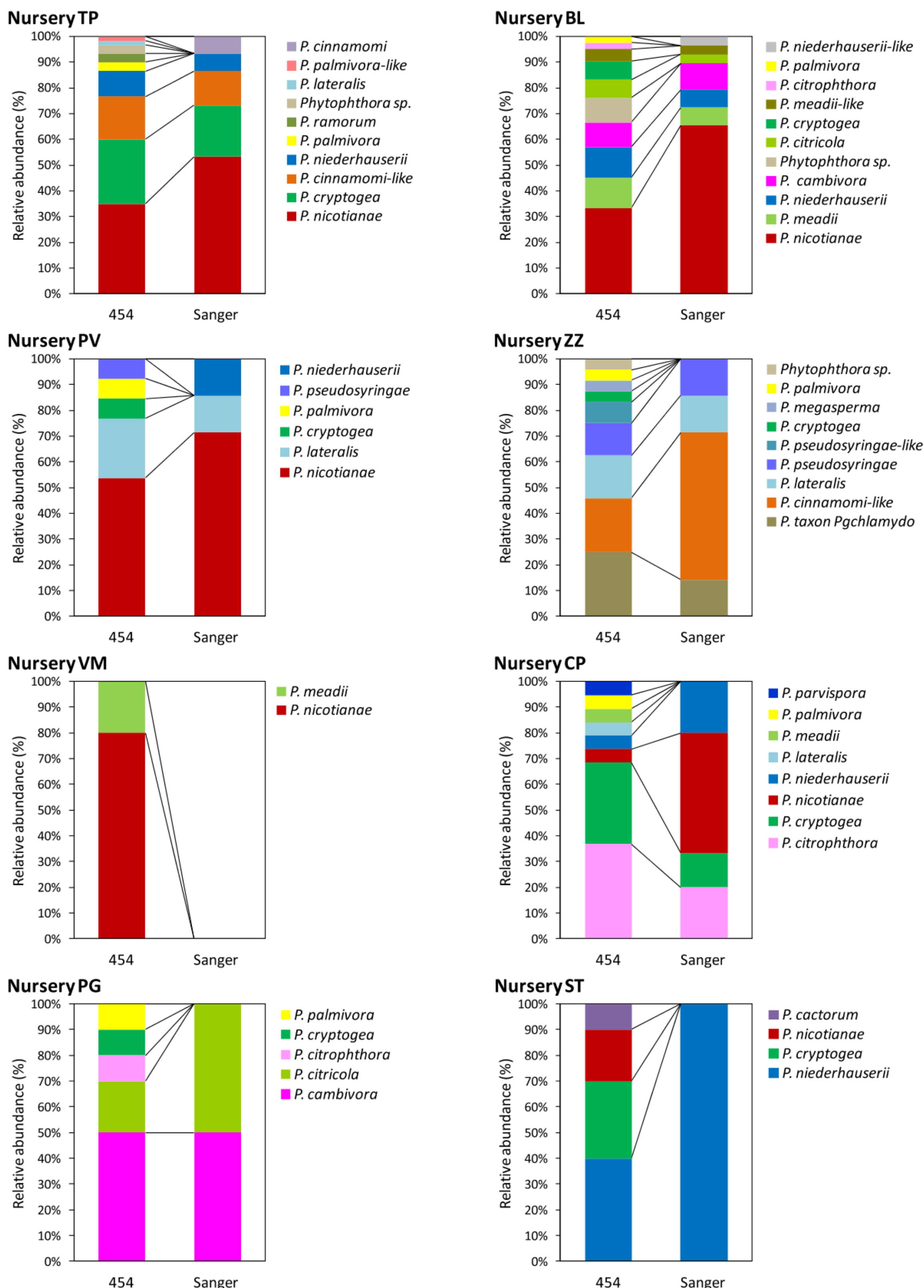

Fig. 3. Histograms showing the relative abundance of the *Phytophthora* phylotypes in the investigated nurseries. Results obtained using 454 pyrosequencing and cloning/Sanger sequencing approaches are compared.

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fungi and oomycetes enable a good identification up to the level of the genus but may become less reliable when used to identify species (Vannini et al. 2013). This is mainly due to the low genetic variation within ITS regions that may be very limited or nonexistent among closely related species. Indeed, the level of similarity (97%) commonly suggested in literature for picking operational taxonomic units would fail to discriminate many *Phytophthora* species (Edgar 2010; Sapkota and Nicolaisen 2015). The use of a higher similarity threshold (98 to 99%) may be useful to increase the level of discrimination among taxa (Vannini et al. 2013; Vettraino et al. 2012). However, whatever the level of similarity used, it may not reflect the current taxonomic status within the genus. Indeed, some accepted *Phytophthora* species are differentiated by single or very few consistent base variations within the ITS regions, while other species are characterized by consistent intraspecific variation (Martin et al. 2012, 2014).

In the present study, the detected 454P sequences were clustered using a 100% similarity threshold and distinct and reproducible ITS1 sequences occurring at least twice (STs) were subjected to specific phylogenetic analyses along with selected validated reference sequences. This approach made it possible to exploit all detected ITS variation and enabled the identification of taxa with the highest possible level of accuracy. Furthermore, the identification of taxa according to specific phylogenetic analyses minimized the impact of indels which are the most frequent type of error during 454 sequencing (Knief 2014). Indeed, the majority of the STs were identified to the level of species and the remaining ones were associated with a restricted number of taxa. This achievement is very important for the analysis of *Phytophthora* species since related species with very similar ITS sequences may be behaviorally distinct from each other. Furthermore, our analyses enabled the phylogenetic placement of a number of phylotypes not associated with any of the currently known species. We anticipate the future use of more variable markers in metabarcoding analyses of *Phytophthora* species to enable a higher level of discrimination among species. However, the single copy nature of possible alternative marker genes (http://www.phytophthoradb.org/) is likely to provide lower levels of sensitivity compared with the multicopy ITS regions. Furthermore, difficulties in designing reliable universal primers and the lower number of available reference sequences in genetic databases may represent an issue in species identification (Schoch et al. 2012).

In agreement with results of our previous study using a CSS approach (Prigigallo et al. 2015), the present investigation highlighted a very complex situation in potted ornamental nurseries with a large number of *Phytophthora* taxa detected in all investigated sites. The reader may refer to our previous study for details about the relevance of taxa detected with both methods and the primary role of nurseries in favoring the dissemination and the evolution of *Phytophthora* species (Brasier 2008; Faedda et al. 2013; Jung et al. 2015; Leonberger et al. 2013). Here we briefly discuss the relevance of some previously undetected taxa to underline the importance of the deeper sequence analysis. The detection of *P. ramorum* was significant. This pathogen is a relatively recently described species causing high mortality of native oak trees in California, where the disease is known as sudden oak death (Garbelotto and Hayden 2012; Hansen et al. 2012). 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 where it is a quarantine pathogen included in the A2 list of the European Plant Protection Organization. Furthermore, *P. ramorum* represents a growing threat to larch and natural ecosystems in the U.K. (http://www.forestry.gov.uk/forestry/INFD-8XLE56). Although, in the present study it was detected in a single site and with a low number of sequence reads, its presence in nurseries represents a great threat to horticulture and natural ecosystems (Croucher et al. 2013; Hansen et al. 2012). In Italy, *P. ramorum* was first detected in 2002 (Gullino et al. 2003) on potted plants of the exotic *Rhodondendron yakushimanum*. The stock was immediately destroyed and the pathogen was considered eradicated. Subsequently, however, it was found on *Viburnum tinus*, a native plant species, in a nursery in the Pistoia province (Tuscany), a major production area of ornamentals (Ginetti et al. 2014). Infected plants were once again destroyed but the detection of *P. ramorum* in a chestnut grove in central Italy (Vannini et al. 2013) and now in an ornamental nursery in southern Italy indicate that phytosanitary measures have so far failed in limiting the spread of this quarantine organism. Very likely the introduction of more sensitive diagnostic methods, such as pyrosequencing, will facilitate controls by phytosanitary regional services.

Particularly relevant is also the detection of *P. lateralis*, a specialized pathogen whose main host plant is Port-Orford Cedar (*Chamaecyparis lawsoniana*). This species was detected in two nurseries (PV and ZZ) using the CSS approach (Prigigallo et al. 2015) and was found in four nurseries (TP, PV, ZZ, and CP) in the present study. The presence of *P. lateralis* in nurseries where Port-Orford Cedar was not grown suggests that the inoculum of this pathogen is also being spread via other host plants. Furthermore, its detection in four of the eight nurseries sampled in this study is important as it suggests a recent widespread dissemination since it was first reported in Italy in 2015 (Prigigallo et al. 2015). It was first recorded in Europe in nurseries in France in 1996 and 1998 and in the Netherlands in 2004 (Hansen et al. 1999). It was considered eradicated until it was reported in shelter-belt trees in Brittany (Robin et al. 2010) and, more recently, in U.K. sites where it represents a serious threat to ornamental *Chamaecyparis* (Green et al. 2012; http://www.forestry.gov.uk/pdf/FCPH-PL-02-2014.PDF/$file/FCPH-PL-02-2014.PDF).

It is also important to highlight the detection of *P. cactorum*, a pathogen occurring worldwide and capable of infecting more than 200 species in 160 genera including many economically important crops such as apple, pear, rhododendron, azalea, and strawberry (Erwin and Ribeiro 1996). Another polyphagous and exotic species, *P. palmivora*, was detected in six different nurseries confirming it as an emerging pathogen in ornamental nurseries in the Mediterranean region (Van Tri et al. 2015). This pathogen is responsible for many different diseases on a wide range of plants worldwide including ornamental species (Erwin and Ribeiro 1996; Hansen et al. 2012). The detection of *P. citricola* s.str. is also relevant considering that isolates with ITS sequences identical to it are only known from *Citrus* species in Taiwan, Japan, and Argentina and have never been recovered from native forest trees in Europe (Jung and Burgess 2009).

In the present study a new phylotype defined as *P. palmivora*-like and five phylotypes clustering within the genus *Phytophthora* but phylogenetically distant from all currently known species were detected. Each of these phylotypes was between 6 and 25 bp different from its closest related taxon. Although, our data suggest these putative new *Phytophthora* species were present at a low frequency and one cannot speculate on their likely host range or specific threats there is no doubt that their presence in nurseries represents a potential new threat to plants in horticultural, agricultural, and/or natural settings. The complementation of metabarcoding with conventional isolation techniques to obtain cultures will be important and in the future other "meta-" data (metatranscriptomic, metaproteomic, and metabolomics) may be useful to obtain a more complete view of microbial communities under a given set of conditions (Segata et al. 2013; Zhang et al. 2010).

On the whole, the present study indicates that the use of genus-specific primers combined with 454P is a valuable tool to investigate *Phytophthora* diversity in different ecosystems. Compared with the CSS approach, it enabled a deeper sequencing at a fraction of the time and cost. This metabarcoding method represents a powerful tool to study *Phytophthora* diversity providing that particular attention is paid to the analysis of raw sequences in order to minimize errors and misinterpretations. Technology is moving

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rapidly and it is now recognized that Illumina sequencing provides a greater accuracy than 454 which has a higher error rate due to homopolymeric miscounts (Knief 2014). Furthermore, a nested approach with a high number of cycles (35) for both the first and second round of amplification was utilized to increase sensitivity since a single amplification round was found to be not sensitive enough to detect the whole *Phytophthora* diversity (Prigigallo et al. 2015; Scibetta et al. 2012). The nested approach could have theoretically increased the incidence of sequencing errors; however, the use of a high-fidelity polymerase has been shown to significantly reduce the risk of sequencing errors and of chimeric amplicons (Oliver et al. 2015). Furthermore, 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 was important to increase the quality of the sequence data. The exclusion of singletons from downstream analyses was considered a good compromise between the need to detect all genetic variation and the need to increase reliability of the sequencing panel. Indeed, according to our analysis the excluded singletons did not represent any additional species or phylotypes and, in most cases, they differed from the reported STs by single indels (data not shown). On the other hand, although the presence of errors in STs cannot be completely excluded, the analysis of the whole data set revealed important indirect confirmation of the consistency of the method since: (i) many identical STs were identified in different nurseries i.e., as a consequence of separate extractions and amplifications, (ii) most STs detected by 454P were identical to STs identified using the CSS sequencing approach, (iii) most STs detected only with 454P were identical to GenBank deposited sequences, and (iv) STs associated to new putative *Phytophthora* species were markedly different from their most closely related taxon and thus not likely a result of simple sequencing or PCR errors.

ACKNOWLEDGMENTS

This research was supported by grant MIUR-FIRB 2010 (RBFR10PZ4N) from the Italian Ministry of Education, University and Research (MIUR) “Metagenomic strategies to assess genetic diversity in soil-borne *Phytophthora* species.” We thank P. Schena for his valuable assistance during nurseries surveys and samplings. David Cooke is supported by the Scottish Government research programme.

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