Università degli Studi di Firenze

Beatrice Ginetti

"Identity, impact and role of *Phytophthora* species in planted forests of north Italy"

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"Identity, impact and role of *Phytophthora* species in planted forests of north Italy"

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Dottorando
Dott. ssa Beatrice Ginetti

Tutore
Prof. Salvatore Moricca

Co-tutore
Prof. Alessandro Ragazzi

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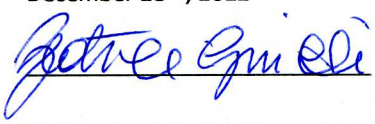
December 28th, 2012 Beatrice Ginetti



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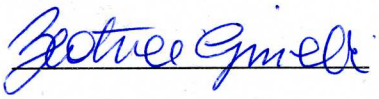

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Parole chiave:
Deperimento delle foreste, *Phytophthora*, essudazioni sul fusto, identificazione convenzionale, identificazione molecolare, analisi filogenetiche, nuovi taxa

Riassunto

Scopo: Scopo di questa ricerca è stato quello di accertare l'identità, l'impatto ed il ruolo ecologico di specie di *Phytophthora* in foreste artificiali del nord Italia ed in una piantagione in Toscana.

Metodi e Risultati: Circa 200 isolati di *Phytophthora* sono stati isolati, nel periodo 2010-2012, da varie matrici nel corso di campionamenti effettuati in piantagioni artificiali nel Parco "Boscoincittà" (Milano) e nei pressi di Alberese (GR). Alcune specie di *Phytophthora* sono state isolate da tessuti di alberi sintomatici su substrato selettivo V8-agar PARPNH. Altre specie congeneri sono state isolate da suolo e da acqua con il metodo dell'esca (mele) e successivamente trasferite sul medesimo substrato selettivo V8-agar PARPNH.

Substrati nutritivi a base di succo di pomodoro V8 (V8-agar) e di piselli (FPM) sono stati utilizzati per indurre la formazione di strutture riproduttive su tasselli di agar con micelio, previamente immersi in acqua di stagno non sterilizzata e filtrata.

Caratteri micro- e macro-morfologici quali aspetto delle colonie su diversi tipi di substrato (V8-agar, MEA, CMA e PDA), morfologia e dimensione degli sporangi, tipologia di anteridio (anfigino o paragino), dimensioni di oogoni, oospore e indice di parete, tasso di crescita a diverse temperature (10, 15, 20, 25, 30, 32, 35, 37, 39 °C) sono stati impiegati per lo screening e l'identificazione tradizionale degli isolati.

Tutti gli isolati sono stati altresì analizzati mediante tecniche molecolari. La regione ITS dell'rDNA, comprente le regioni spaziatrici ITS1 ed ITS2 ed il gene interno 5.8S, una regione del gene mitocondriale *cox*1, e una regione del gene della beta-tubulina sono state amplificate mediante PCR. La regione ITS è stata sottoposta a digestione con gli enzimi di restrizione MspI e AluI, mentre il gene mitocondriale *cox*1 è stato digerito con l'enzima RsaI.

Gli ampliconi della regione ITS, del gene *cox*1 e della beta-tubulina di isolati da ospiti, matrici e aree geografiche diverse, o mostranti variabilità nei caratteri morfologici, o che presentavano differenti profili su gel a seguito di digestione con enzimi di restrizione, sono stati sequenziati, al fine di poter condurre un'analisi filogenetica.

La combinazione di metodi convenzionali e moleculari ha portato all'identificazione inequivocabile delle seguenti specie: *P. gonapodyides*, *P. inundata*, *P. lacustris*, e *P. humicola* (quest'ultima nuova per l'Europa); dei taxa, solo parzialmente descritti ma non formalmente riconosciuti, *P.* taxon PgChamydo e *P.* taxon walnut (entrambi nuovi per l'Italia); alcuni ibridi tra *P. lacustris* and *P.* taxon PgChlamydo; ed una nuova specie, *P. acerina* prov. nom.

Sono state infine condotte delle prove di inoculazione artificiale sotto corteccia allo scopo di saggiare la virulenza delle specie di *Phytophthora* in collezione. Le prove sono state condotte su rametti di un anno di età raccolti in campo da piante adulte di *Acer pseudoplatanus* e *Fagus sylvatica*. Ogni replica è consistita di materiale geneticamente identico (un medesimo albero) al fine di ottenere una risposta omogenea. La lunghezza delle lesioni è stata valutata dopo 3 settimane previa rimozione della corteccia esterna. Re-isolamenti sono stati effettuati a random dai campioni inoculati su substrato selettivo V8-agar-PARPNH onde soddisfare i postulati di Koch e avere quindi conferma che fossero le specie di *Phytophthora* gli agenti causali.

Conclusioni: specie note e sconosciute di *Phytophthora* sono state identificate come la causa del deperimento degli alberi nel Parco Boscoincittà (Milano) e in una piantagione ad Alberese (GR).
Significato ed impatto dello studio: importanti membri del genere *Phytophthora* sono risultati essere i responsabili del declino e della morte delle piante nelle aree investigate. La vulnerabilità degli alberi, così come l'insorgenza, la diffusione e la gravità dei danni provocati dalle specie di *Phytophthora* sono sembrate strettamente correlate ai cambiamenti climatici. Alcune di queste specie si connotano come patogeni nuovi ed invasivi, che stanno seriamente compromettendo la salute delle piante e la produttività delle foreste.

Lavori correlati alla Tesi.

Ginetti B, Uccello A, Bracalini M, Ragazzi A, Jung T, Moricca S, 2012. Root Rot and Dieback of *Pinus Pinea* caused by *Phytophthora humicola* in Italy. Plant Disease **96,** 1694. http://dx.doi.org/10.1094/PDIS-05-12-0451-PDN

Ginetti B, Moricca S, Squires J, Cooke DEL, Ragazzi A, Jung T, 2013. *Phytophthora acerina* sp. nov., a new species causing bleeding cankers and dieback of *Acer pseudoplatanus* trees in planted forests in Northern Italy**,** manoscritto sottomesso a: Plant Pathology.

Ginetti B, Ragazzi A, Moricca S, 2013. The impact of *Phytophthora acerina* on *Acer pseudoplatanus* stands in a park of north Italy, manoscritto sottomesso a: Plant Pathology.

Ginetti B, Squires S, Cooke DEL, Ragazzi A, Moricca S, 2013. Multiple *Phytophthora* species from ITS Clade 6 recovered from semi-natural ecosystems and streams*,* manoscritto sottomesso a: Plant Pathology.

Keywords:

Forest decline, *Phytophthora sp.*, bleeding cankers, conventional identification, molecular identification, phylogenetic analyses, new taxa

Summary

Aims: The aim of this research was to ascertain the identity, impact and ecological role of species of *Phytophthora* in planted forests of north Italy and in a plantation in Tuscany.

Methods and Results: About 200 *Phytophthora* isolates were recovered in the period 2010-2012 from a number of matrices during forest surveys in north Italy in the Boscoincittà Park (Milan) and in a plantation in Alberese (GR), in Tuscany. Isolates from plant tissue of symptomatic trees were recovered on the selective V8-PARPNH agar medium. Isolations were also carried out from soil and water samples on the same selective medium following apple fruit-baiting.

Nutrient media V8 Juice agar and Frozen Pea (FPM) were used to induce formation of reproductive structures after flooding *Phytophthora* agar squares with non-sterilized, filtered pond water.

Morpho-physiological characters like colony phenotypes on different media (V8-agar, MEA, CMA and PDA), morphology and size of sporangia, antheridium type (amphigynous or paragynous), sizes of oogonia, oospores and wall index, growth rates under a range of temperatures (10, 15, 20, 25, 30, 32, 35, 37, 39 °C), were employed for the traditional screening and identification of isolates.

All isolates were also analysed by means of molecular methods. The ITS region, including the ITS1 and ITS2 spacers and the internal 5.8S gene of the rRNA operon, a portion of the mitochondrial *cox*1 gene region and one of the beta-tubulin nuclear gene region were PCR-amplified.

The ITS region was subjected to digestion with MspI and AluI restriction enzymes, while the cox1 gene was digested with RsaI.

Amplicons of ITS region and *cox*1 gene of isolates from different hosts, matrices and geographic origin, or which displayed different morphological characters and restriction endonuclease cleavage data, were also sequenced for making phylogenetic inferences.

This combined (conventional/molecular) approach lead to the unequivocal detection and identification of the following species: *P. gonapodyides*, *P. inundata*, *P. lacustris*, and *P. humicola* (the latter new for Europe); the taxa previously only partially described but not formally named *P.* taxon PgChamydo and *P.* taxon walnut (both new for Italy); some hybrids between *P. lacustris* and *P.* taxon PgChlamydo; and a new species, *P. acerina* prov. nom.

Under-bark inoculation trials were carried out to test the virulence of the whole *Phytophthora* set. Inoculation tests were performed on one-year-old twigs collected in the field from mature *Acer pseudoplatanus* and *Fagus sylvatica* trees. In order to get homogeneous responses, each replicate was on same-genotype material (a unique tree). Lesion lengths were assessed 21 days after removal of the outer bark. Random reisolations were made on selective V8-PARPNH-agar to satisfy Koch's postulates and thus confirm the *Phytophthora* species as the causal agents.

Conclusions: known and unknown species of *Phytophthora* were identified as the cause of the decline of trees in the Boscoincittà Park (Milan) and in a plantation in Alberese (GR).

Significance and Impact of the Study: prominent members of the genus *Phytophthora* were found to be responsible for the decline and death of trees in the investigated areas. Tree vulnerability, as well as the emergence, spread and severity of diseases induced by the *Phytophthora* species appeared to be strongly related to climate change. Some of these species are new invasive pathogens which are seriously impacting tree health and forest productivity.

Papers related to the Thesis.

Ginetti B, Uccello A, Bracalini M, Ragazzi A, Jung T, Moricca S, 2012. Root Rot and Dieback of *Pinus Pinea* caused by *Phytophthora humicola* in Italy. Plant Disease **96,** 1694. http://dx.doi.org/10.1094/PDIS-05-12-0451-PDN

Ginetti B, Moricca S, Squires J, Cooke DEL, Ragazzi A, Jung T, 2013. *Phytophthora acerina* sp. nov., a new species causing bleeding cankers and dieback of *Acer pseudoplatanus* trees in planted forests in Northern Italy**,** manuscript submitted to: Plant Pathology.

Ginetti B, Ragazzi A, Moricca S, 2013. The impact of *Phytophthora acerina* on *Acer pseudoplatanus* stands in a park of north Italy, manuscript submitted to: Plant Pathology.

Ginetti B, Squires S, Cooke DEL, Ragazzi A, Moricca S, 2013. Multiple *Phytophthora* species from ITS Clade 6 recovered from semi-natural ecosystems and streams*,* manuscript submitted to: Plant Pathology.

Chapter I
Introduction

Introduction

Acer psudoplatanus trees growing in an artificial forest in the Boscoincittà park (Milan), in the north of Italy, showed visible symptoms such as bark cankers, production of mucilaginous exudates along the stem, stunted growth and general crown dieback. Surveys were conducted with the aim of understanding the possible cause of such dieback. The symptomology observed on declining trees induced to hypothesize that the cause could be ascribed to the occurrence of one or more *Phytophthora* species. For this reason, a number of identification protocols (both traditional and molecular) were applied in order to discover the possible etiologic agent(s).

Isolations (on the selective V8-PARPNH agar medium) were carried out during winter and summer seasons of 2010-2011-2012 from plant tissue of symptomatic trees, from soil samples (collected near infected trees) and from water bodies on the same selective medium following apple fruit-baiting.

More than 200 *Phytophthora* isolates were recovered from the total of material sampled.

Research was then addressed to ascertain the identity, impact and ecological role of all the sampled *Phytophthora* species in the forests of Boscoincitta park. To this purpose, morphological, physiological, molecular and phylogenetic analyses were performed on these species. Infection trials were also carried out with *Phytophthora* isolates recovered from host tissue, soil and water. Concurrently, similar surveys were conducted in central Italy, in a plantation in Alberese (GR) in Tuscany.

The first and the second articles (Chapter II and III respectively) are focused on the discovery of a new *Phytophthora* species, *Phytophthora acerina* nom. prov. on *Acer pseudoplatanus* stands. The name given refers to the tree species from which all known isolates were isolated. This new species belongs to Clade 2 and it is included within the so called "*Phytophthora citricola* complex", that comprises several species such as *P. citricola* s. str., *P. citricola* II, III and E, *P. plurivora*, *P. multivora*, *P. pini*. The first of these papers is intended to give a morphological, physiological and taxonomic description of the species while the second is centred on the ecological role and epidemiological aspects of the oomycete.

The third article (Chapter IV) analyzes the species detected from water bodies, ponds and canals in the Boscoincittà park. Isolates of *P. gonapodydes*, *P. inundata*, *P. lacustris*, *P*. taxon Pg Chlamydo, *P*. taxon walnut and a possible hybrid between *P. lacustris* and *P.* taxon Pg Chlamydo were baited with apple fruits from several water bodies in the park. All these species belong to Clade 6, a group made of species that are usually isolated from water bodies such as rivers, canals, lakes and ponds. They have never been reported to play a serious pathogenic action against plants species; however, their ecology and role in the investigated ecosystem need to be studied through extensive baiting and artificial infection trials. Indeed most of the *Phytophthora* species baited in the Boscoincittà park showed a potential aggressiveness towards tested plants.

The fourth paper (Chapter V) is the first report of *P. humicola* in Europe. The finding was in a ten-year-old plantation near Aberese (GR) in Tuscany. This *Phytophthora* species was isolated from soil of symptomatic *Pinus pinea* by apple fruit baiting.

Taxonomic placement of the *Phytophthora* genus

Although the members of the genus *Phytophthora* and other oomycetes have been included for a long time in the Fungi kingdom, they were found to belong, following a review of their phylogenesis,

to the new kingdom *Straminipila* (Jung & Burgess, 2009). The genus *Phytophthora* is closely related to the genus *Pythium* and together they are classified in the *Pythiaceae* family, so named because the *Pythium* taxa was first to be described; currently the genus is assigned to the Order *Peronosporales* and Phylum *Oomycota* within the heterokonts, a group that includes biflagellate organisms.

Until the mid-90s no more than 54 species of *Phytophthora* had been described worldwide (Erwin & Ribeiro 1996). Since that time the knowledge about the different species of *Phytophthora* and diseases induced by these pathogens in plants have increased considerably. Only in the last 15-20 years, about 25 new *Phytophthora* taxa have been described from both forest environments and semi-natural ecosystems, 13 of which have been officially recognized and described. The taxonomic increase within the *Phytophthora* genus finds its justification in the growing number of samplings in search for these pathogens. These microorganisms have been searched in forests, semi-natural environments and nurseries where they often caused devastating declines and generalized die-offs of most important tree species (Jung *et al*. 2000; Vettraino *et al.* 2002; Jung & Blaschke, 2004; Jung, 2009). Furthermore, the development and availability of new advanced molecular tools of investigation have contributed to the discovery of new *Phytophthora* species not previously identified because they were indistinguishable morphologically and physiologically from each other with traditional survey methods (Man in't Veld *et al.* 2002; Jung *et al.* 2003; de Cock & Levesques, 2004; Burgess *et al.* 2009).

Fifty described *Phytophthora* species were phylogenetically analyzed by Cooke *et al*. (2000). On the basis of phylogenetic results of ITS1 and ITS2 rDNA sequences the majority of the *Phytophthora* taxa clustered into eight main groups or lineages, defined Clade 1-8, which formed a recently evolved monophyletic group. Three different *Phytophthora* sp. represented two groups apart, *P. macrochlamydospora* and *P. richardie* (Clade 9) and *P. insolita* (Clade 10). These two Clades were the nearest neighbours for Clade 1-8. Clade 1-5 mainly contains *Phytophthora* with papillate, caducous sporangia with airborne dispersion, whilst Clade 6-8 principally comprised *Phytophthora* that are non-papillate and present a widely soil-borne root-infecting habit (Fig. 1)**.**

The Clade 6

When first described, prior to the advent of molecular tools in *Phytophthora* systematic, Clade 6 consisted of just three species: *Phytophthora gonapodyides*, *P. humicola* and P. *megasperma* (Erwin & Ribeiro, 1996). To date, due to an extensive review on the evolution, ecology, reproduction and impact of Clade 6 *Phytophthora,* more than 20 species (most of which not yet formally described) have been included. Several *Phytophthora* species belonging to ITS Clade 6 were found living as assemblages in riparian ecosystems in Europe (Brasier *et al*., 2003a), in Oregon and Alaska (Reeser *et al*., 2011) and in Australia (Jung *et al*., 2011). In any case, with the exception of *P.* taxon asparagi, *P. gonapodyides*, *P. megasperma* and *P. rosacearum*, all the species belonging to this clade have limited association with agriculture and horticulture. With the newest advent of molecular tools in the identification of *Phytophthora* species many new, not described taxa were identified within this ITS Clade. On the basis of the ITS data, Clade 6 has been divided into three sub-clades; sub-clade I, with *P. humicola*, *P. inundata*, *P. rosacearum* and several other undescribed taxa, *P.* taxon walnut included; sub-clade II contains, among many others, *P. gonapodyides*, *P. megasperma* and the recently described *P. lacustris* (Nechwatal *et al*., 2012) and some other undescribed taxa such as *P.* taxon PgChlamydo, *P.* taxon hungarica and *P.* taxon forestsoil; sub-clade III just contains *P.* taxon

asparagi (Fig. 2). Most of the species belonging to the ITS Clade 6 are usually isolated from riparian and stream ecosystems and generally don't represent a threat to agriculture and horticulture but the ecological function of most of these taxa in ecosystems is still unclear (Jung *et al*., 2011). Brasier *et al*., (2003 a,b) assumed that several members of this ITS Clade changed to a saprophytic lifestyle, a conjecture supported by their, often dominant, presence in most of the water bodies monitored. Despite this, some of these species can be opportunistic and even aggressive to plants (Brown & Brasier, 2007; Duran *et al*., 2008; Jung, 2009). Species such as *P. gonapodydes* and *P. inundata* sporadically caused root and collar rot and aerial cankers in Europe, especially during very humid periods, on *Fagus sylvatica*, *Quercus robur* and *Alnus glutinosa* (*P. gonapodyides*; Jung *et al*., 1996; Jung & Blaschke 2004; Brown & Brasier 2007) and on *Aesculus, Salix* and olive trees (*P. inundata*; Sanchez-Hernandez *et al*., 2001; Brasier *et al*., 2003b). Furthermore, inoculation trials conducted with *P. lacustris*, *P. gonapodydes* and *P. megasperma* demonstrated their capability to induce significant fine root damages to flooded *Alnus glutinosa* and *Prunus persica* seedlings; weak to moderate aggressiveness of *P. lacustris* on wounded stems or twigs of *Alnus*, *Prunus* and *Salix* species was established too (Nechwatal *et al*., 2012).

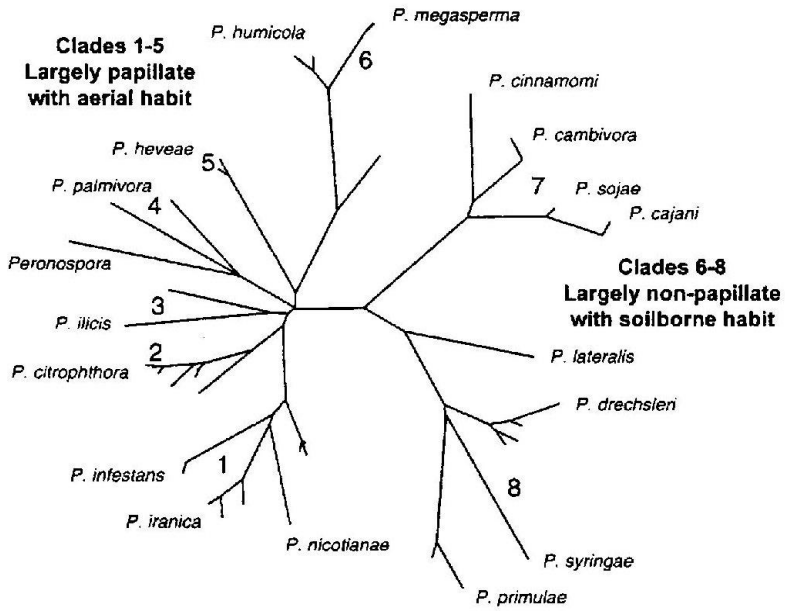

Figure 1 Radial phylogenetic tree showing the broad evolutionary trends among clades 1-8 of the main *Phytophthora-Peronospora* cluster and selected individual taxa at the clade termini (from Cooke et al., 2000).

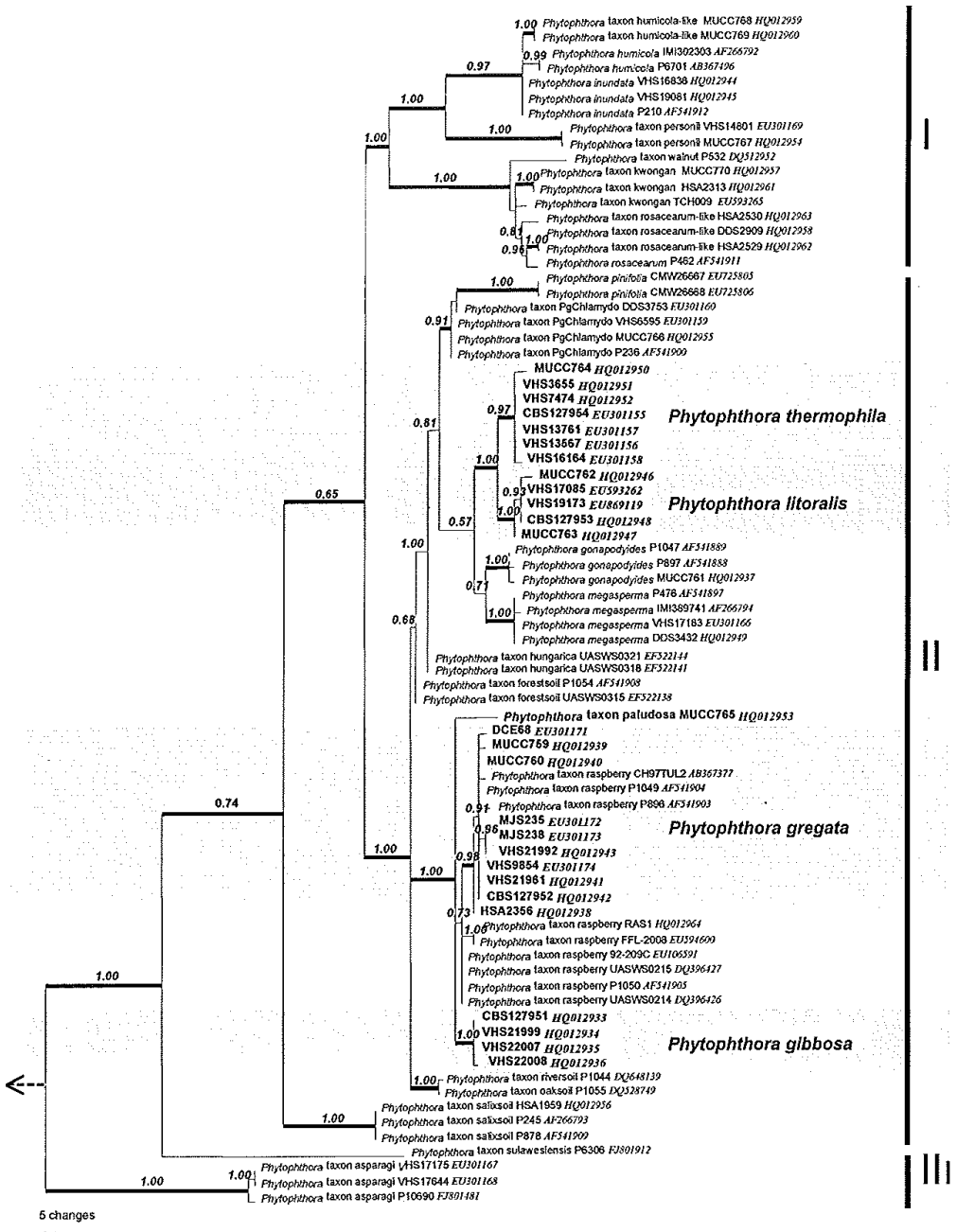

Figure 2 Bayesian inference tree based on rDNA ITS sequences showing phylogenetic relationships within *Phytophthora* ITS Clade 6 (from Jung *et al.* 2011).

The Clade 2: The "*Phytophthora citricola* complex"

Phytophthora citricola, isolated from citrus fruits showing brown rot, was described for the first time in Taiwan by Sawada in 1927.

Unfortunately, the lack of a formal classification caused much confusion in the identification and, in the first instance, *P. citricola* was considered to be conspecific of *P. cactorum* (Tucker, 1931; Leonian, 1925).

In 1932, homothallic isolates of *Phytophthora* showing sporangia characterized by flat and wide papillae were described as *P. cactorum* var. *applanata* (Chester, 1932). Finally Waterhouse (1957) investigated both isolates, (*P. citricola* by Sawada and the designated *P. cactorum* var. applanata by Chester) concluding that they belonged to the same species "*P. citricola*", so named because this was the first species to be described.

However, morphological variations in the isolates belonging to the *P. citricola* group have been repeatedly reported (Zentmyer *et al.* 1974, Oudemans *et al.* 1994, Balci & Halmschlager 2003, Jung *et al.* 2005).

Subsequent researches based on both morphological and molecular studies using a broad range of *P. citricola* isolates showed that *P. citricola* has different characteristics and is very diverse (Oudemans *et al.* 1994, Bhat & Browne 2007, Moralejo *et al.* 2008). Due to an isoenzyme study conducted by Oudemans *et al.* (1994) a global collection of 125 isolates of *P. citricola* has been grouped into five distinct subgroups (CIT1-5).

Furthermore, using the SSCP fingerprinting technique, *P.citricola* has been divided into 4 different groups, from *P. citricola* I to IV (Kong *et al.* 2003, Gallegly & Hong 2008). These observations, associated with both the wide range of hosts and the geographical distribution of *P. citricola* (Erwin & Ribeiro 1996, Fontaneto *et al.* 2008), have strongly suggested the existence of a species complex, acknowledged as *P. citricola* complex, which includes morphologically similar but genetically distinct species.

This condition led researchers to reassess *Phytophthora* isolates belonging to this complex. Recently a large group of isolates from Western Australia, isolated from tissues of symptomatic or dead plants in natural ecosystems by the Vegetation Health Service (VHS) and misidentified as *P. citricola* for about 35 years, have been described as *P. multivora* (Scott *et al*., 2009). Some sequences in GenBank identified as *P. citricola* have sequences identical to *P. multivora*. For the same reason many isolates obtained from 39 host species belonging to 16 families during a large-scale sampling in semi-natural forest ecosystems and nurseries throughout Europe during the decade 1999-2009, and already identified in GenBank as *P. citricola*, have been described by Jung & Burgess (2009) as a new species, *P. plurivora* sp. nov. on the basis of their unique combination of morphological, physiological and molecular character.

This latter study was based on results obtained thanks to sequence analysis of internal transcribed spacer region (ITS) and mitochondrial genes *cox*1 and β-tubulin, in combination with the morphological and physiological characteristics of the investigated individuals.

In 2010 two other species isolated from cultivated *Agathosma* were revealed in the *P. citricola* complex. They were described as the new species *P. capensis* and the putative novel species *P. taxon emzansi* by Bezuidenhout *et al.* (2010).

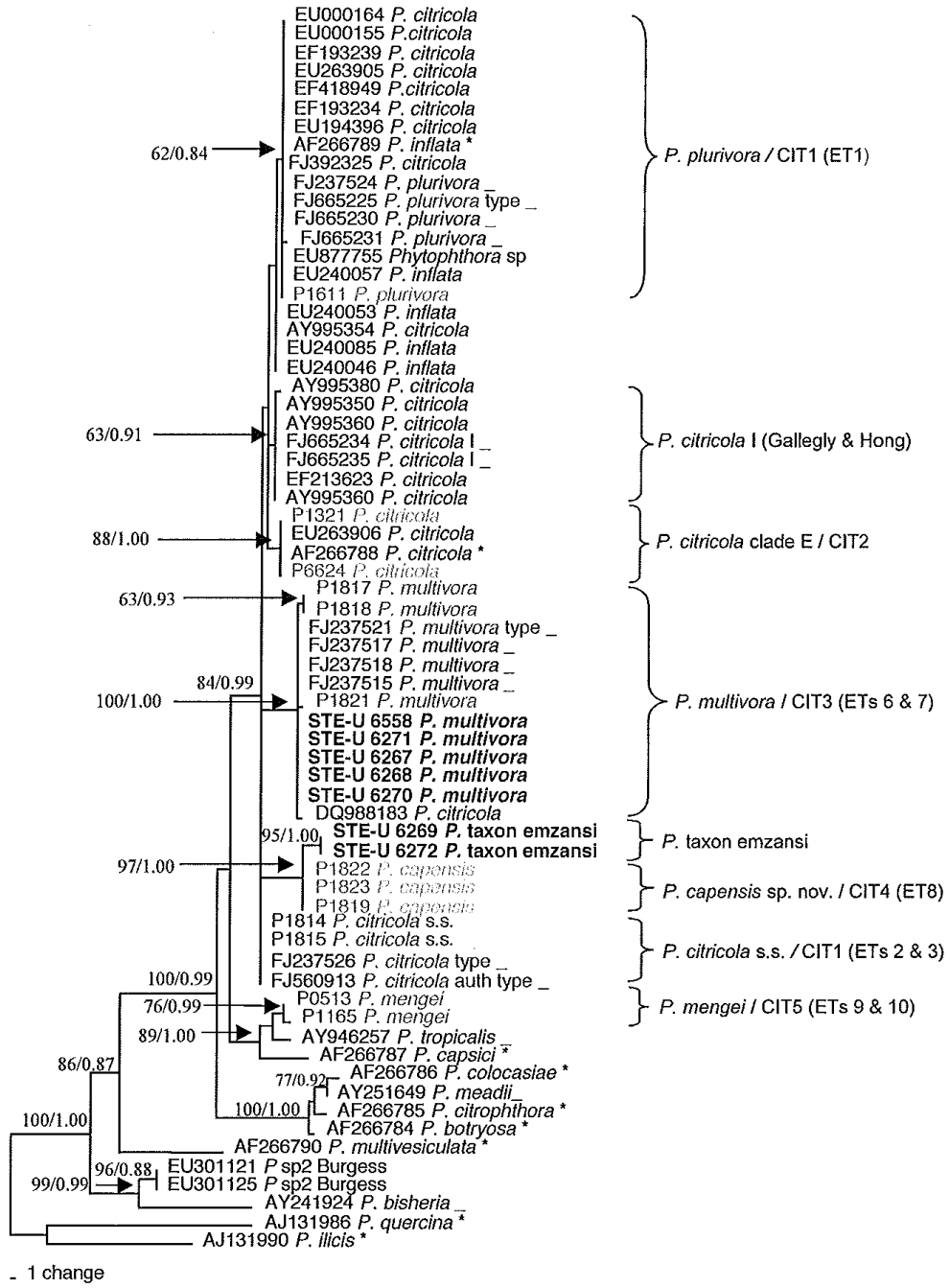

Figure 3 Phylogeny obtained from a heuristic search of *Phytophthora* Clade 2 species based on the ITS gene region (from Bezuidenhout *et al*. 2010).

A different story regards *P. pini*: this species was described first by Leonian in 1925 but it was widely disregarded until 1963, when it was identified as *P. citricola* by Waterhouse (1957). Examination of these isolates revealed that the ex-type culture of *P. pini* is identical to *P. citricola* I and for this

reason, Hong *et al.* (2011) resurrected *P. pini* to distinct species status within the *P. citricola* complex replacing *P. citricola* I.
Many problems can emerge as a result of these continuous changes in the *P. citricola* complex taxonomy and a special attention is needed because many sequences are still incorrectly entered in GenBank as *P. citricola* even if belonging to different species.

Hystory and phytopathological importance of the *Phytophthora* genus

"*Phytophthora*" is a name that derives from the Greek language and it means literally *Phyto* (plant) and *phthora* (destroyer); in fact, this taxon includes species that are mainly parasitic on various host plants. Some species are host specific while others are defined generalist, being able to affect a wide range of host species. These pathogens are known worldwide as primary parasites of fine roots and causative agents of root and collar rot, bark cankers and bleeding bark cankers on both mature and young individuals of hundreds of tree and shrub species. It can be affirmed that many *Phytophthora* species are among the most aggressive and dangerous plant pathogens in the world. As a consequence of the bark and root damages, the crowns of affected trees develop typical symptoms such as increased transparency, sparse ramification and stunted growth of lateral twigs and clustering of leaves at the end of branches. Finally, small-sized and often chlorotic foliage, dieback of branches and crown dieback could be shown.
The potato blight caused by the pathogen *P. infestans* decimated the entire Irish population in a few years (1845-1849); this was because *P. infestans* totally destroyed Ireland's potato crops which represented the staple food of the population. Effects were poverty and mass starvation with deep sociological and economic changes that induced the emigration of large numbers of people to the United States. It is estimated that 8 million Irish people were struck by this scourge and that probably no other plant disease ever in history played a so devastating impact on a population. This event is an example of the infectiveness and destructive capability of the species which can cause, when environmental factors favour a rapid increase of the pathogen's biomass, a severe and serious epidemic.
Another important example of the hazard represented by the members of the genus *Phytophthora* is the syndrome commonly known as "Sudden Oak Death", caused by *P. ramorum*. Fourteen isolates of this species were first isolated from German and Dutch diseased *Rhododendron* sp. and *Viburnum* sp. between 1993 and 1999 (Werres *et al.* 2001). From about 1995 to 2002 this species has been indicated as the cause of extensive mortality of *Quercus spp*. and *Lithocarpus densiflorus* in California. During that period this disease reached such a huge proportion to be described as an epidemic, having affected approximately 300 km of the Central Cost of California. Symptoms on trees were cankers with brown or black discolored outer bark and production of dark red sap developed before foliage symptoms became evident (Rizzo *et al.* 2002). The pathogen was then introduced into native forests of California and Oregon where it was able to infect a lot of woody plants and some herbaceous species. Pacific madrone (*Arbutus menziesii*) seedlings and saplings seemed to be one of the preferred susceptible species (Maloney *et al.* 2004). This quarantine pathogen was detected a few years later in Europe, exactly in the UK where it infected foliage of *Rhododendron ponticum* first to be transmitted to *Fagus*, *Quercus* and other tree hosts later (recently *Larix* plantations in Scotland), causing aerial stem bleeding cankers (Brown & Brasier, 2007).

During the spring of 2002 *P. ramorum* was isolated from a plant of *Rhododendron yakushimanum* in a nursery in the north of Italy (Verbania, Piemonte). Control measures were implemented immediately and subsequent examinations on the Verbania nursery identified the outbreak of the pathogen in a young pot plant of *R. yakushimanum* of Belgian origin (Gullino *et al.,* 2003). Fortunately, since then no other alert was issued in Italy.

Phytophthora species are able to cause these kinds of pandemics due to their biological reproductive style. In fact they are diploid organisms that produce heterokonta biflagellate zoospores. Many species are able to produce oospores that, in addition to be useful to survive for long periods in soil, giving a greater chance of survival during adverse periods, represent a genetic variability resource for the progeny. Furthermore Phytophthoras have a really short generation time caused by the rapid production of biflagellate zoospore, making it possible for a rapid spread of the disease from a single initial infection (Dick, 1992). The major part of soil bearing pathogenic fungi is considered monocyclic and their inoculum does not increase considerably during the year (Vanderplank, 1963). Inocula of *Phytophthora* species are on the contrary able to increase suddenly from really low levels (often undetectable) to very high levels in a period of some weeks or just few days, causing destructive foliar as well as root diseases in the population of the attacked host (MacKenzie *et al.,* 1983). Within the Oomycetes there are other members which share this capability and the most similar to the Phytophthoras are species of *Pythium*. Analyses based on the relationship between climatic conditions and biological cycle reveal that the success of Phytophthoras is mainly due to the more ephemeral life form such as zoospores and sporangia rather than to the resting structures or the survival propagules such as hyphal aggregation and clamydospores.

The swift production of sporangia and zoospores from infected plant organs increase the charge of inoculum of *Phytophthora* species especially when environmental conditions, the most important of which is the presence of free water, are favorable (Dick, 1992). For this reason pathogenic *Phytophthora* are commonly considered to be polycyclic (Fry, 1982; MacKenzie *et al.,* 1983).

Phytophthora life cycle

The life cycle of *Phytophthora* species is illustrated in Figure 4. *Phytophthora* species are able to survive under adverse conditions for many years thanks to resting structures such as oospores and clamydospores in the soil and in infected root tissue till environmental conditions became suitable and favourable permitting their germination in sporangia or in a coenocytic mycelium. The growing hyphae are able, in turn, to origin the infective cycle by forming sporangia that release zoospores with a reniform shape. Zoospores are provided with two flagella which allow them to swim for hours (Bimpong & Clerk, 1970) but that can also stop them from swimming. The zoospore thus rounds off, develops a cell wall (Bartnicki-Garcia, 1973) and turnsin a structure called “cyst”.

Cysts can germinate by producing germ tubes and mycelia or, occasionally, thanks to a process called “repeated emergence”, a zoospore that was previously formed inside the cyst (Drechsler, 1930; Blackwell, 1949). Motile zoospores are very important and are considered the major infectious propagules (Erwin & Ribeiro, 1996).

The just described process represents the asexual stage of the cycle. In order to give origin to the sexual phase, the two different sexual structures represented by an antheridium (male component) and by an oogonium (female component) have to meet each other.

Some *Phytophthora* species are homothallic and for this reason are self-fertile, whereas others are heterothallic (self-steril) and need the two opposite mating types, A1 and A2, to encounter. The oogonial incept can grow through the antheridial incept, forming an antheridium classified as amphigynous, or in some other species it attaches to the oogonium by contact usually near the bearing hypha resulting in a paragynous antheridia. Mature fertilized oogonia produce sporangia and the cycle repeats itself.

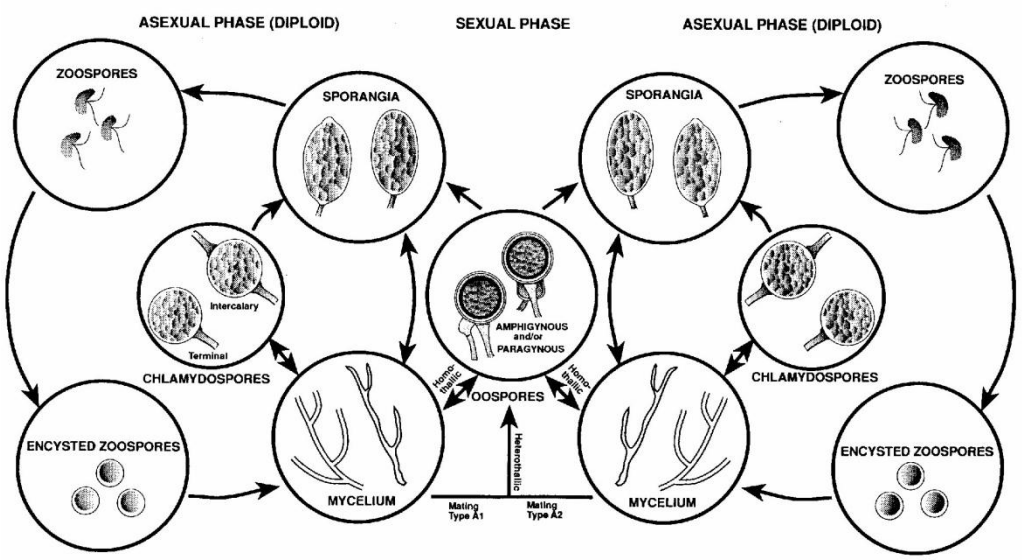

Figura 4 Life cycle of soil born *Phytophthora* species (from Erwin & Ribeiro, 1996).

Significance and impact of the present study

Prominent members of the genus *Phytophthora* were found to be responsible for the decline and death of trees in the investigated forest areas (Boscoincittà park). Already known and unknown species of *Phytophthora* were identified and, among these, the newly described species *P. acerina* sp. nov. This species was unequivocally ascertained to be the causative agent of the dieback of *A. pseudoplatanus* in the park.

Besides this new pathogen, the combined (conventional/molecular) approach adopted led to the unequivocal detection and identification of several interesting species: *P. gonapodyides*, *P. inundata*, *P. lacustris*, and *P. humicola* (the last one new for Europe); the taxa previously only partially described but not formally named *P.* taxon PgChamydo and *P.* taxon walnut (both new for Italy); and some hybrids between *P. lacustris* and *P.* taxon PgChlamydo.

Tree vulnerability, as well as the emergence, spread and severity of diseases induced by *P. acerina* appeared to be related to climate change.

Some of the species found are new invasive pathogens which have potential to seriously impact tree health and forest productivity.

Future prospects: assessment of the interactions between *Phytophthora* species and endophytic fungi

A possible future direction of the research could be to investigate whether *Oomycetes* and endophyte may form a new pathogenic complex. Hypotheses of a possible vicariance between these two groups of microorganisms have been formulated, and thus it could be worthwhile to investigate whether any additive, or synergistic effect exists among them.

Mediterranean countries have faced two opposite climatic situations during the last decades: extreme water deficit during the summer period, and excess of rainfall during the winter season. It is well known that water is fundamental for the reproduction and active or passive mobility of *Phytophthora* species. The concentration of rainfall during the coldest months of the year and mild winters increases the presence of free water in the soil and promotes the development of zoospores, greatly enhancing their infectivity. Moreover, due to a permanent soil saturation, resistance mechanisms of trees are reduced so that *Phytophthora* species can invade more easily their root systems. Then they move upwards to the stem base, causing collar rot and aerial bleeding cankers. *Phytophthora*-infected trees are weakened and appear to be more prone to the attacks of secondary parasites.

Hot and dry summers predispose also plants to water stress and to the attack of pathogenic endophytes (Moricca & Ragazzi, 2008). These conditions in fact increase the aggressiveness of endophytic fungi. These microorganisms are natural, asymptomatic host tissues colonizers and their presence is virtually documented in all tree species. Climate anomalies, such as extreme events, windstorms, damages caused by hail, (which are increasingly reported during summer time), favour the penetration of endophytic fungi inside the plants (Smith *et al.* 1996). Moreover, whereas oomycetes are more vital and active during cold periods, fungal endophytes become more aggressive during the hottest months. All these factors and conjunctures lead to hypothesize a possible vicariance over time and / or space between *Oomycetes* and endophytic fungi in forested areas. This possibility could explain the variety of symptoms observed in the decline and dieback of tree species in deciduous forests of the Lombard plain.

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Chapter II

Phytophthora acerina sp. nov., a new species causing bleeding cankers and dieback of *Acer pseudoplatanus* trees in planted forests in Northern Italy

Phytophthora acerina sp. nov., a new species causing bleeding cankers and dieback of *Acer pseudoplatanus* trees in planted forests in Northern Italy

B. Ginetti[1], S. Moricca[1], J. Squires[2], D. Cooke[2], A. Ragazzi[1], T. Jung[3,4]

[1]University of Florence, Agricultural and Biotechnology Department, Section of Plant Protection, Cascine Square 28 -50144, Florence, Italy.
[2]The James Hutton Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK.
[3]*Phytophthora* Research and Consultancy, Thomastrasse 75, D-83098 Brannenburg, Germany.
[4]Institute for Biotechnology and Bioengineering, Centre of Genomics and Biotechnology, (IBB/CGB), Plant and Animal Genomic Group, Laboratório de Biotecnologia Molecular e Fitopatologia, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.

Abstract

In planted forest stands in Northern Italy a severe dieback of *Acer pseudoplatanus* trees was noticed in 2010. Affected trees showed collar rot and aerial bleeding cankers along the stems leading to crown dieback and eventually death. An unknown *Phytophthora* species was consistently isolated from necrotic bark and xylem tissue and from rhizosphere soil. Based on its unique combination of morphological and physiological characters and phylogenetic analysis this new taxon is here described as *Phytophthora acerina* sp. nov. Phylogenetic analyses of ITS, *cox*1 and ß-tubulin gene regions demonstrated that *P. acerina* is unique and forms a separate cluster within the '*P. citricola* complex' with *P. plurivora* being its closest known relative. *Phytophthora acerina* is homothallic with smooth walled oogonia, thick-walled, mostly aplerotic oospores with a high abortion rate, paragynous antheridia, and persistent, morphologically quite variable semipapillate sporangia. Four to five weeks old cultures produced globose to subglobose, appressoria-like and coralloid hyphal swellings and characteristic stromata-like hyphal aggregations. Optimum and maximum temperatures are 25°C and 32 °C, respectively. Genetic uniformity of all 15 analysed isolates and the apparent absence of this species in the extensive surveys of nurseries, forests and seminatural ecosystems conducted in the previous two decades across Europe indicate a recent clonal introduction to Northern Italy and Europe. Underbark inoculation tests demonstrated the aggressiveness of *P. acerina* to *A. pseudoplatanus* and *Fagus sylvatica*, indicating this pathogen might be a serious risk to maple and beech plantations and forests in Europe.

Introduction

Phytophthora is a major genus of plant pathogens causing more than 66 % of all known fine root diseases and more than 90 % of all collar rot diseases of woody plant species (Tsao, 1990). Many devastating declines of trees and natural ecosystems have been and continue to be driven by non-native *Phytophthora* species which remain unnoticed in their native environment. After their introduction to other continents they became invasive and started to threaten a non-adapted flora which due to a lack of co-evolution contains a high number of susceptible species. Most renowned examples include various diebacks of eucalypt forests, *Banksia* woodlands and heathland ecosystems in Australia and littleleaf disease of pines in the USA caused by *P. cinnamomi* (Cahill *et al.* 2008; Shearer & Tippett, 1989), ink disease of *Castanea* spp. in Europe and the USA caused by *P. cinnamomi* and *P. cambivora* (Jung *et al*. 2012a; Vettraino *et al.* 2005), root rot of *Chamaecyparis*

lawsoniana in Oregon caused by *P. lateralis* (Jules *et al.* 2002; Hansen *et al.* 2000), and the extensive mortality of oaks and tanoaks in California and of Japanese larch in the UK caused by the airborne *P. ramorum* (Brasier & Webber 2010; Rizzo *et al.* 2002). In the past 15 years the involvement of *Phytophthora* species in widespread declines of *Acer* spp., *Aesculus hippocastanum*, *Alnus* spp., *Betula* spp., *Fagus sylvatica*, *Juglans regia, Quercus* spp. and *Tilia* spp. across Europe has been demonstrated (Jung *et al.* 1996, 2000, 2005, 2009, 2012a; Jönsson *et al.* 2005; Jung & Blaschke 2004; Balci & Halmschlager 2003;. Vettraino *et al.* 2002, 2003; Gibbs *et al.* 1999; Brasier *et al.* 1993). The high aggressiveness of the most frequently involved *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. plurivora*, *P. quercina* and *P. ramorum* to a range of most common European tree species such as *Acer platanoides* and *A. pseudoplatanus*, *A. hippocastanum*, *Castanea sativa*, *F. sylvatica*, *J. regia* and several *Quercus* spp. (Jung & Burgess 2009; Fleischmann *et al.* 2004; Vettraino *et al.* 2003; Brasier & Jung 2003; Jung *et al.* 1996, 1999, 2002, 2003; Robin *et al*. 1998, 2006) strongly indicate that these *Phytophthora* species have not co-evolved with these tree species and were introduced to Europe. Synergistic interactions between root losses and bark infections caused by introduced soilborne *Phytophthora* species and the increasing frequency of climatic extremes are a major cause for the severe declines of oak and beech forests. As current models of climate change are predicting a further intensification of the underlying climatic trends, a proliferation of *Phytophthora* damages may be expected, increasing the instability and vulnerability of forest ecosystems dominated by tree species susceptible to *Phytophthora* (Jung, 2009; Jung *et al*. 1996, 2000, 2012a; Desprez-Loustau *et al.* 2007; Brasier & Scott 1994).

Another risk of the introduction of alien *Phytophthora* species is the possibility of creating species hybrids with host ranges and aggressiveness totally different from those of their parents as demonstrated by *Phytophthora alni* ssp. *alni* the causal agent of alder mortality across Europe (Brasier *et al.* 2004).

Before 2000, 55 morphospecies of *Phytophthora* were known to science of which 33% had been found associated with damages of trees in nurseries, plantations and forests (Erwin & Ribeiro 1996; Brasier, 2009). Between 2001 and 2012, 103 new *Phytophthora* taxa have been described as species or informally designated, and 64% of them came from nursery trees, forests or other natural ecosystems (Kroon *et al.* 2012; Hansen *et al.* 2012; Jung *et al.* 2009, 2011; Brasier, 2009). On a global scale, a conservative calculation is predicting another 100 to 500 unknown *Phytophthora* species (Brasier, 2009). Alone in Europe, in the previous two decades an array of 25 new *Phytophthora* taxa were detected from forests, seminatural ecosystems and plantations, of which 17 have been formally described (Aghighi *et al.* 2012; Grünwald *et al.* 2012; Nechwatal *et al.* 2012; Man In't Veld *et al.* 2011; Jung *et al.* 2009, 2012a; Jung & Burgess, 2009). This dramatic development has been caused by (i) the exponential increase in the international trade of rooted nursery stock, (ii) the rapid development and application of new molecular detection and identification tools and (iii) the increased interest in environmental surveys for *Phytophthora* species.

Planting of infested nursery stock is one of the major pathways of *Phytophthora* species into agricultural and horticultural systems, forests and other natural ecosystems (Jung *et al.* 2012b; Hansen *et al.* 2012; Hansen, 2008; Brasier, 2008; Erwin & Ribeiro 1996). A series of extensive surveys of field- and container-grown nursery stock of forest trees, horticultural and ornamental plants in more than 600 nurseries in 16 European countries have detected widespread *Phytophthora*

infestations with 40 *Phytophthora* taxa of which 32 are considered exotic invasives (Jung *et al*. 2012b). Similar results are coming from Australia and the USA (Schwingle *et al.* 2007; Ferguson & Jeffers 1999; Hardy & Sivasithamparam 1988). The devastating impact of a large-scale use of infested nursery stock on natural ecosystems was demonstrated by the rapid spread of the root and collar rot epidemic of alders in Europe caused by *P. alni* (Jung *et al.* 2012a; Jung & Blaschke 2004; Brasier *et al*. 2004; Gibbs *et al.* 1999).

Novel molecular methods increased the understanding of the true relationships between the different *Phytophthora* species and between *Phytophthora* and other oomycete genera and enabled the development of natural phylogenies for the genus and related oomycetes (Blair *et al.* 2008; Villa *et al*. 2006; Kroon *et al.* 2004; Martin & Tooley 2003; Cooke *et al.* 2000). It was shown that several wellknown morphospecies like *P. citricola*, *P. gonapodyides*, *P. megasperma*, *P. porri* or *P. syringae* were complexes of morphologically and physiologically almost indistinguishable species (Burgess *et al*. 2009; Jung & Burgess 2009; de Cock & Lévesque 2004; Brasier *et al.* 2003, Jung *et al.* 2003; Man in't Veld *et al.* 2002; Oudemans *et al.* 1994; Hansen & Maxwell 1991). After unravelling of these complexes many new species were described which in some cases are not even closely related to each other, eg. *P. syringae* and *P. pseudosyringae* or *P. megasperma* and the new species *P. sojae*, *P. trifolii* and *P. medicaginis*. The morphospecies *P. citricola sensu lato* was divided into *P. citricola s. str.* and seven new closely related taxa that together form the '*P. citricola* complex', ie. *P. capensis*, *P. multivora*, *P. pini*, *P. plurivora*, *P. citricola* III, *P. citricola* E and *P.* taxon 'emzansi'; and the two new species *P. mengei* and *P. elongata* which also belong to ITS Clade 2 but are more distantly related to the other taxa (Hong *et al.* 2009, 2011; Bezuidenhout *et al.* 2010; Rea *et al.* 2010; Jung & Burgess 2009; Scott *et al.* 2009). The combined use of standardized morphological and physiological studies and analyses of sequence data from different nuclear and mitochondrial genes has become indispensable for a correct identification of *Phytophthora* isolates and a characterisation of new *Phytophthora* species.

In Northern Italy, in recent years a severe decline and dieback of *Acer pseudoplatanus* trees was observed in several planted forest stands. Affected trees showed thinning and dieback of the crown and bleeding cankers at the stem base and along the stem, with tongue-shaped, orange-brown lesions of the inner bark and brown to greenish staining of the cambium and adjacent xylem tissue. In June and December 2010 and in February 2011 *Phytophthora* isolates were consistently isolated from necrotic bark and stained xylem tissue and from rhizosphere soil of symptomatic *A. pseudoplatanus* trees in planted forest stands of the Boscoincittà park near Milan (45° 27' N 09° 11' E). Being homothallic with persistent semipapillate sporangia the isolates resembled species from the '*Phytophthora citricola* complex'. Detailed morphological and physiological studies and a phylogenetic analysis of ITS, *cox*1 and β-tubulin sequence data revealed that all isolates belong to an unknown unique taxon of the '*P. citricola* complex' which is here described as *Phytophthora acerina* sp. nov.

Materials and methods

Sampling and isolation procedures

Sampling was carried out from ca 30-35 years old planted *Acer pseudoplatanus* trees in four forest stands of the Boscoincittà park near Milan, Italy during both summer (June 2010) and winter (December 2010 and February 2011).

Affected trees showed symptoms characteristic for infections by *Phytophthora* spp. such as general decline and dieback of the crown, bleeding cankers at the collar and along the stems with greenish-brown or reddish streaks at the cambium and the outer xylem layers (Fig. 1). Severely damaged trees eventually died.

Figure 1 Symptoms caused by *Phytophthora acerina* on 30-35 years old *Acer pseudoplatanus* trees in forest stands of the Boscoincittà park near Milan; a-b. thinning and dieback of crowns; c. mortality and crown dieback; d-e. bleeding canker on lower stem with tarry spots on the surface of the bark (d) and a tongue-shaped orange necrosis of the inner bark (e); f. collar rot lesion with tarry spots; g. tongue-shape brownish necrosis of the inner bark; h-i. greenish to greyish discoloration of the xylem underneath a bark lesion.

Bark samples including the cambium and adjacent stained xylem tissue were taken from active lesions of five trees using a hatchet, a knife and a scalpel. The samples were stored in polyethylene envelopes for a maximum of 24 h. Small pieces (c.8x3x3 mm) were cut from different parts and depths of the phloem and xylem samples, blotted on filter paper, and plated onto selective PARPNH agar (V8-agar (V8A) amended with 10 µg/mL pimaricin, 200 µg/mL ampicillin, 10 µg/mL rifampicin, 25 µg/mL pentachloronitrobenzene (PCNB), 50 µg/mL nystatin, and 50 µg/mL hymexazol; (Tsao, 1983). The plates were incubated at 20 °C in the dark and examined daily under the stereomicroscope for *Phytophthora* –like hyphae which were transferred to V8A for initial confirmation as *Phytophthora* species.

Aliquots of 250 ml soil were taken from the same five *A. pseudoplatanus* trees at 5-20 cm depth in a distance of 1-1.5 m from the stem base at four points around each tree. In addition, each one sample of soil was taken from four sites along the canal that crosses the park at three different distances (1, 5, and 10 m) from the canal at each site. For the isolation of *Phytophthora* from the soil samples green apple fruits were used as baits. After surface disinfection with 95% ethyl alcohol four equidistant holes with 1 cm diameter and ca 2 cm depth were cut into each apple with a sterilised scalpel. Each hole was completely filled with subsamples of soil from one tree and subsequently wetted with sterile deionised water in order to induce the germination of potential *Phytophthora* resting structures. Each apple was finally covered with a transparent tape (Parafilm) and incubated at 18 °C in the dark for about 5-7 days after which the apples were examined for the development of a fruit rot. Pieces were cut from the interface between the necrotic area and the healthy tissue and plated onto PARPNH. The plates were incubated at 20 °C in the dark and outgrowing *Phytophthora* hyphae were subsequently transferred to V8A.

In December 2010 four apple baits were placed along the entire route of a canal that runs through the Boscoincittà park. The apples were first inserted into jute bags, then placed in small cages as protection against animals and finally immersed in the water.

A second survey was conducted in February 2011 in four little ponds located in the park.

After seven days the apples were collected and transported to the laboratory where isolations were carried out as described before.

***Phytophthora* isolates**

The isolates used in the phylogenetic, morphological and physiological studies are given in Table 1.

DNA isolation, amplification and sequencing

The 24 *Phytophthora* isolates obtained were subsequently transferred on Potato Dextrose Agar (39 g of DIFCO PDA and 5 g of agar in 1 liter of deionized water) and cultivated at a temperature of 22 °C for 1 week. The mycelium was collected in sterile 1.5 ml Eppendorf, gently scratching the surface of the colony with the aid of a sterile scalpel and placed in the freezer (-20 °C) for not less than 12 hours, up to complete freezing. DNA was then extracted following the protocol recommended by the extraction GenElute plant Genomic DNA Miniprep Kit (Sigma Aldrich) and finally stored at -20 °C.

Table 1 Isolates of *"Phytophthora citricola* complex" considered in the phylogenetic study

Culture no.[1]	Identification	Host	Location, year	Reference	ITS	*cox*1	β-tubulin
IMI 021173, CBS 221.88	*P. citricola* (type)	*Citrus sinensis*, fruit	Taiwan, 1927	Scott et al. (2009)	FJ237526	FJ237512	FJ665255
CBS 295.29	*P. citricola* (authentic type)	*Citrus* sp., leaf	Japan, 1929	Jung & Burgess (2009)	FJ560913	FJ665244	FJ665256
CH98U121C	*P. citricola*	-	Japan	Uddin et al. (unpubl.)	AB367378	-	-
Citri-P0713[3]		-	Japan, (Argentina)	Uddin et al. (unpubl.)	AB367492	-	-
CBS 181.25, IMI 077970	*P. pini*	*Pinus resinosa*, roots	Minnesota, USA, 1925	Hong (unpubl.)	FJ392322	-	-
22F3, P33		-	Ohio, USA	Hong (unpubl.)	FJ392321	-	-
CIT-US1[2]		*Fagus sylvatica*, canker	New York State, USA, 2003	Jung & Burgess (2009)	FJ665234	FJ665242	FJ665253
CIT-US10[2]		*F. sylvatica*, canker	New York State, USA, 2004	Jung & Burgess (2009)	FJ665235	FJ665243	FJ665254
91-309		*Thuja* sp., canker	Maumens, Switzerland	Lefort et al. (unpubl.)	EU000125	-	-
CBS 369.61	*P. citricola* II	*Rhododendron* sp.	Germany, 1958	Hong (unpubl.)	FJ392325	-	-
22F2, P52		-	New York State, USA, 1987	Hong (unpubl.)	FJ392324	-	-
15C9	*P. citricola* III	*Acer saccharum*	Wisconsin, USA, 1985	Hong (unpubl.)	FJ392327	-	-
1E1		Irrigation water	Oklahoma, USA	Hong (unpubl.)	FJ392326	-	-
P11835.2[4]		-	Spain	Moralejo (unpubl.)	DQ648146	-	-
OH6/5		*Quercus rubra*, soil	Ohio State, USA, 2004	Balci et al. (2007)	EF032477	-	-
IMI 031372[4]	*P. citricola* E	*Rubus idaeus*	Ireland	Cooke et al. (2000)	AF266788	-	-
112		-	Switzerland	Bragante et al. (unpubl.)	EU263906	-	-
87-302		*Rubus idaeus*	Switzerland,	Lefort et al. (unpubl.)	EU000100.1		

			Grandcours				
83-41[4]		-	Switzerland, Angers	Lefort et al. (unpubl.)	EU000081	-	-
WAC 13201, CBS 124094[2]	*P. multivora* (type)	*Eucalyptus marginata*	Yalgorup, WA, 2007	Scott et al. (2009)	FJ237521	FJ237508	FJ665260
WAC 13200[2]	*P. multivora*	*E. gomphocephala*	Yalgorup, WA, 2007	Scott et al. (2009)	FJ237522	FJ237509	FJ665261
WAC 13204[2]		*E. gomphocephala*	Yalgorup, WA, 2007	Scott et al. (2009)	FJ237518	FJ237507	FJ665259
WAC 13205, CBS124095[2]		*E. marginata*	Jarrahdale, WA, 1998	Scott et al. (2009)	FJ237517	FJ237506	-
VHS 16168[2]		*Banksia grandis*	Pemberton, WA	Scott et al. (2009)	FJ237513	-	FJ665257
IMI 329674[2]		Soil	Walpole, WA	Scott et al. (2009)	-	FJ237504	-
VHS 16439[2]		*B. littoralis*	Mandarah, WA	Scott et al. (2009)	FJ237516	FJ237505	FJ665258
P1817[4]		*Medicago sativa*	South Africa	Kroon et al. (2004)	AB367494	-	AY564055
P10458[4]		-	-	Blair et al. (2007)	-	-	EU079582
P7902[4]		*Pinus radiata*	USA, 1992	Blair et al. (2007)	-	-	EU080236
PLU-A5, CBS 124093[2]	*P. plurivora* (type)	*F. sylvatica,* root lesion	Irschenberg, Germany, 2004	Jung & Burgess (2009)	FJ665225	FJ665236	FJ665247
PLU-A9[2]	*P. plurivora*	*F. sylvatica*, canker	Irschenberg, Germany, 2004	Jung & Burgess (2009)	FJ665226	-	-
PLU7		*Q. robur,* soil	Pulling, Germany, 1994	Schubert et al. (1999)	AJ007370	-	-
PLU9, CBS 124087		*Q. robur,* soil	Pulling, Germany, 1994	Scott et al. (2009)	FJ237523	FJ237510	FJ665245
PLU30, CBS 124089[2]		*Q. robur,* soil	Cornuda, Italy, 1995	Jung & Burgess (2009)	FJ665227	FJ665237	FJ665248
PLU35, CBS 124090		*Q. petraea,* soil	Ljubliana, Slovenia, 1995	Scott et al. (2009)	FJ237524	FJ237511	FJ665246
PLU36[2]		*F. sylvatica*, canker	Munich, Germany, 1995	Jung & Burgess (2009)	FJ665228	-	-
PLU41, CBS 124091[2]		*Ac. Saccharum,* root	Mount Royal, Canada, 1996	Jung & Burgess (2009)	FJ665229	FJ665238	FJ665249

PLU77[2]		*Q. robur,* nursery soil	Nettetal, Germany, 1999	Jung & Burgess (2009)	FJ665230	FJ665239	FJ665250
PLU92[2]		*Quercus sp.,* soil	Turkey, 2000	Jung & Burgess (2009)	FJ665231	FJ665240	FJ665251
PLU255[2]		*F. sylvatica*, canker	Sumuva, Czech Republic, 2007	Jung & Burgess (2009)	FJ665232	-	-
PLU276, CBS 124092[2]		*Carpinus betulus,* soil	Snagov, Romania, 2008	Jung & Burgess (2009)	FJ665233	FJ665241	FJ665252
P10338[4]				Blair et al. (2007)	-	-	EU079526
MN21HH[4]		*Rhododendron* sp.	USA	Schwingle et al. (20079	DQ486661	-	-
InfGaul		*Gaultheria shalon*	Scotland	Schlenzig (2005)	AY879291	AY894685	-
IMI 342898		*Syringa vulgaris*	UK	Cooke et al. (2000) - ITS, Kroon et al. (2004) - *cox*1	AF266789	AY564187	-
P1822	*P. capensis*	Stream water	South Africa	Bezuidenhout et al. (2010)	GU191219	GU191277	GU191325
P1823		*Olea capensis*	South Africa	Bezuidenhout et al. (2010)	GU191231	GU191298	GU191327
P1819		*Curtisia dentate*	South Africa	Bezuidenhout et al. (2010)	GU191232	GU191275	GU191328
STE-U 6269	*P.* taxon emzansi	*Agathosma betulina*	South Africa	Bezuidenhout et al. (2010)	GU191228	GU191270	GU191317
STE-U 6272		*A. betulina*	South Africa	Bezuidenhout et al. (2010)	GU191220	GU191269	GU191316
B057[6]	*P. acerina*	*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951285	KC156134	KC201283
B080[6]		*Ac. Pseudoplatanus* soil	Milan, Italy, 2010	Ginetti et al. (2012)	JX951291	KC156140	KC201289
B035[6]		*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951282	KC156131	KC201281
B053[6]		*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951283	KC156132	KC201282
B054[6]		*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951292	KC156141	KC201290
B055[6]		*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951293	KC156142	KC201291
B056[6]		*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951284	KC156133	-
B058[6]		*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951286	KC156135	KC201284
B060[6]		*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951287	KC156136	KC201285

B062[6]	*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951294	KC156143	KC201292
B063[6]	*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951295	KC156144	KC201293
B064[6]	*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951288	KC156137	KC201286
B071[6]	*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951289	KC156138	KC201287
B077[6]	*Ac. pseudoplatanus*	Milan, Italy, 2010	Ginetti et al. (2012)	JX951290	KC156139	KC201288
B081[6]	*Ac. Pseudoplatanus* soil	Milan, Italy, 2010	Ginetti et al. (2012)	JX951296	KC156145	KC201294
CH95PHE28[4]	*Eustoma grandiflorum*	Japan	Villa et al. (2006)	AB217676	-	-
CH95PHE[4]	*E. grandiflorum*	Japan	Villa et al. (2006)			
TARI23044[4]	*Prunus persica*	Taiwan, 2009	Ann et al. (2009)		-	-
P10366[4]	-	-	Coffey et al.		-	-

[1] Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; IMI = CABI Bioscience, UK; WAC = Department of Agricolture and Food Western Australia Plant Pathogen Collection, Perth, Australia; VHS = Vegetation Health Service of the Department of Enviroment and Conservation, Perth, Australia; Other isolate names and numbers are as given on GenBank

[2] Isolates used in statistical analysis.

[3] Same code as isolate of Oudemans et al. (1994) which was collected in Argentina.

[4] Submitted to GenBank as P. citricola.[3] Same code as isolate of Oudemans et al. (1994) which was collected in Argentina.

[5] Submitted to GenBank as P. inflata.

[6] Isolates used in the morphological and growth-temperature studies

The ITS region (Internal Transcribed Spacer) of the ribosomal DNA was amplified using the primers ITS-6 (5' GAA GGT GAA GTC GTA ACA AGG 3 ') (Cooke *et al.* 2000) and ITS-4 (5 'TCC TCC GCT TAT TGA TAT GC 3') (White *et al.* 1990). The program used for amplification of the ITS region was: 95 °C for 3min, 35 cycles at 95 °C for 30 s, 55 °C 30s, 72 °C for 1 min. The final extension was carried out at 72 °C for 5 min.

The mitochondrial region of the *cox*1 gene was amplified with primers OomCoxILevup (MGA TGG CTT TTT 5'TCA WCW TCA AC 3') and Fm85mod (5 'RRH WAC KTG DAT RAT ACT ACC AAA 3'), as reported by Martin & Tooley (2003). The program used for amplification of the *cox*1 region was: 95 ° C for 2 min followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min. The final extension was carried out at a temperature of 72 °C for 10 min.

The β-tubulin region was amplified using the primers Btub F1 (5' GCC AAG TTC TGG GAG GTC ATC) (Blair *et al.* 2008) and βtub R1 (5' CCT GGT ACT GCT GGT ACT CAG) (Kroon *et al.* 2004). PCR reaction mixture and conditions were the same as for the ITS region.

Phylogenetic analysis

Phytophthora isolates obtained in this work were compared with the closely related species (ITS clade 2; Jung & Burgess, 2009) and other *Phtyophthora* species representative of other ITS clades as outgroups. Less sequences were available for *cox* 1 region and for this reason the dataset for this region is smaller.

The obtained sequences were inspected, analyzed and corrected with the program Sequencer 4.9 and then aligned with Clustal X2. Manual adjustments were made visually by inserting gaps where necessary in BioEdit Sequence Alignment Editor. The aligned sequences were subsequently processed with the program Topali 2.5 for the construction of phylogenetic Neighbour Joining trees and with the program GeneDoc to highlight the polymorphic nucleotides.

Colony morphology, growth rates and cardinal temperatures

Morphology of hyphae and colony growth patterns were described from 7-d-old cultures grown at 20 °C in the dark on V8A (16 g agar, 3 g $CaCO_3$, 100 mL Campbell's V8 juice, 900 mL distilled water), malt extract agar (MEA), and half strength PDA (19.5g PDA, 7.5g agar, 1l distilled water) (all from DIFCO, Sparks, MD, USA). Colony morphologies were described according to Erwin & Ribeiro (1996) and Jung & Burgess (2009).

For temperature-growth relationships, three replicate V8A plates per isolate were incubated at 10, 15, 20, 25, 30, 32 and 35 °C. Radial growth rate was recorded after 5–7 d along two lines intersecting the centre of the inoculum at right angles (Hall, 1993).

Morphology of sporangia and gametangia

Sporangia and gametangia were measured on V8A as described by Jung *et al.* (1999). Sporangia were produced by immersing 15×15 mm square agar discs taken from growing margins of 3–5-d-old colonies in 90 mm Petri dishes filled with deionised water, and young leaves of *Quercus ilex* floating at the surface. After 6h the water was replaced by non-sterile pond water. The plates were incubated at 20-25 °C. After 24-36h dimensions and characteristic features of 50 mature sporangia per isolate chosen at random were determined at × 400 magnification (ZEISS, West Germany). For each isolate dimensions and characteristic features of 50 mature oogonia, oospores and antheridia chosen at random were measured at ×400 magnification at the surface of 20 × 15 mm square agar

discs cut from the centre of 14–21-d-old V8A cultures grown in the dark at 20 °C. The oospore wall index was calculated as the ratio between the volume of the oospore wall and the volume of the entire oospore (Dick, 1990).

Under-bark inoculation test

One-year-old twigs (diameter approx. 5-10 mm) were collected in the field from single mature trees of *A. pseudoplatanus* and *Fagus sylvatica* in March and May, respectively, shortly after bud burst; leaves were removed and the twigs were cut into sections of ca 12 cm length.

In the centre of each cutting a ca 0.5 cm diameter piece of bark was removed aseptically with a razor blade. An even-sized V8A disc cut from the margins of freshly (4-5 d) growing cultures of a *P. acerina* isolate was placed on the wound, covered with the removed bark piece and autoclaved wet cotton and sealed with a tape (Parafilm) and aluminium foil. Controls received only sterile V8A discs. Five isolates were tested. Ten twigs were inoculated per isolate or control and placed in autoclaved glass Petridishes containing two layers of moist filter paper. The plates were sealed with tape (Parafilm) and incubated at 20 °C in the dark. After 21 days lesion lengths were measured after removal of the outer bark. Random re-isolations were made using PARPNH to fulfill Koch' s postulates.

Statistical analysis

Statistical analyses were carried out using STATA11 (Stata Statistical software, College Station, Texas, USA) to determine if morphometric and growth rate differences between *P. acerina* and other taxa of the '*P. citricola* complex' were statistically significant. Due to the number of 50 measurements for every character in each isolate data showed a normal distribution allowing the use of parametric tests. Data were tested for homogeneity of variances between independent data. In case of homogeneity a two-sided t-test was applied. If the hypothesis of homoskedasticity was violated, a modified t-test for heterogeneous variances was used.

Results

Isolation results

In December 2010, *P. acerina* was isolated from necrotic bark and underlying stained xylem of all five mature *A. pseudoplatanus* trees sampled in planted forest stands of the Boscoincittà park. *Phytophthora acerina* was also isolated from rhizosphere soil of one of these five trees. No other *Phytophthora* species was recovered from bleeding cankers and rhizosphere soil of declining maple trees.

In contrast, *P. acerina* could not be isolated from water bodies or from soil sampled near the canal. However, five other *Phytophthora* spp. were found, ie. *P. lacustris*, *P.* taxon 'PgChlamydo', *P. gonapodyides*, *P. inundata* and *P.* taxon 'Walnut'.

Phylogenetic analysis

Identity, host, location, isolation information and GenBank accession numbers for "*Phytophthora* citricola complex isolates" used in this study are shown in Table 1.

For the sequencing of the ITS region, the *cox*1 gene and the β-tubulin region 15 *Phytophthora* isolates were selected, by considering 1) all the different profiles on the gel obtained by digestion

with restriction enzymes; 2) the morphology of the colonies on V8-agar cultures and 3) the source of the isolates.

Phylogenetic analyses results are shown in Neighbor-Joining tree calculated with 500 bootstrap runs (Fig 2-3). Excluding outgroups, the aligned dataset for ITS (66 sequences) and *cox*1 (32 sequences) consisted of 963 and 687 characters, respectively. The number of phylogenetic informative sites of the nucleotide alignment data was of 298 (30,94 %) characters for the ITS and 71 (10,33 %) nucleotides for the *cox*1.

Polymorphic nucleotides obtained aligning the analyzed *Phytophthora* sequences are shown in Table2.

Taxonomy

Phytophthora acerina B. Ginetti, S. Moricca, J. Squires, D. Cooke, A. Ragazzi & T. Jung, sp. nov. — CBS accession nr. CBS 133931. Figs. 4–6

Sporangia abundantia in cultura liquida, persistentia, terminalia, interdum lateralia aut intercalaria, semi-papillata vel rare bipapillata, ovoidea, limoniformia vel obpyriformia, rare ellipsoidea, obovoidea vel distorta, saepe cum obturamento conspicuo basale, apex interdum arcuatus, 50.2 ± 11.2 × 33.2 ± 7.3 µm, ratio longitudo ad altitudinem 1.5 ± 0.2. Sporangiophora simplicia vel ramosa sympodiis laxis irregularibus; interdum inserta lateraliter ad sporangia, inflationes ad nodos rarae. Systema sexus homothallica, solum partim functionalis; oogonia in medio 50 % abortiva, terminalia, lateralia vel sessilia, globosa, rare subglobosa vel excentrica, 31.3 ± 3.8 µm. Oosporae apleroticae vel pleroticae, 27.8 ± 3.3 µm, paries 2.0 ± 0.3 µm, maturitate frequenter pigmentati lutei ad luteifusci. Antheridia paragynosa, 12.5 ± 2.3 × 9.8 ± 1.6 µm. Aggregationes hypharum frequenter in agaro 'V8A' et in cultura liquida, diameter 15-150 µm. Inflationes hypharum subglobosae. Chlamydosporae non observatae. Temperaturae crescentiae in agaro 'V8A', optima c. 25 °C et maxima c. 32 °C. Coloniae in agaro 'V8A' chrysanthemum cum mycelio aerio restricto. Regiones 'rDNA ITS', '*cox*I' et 'β-tubulin' cum unica sequentia (GenBank JX951285, FJ665236, FJ665247).

Etymology: Name refers to *Acer pseudoplatanus* from which all known isolates had been isolated.

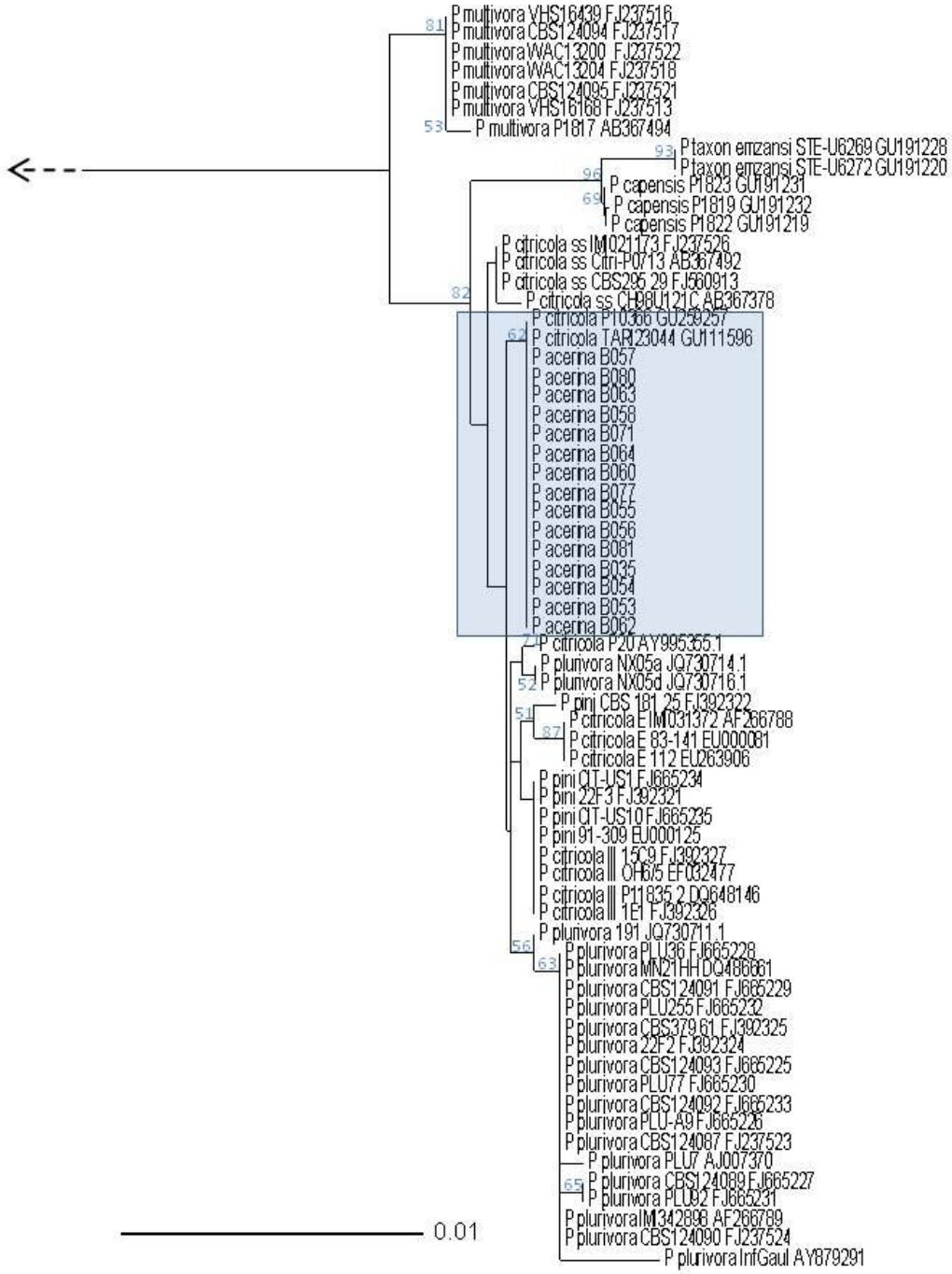


Figure 2 Neighbour Joining tree using rDNA ITS sequences showing phylogenetic relationships within the *P. 'citricola'* complex. Numbers in blue represent bootstrap support for the nodes. *P. cryptogea* was used as outgroup taxon (not shown).

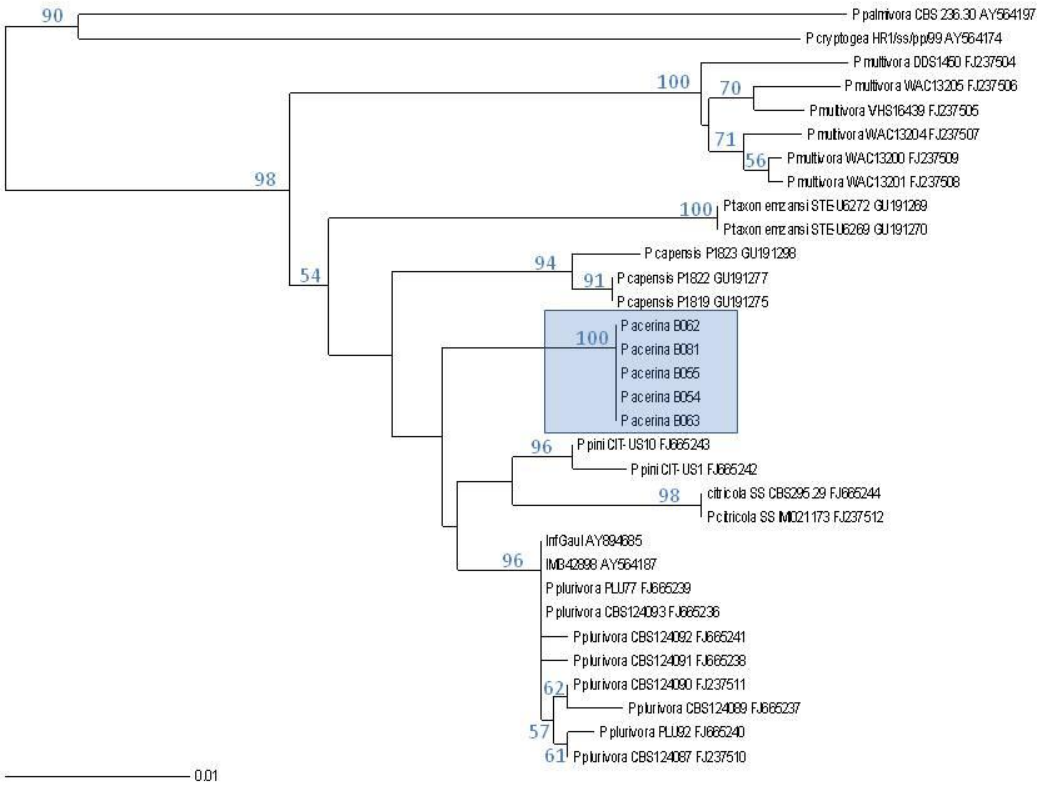


Figure 3 Neighbour Joining tree based on analysis of mitochondrial gene *cox*I sequence, showing phylogenetic relationships within the *P. 'citricola'* complex. Numbers in blue represent bootstrap support for the nodes.

Table 2 Polymorphic nucleotides from aligned sequence data of ITS, cox1 and b-tubulin gene regions showing the variation between isolates of *Phytophthora acerina*, *P. citricola* s.str., *P. plurivora* and *P. citricola* s.l.(including *P. citricola* I, III and E). Blue shading denotes polymorphisms found in *P. citricola* s. str., green shading is for those found in *P. plurivora*, orange shading is for polymorphisms found in *P. citricola* I, III and E and yellow shading underlines polymorphisms only found in *P. acerina*. Grey shading denotes no data available.

ITS

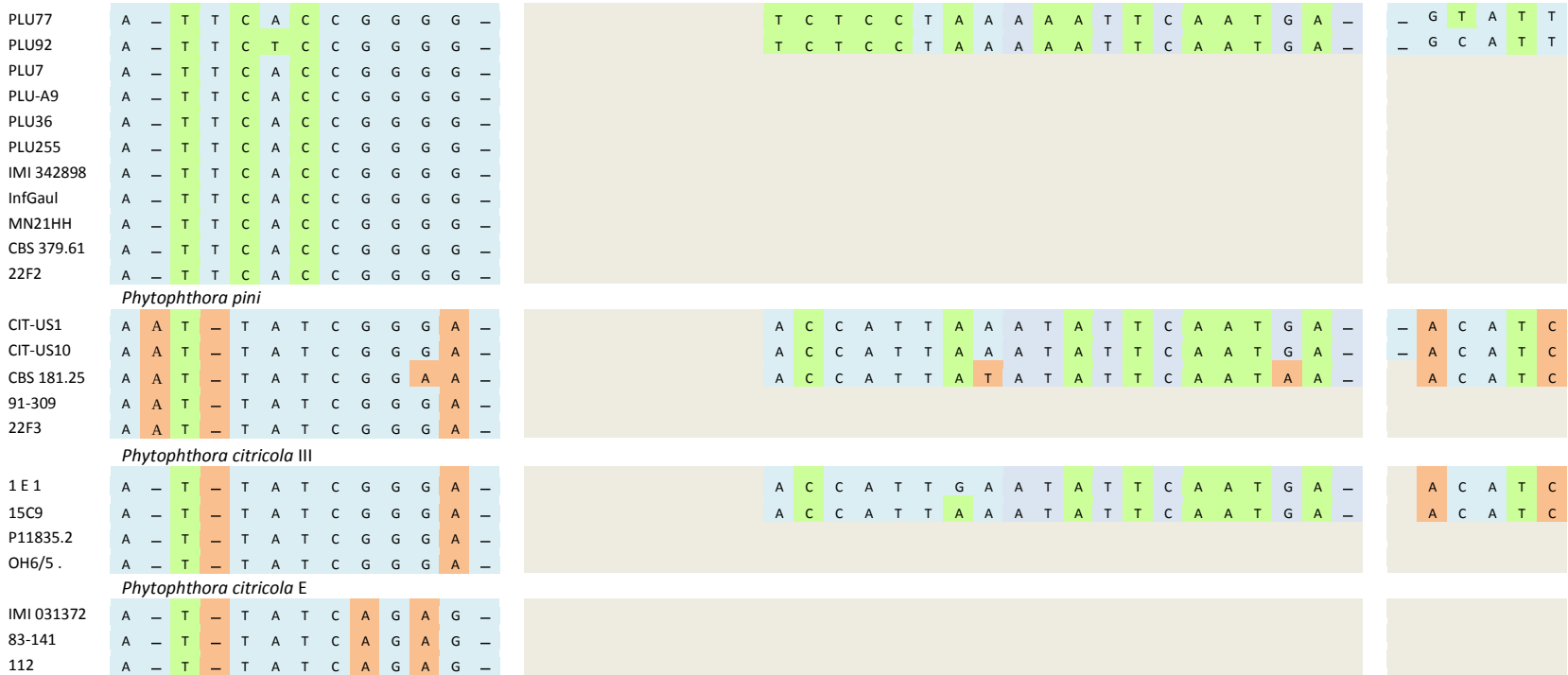
Culture no.	9	15	20	66	154	351	397	541	542	564	650	698	752
Phytophthora acerina[1]													
B057	A	–	T	–	T	A	T	T	G	R	G	G	–
B054	A	–	T	–	T	A	T	T	G	R	G	G	–
B055	A	–	T	–	T	A	T	T	G	R	G	G	–
B062	A	–	T	–	T	A	T	T	G	R	G	G	–
B063	A	–	T	–	T	A	T	T	G	R	G	G	–
B081	A	–	T	–	T	A	T	T	G	R	G	G	–
Phytophthora acerina[2]													
TARI 23045	A	–	T	–	T	A	T	T	G	G	G	G	–
WPC10366A631	A	–	T	–	T	A	T	T	G	G	G	G	–
CH95PHE28	A	–	T	–	T	A	T	T	G	A	G	G	C
CH95PHE31	A	–	T	–	T	A	T	T	G	A	G	G	C
P20	T	A	T	–	T	A	T	C	G	G	G	G	–
Phytophthora acerina[3]													
191	A	–	T	–	T	A	C	C	G	G	G	G	–
NX05a	A	–	T	–	T	A	T	C	G	G	G	G	–
NX05d	A	–	T	–	T	A	T	C	G	G	G	G	–
Phytophthora citricola s.str.													
CBS 221.88	A	–	C	T	T	A	T	C	G	G	G	G	–
CBS 295.29	A	–	C	T	T	A	T	C	G	G	G	G	–
Citri-P0713	A	–	C	T	T	A	T	C	G	G	G	G	–
CH98U121C	A	–	C	T	T	A	T	C	G	G	G	G	–
Phytophthora plurivora													
CBS 124087	A	–	T	T	C	A	C	C	G	G	G	G	–
CBS 124090	A	–	T	T	C	A	C	C	G	G	G	G	–
CBS 124089	A	–	T	T	C	T	C	C	G	G	G	G	–
CBS 124091	A	–	T	T	C	A	C	C	G	G	G	G	–
CBS 124092	A	–	T	T	C	A	C	C	G	G	G	G	–
CBS 124093	A	–	T	T	C	A	C	C	G	G	G	G	–

***cox*1**

Culture no.	99	153	204	210	372	393	399	420	549	561	564	603	621	660	702	711	753	774	864	912	969	1036	1092	1119	1128	1200	1248	1279
Phytophthora acerina[1]																												
B057	G	A	C	T	A	G	T	T	A	C	C	A	C	C	A	C												
B054	G	A	C	T	A	G	T	T	A	C	C	A	C	C	A	C	G	A	A	C	T	T	G	A	T	A	A	T
B055	G	A	C	T	A	G	T	T	A	C	C	A	C	C	A	C	G	A	A	C	T	T	G	A	T	A	A	T
B062	G	A	C	T	A	G	T	T	A	C	C	A	C	C	A	C	G	A	A	C	T	T	G	A	T	A	A	T
B063	G	A	C	T	A	G	T	T	A	C	C	A	C	C	A	C	G	A	A	C	T	T	G	A	T	A	A	T
B081	G	A	C	T	A	G	T	T	A	C	C	A	C	C	A	C	G	A	A	C	T	T	G	A	T	A	A	T
Phytophthora acerina[2]																												
TARI 23045																												
WPC10366A631																												
CH95PHE28																												
CH95PHE31																												
P20																												
Phytophthora acerina[3]																												
191																												
NX05a																												
NX05d																												
Phytophthora citricola s.str.																												
CBS 221.88	T	T	A	C	A	T	A	A	A	T	C	A	T	T	G	A	A	T	T	T	A	C	G	T	C	G	T	–
CBS 295.29									A	T	C	A	T	T	G	A	A	T	T	T	A	C	G	T	C	G	T	–
Citri-P0713																												
CH98U121C																												
Phytophthora plurivora																												
CBS 124087	T	A	A	T	G	T	T	T	T	C	T	C	C	T	A	A	A	A	A	T	T	C	A	A	T	G	A	–
CBS 124090	T	A	A	T	G	T	A	T	T	C	T	C	C	T	A	A	A	A	A	T	T	C	A	A	T	G	A	–
CBS 124089									T	C	T	C	C	T	A	A	A	A	A	T	T	C	A	A	T	G	A	–
CBS 124091									T	C	T	C	C	T	A	A	A	A	A	T	T	C	A	A	T	G	A	–
CBS 124092									T	C	T	C	C	T	A	A	A	A	A	T	T	C	A	A	T	G	A	–
CBS 124093									T	C	T	C	C	T	A	A	A	A	A	T	T	C	A	A	T	G	A	–

β-tubulin

Culture no.	60	308	382	414	829	1076
Phytophthora acerina[1]						
B057	A	G	C	A	T	T
B054	A	G	C	A	T	T
B055	A	G	C	A	T	T
B062	A	G	C	A	T	T
B063	A	G	C	A	T	T
B081	A	G	C	A	T	T
Phytophthora acerina[2]						
TARI 23045						
WPC10366A631						
CH95PHE28				T	T	
CH95PHE31				A	T	
P20						
Phytophthora acerina[3]						
191						
NX05a						
NX05d						
Phytophthora citricola s.str.						
CBS 221.88	–	G	C	A	C	T
CBS 295.29	–	G	C	A	C	T
Citri-P0713						
CH98U121C						
Phytophthora plurivora						
CBS 124087	–	G	C	A	T	T
CBS 124090	–	G	T	A	T	T
CBS 124089	–	G	C	A	T	T
CBS 124091	–	G	T	A	T	T
CBS 124092	–	G	T	A	T	T
CBS 124093	–	G	T	A	T	T



[1] Isolate used in this study

[2] Submitted to GenBank as *P.citricola*

[3] Submitted to GenBank as *P. plurivora*

Sporangia of *P. acerina* were rarely observed on solid agar but were produced abundantly in non-sterile pond water. Sporangia were typically borne terminally on unbranched sporangiophores. Small subglobose hyphal swellings were sometimes formed on the sporangiophore. Sporangia were persistent, semipapillate, less frequently bi- or tripapillate or bilobed (over all isolates < 1%) and sometimes formed a conspicuous basal plug that protruded into the sporangium (Fig. 4r-s). In all isolates sporangial shapes showed a wide variation including ovoid (over all isolates 54.9 %; Fig. 4a-b, d), limoniform (14.1 %; Fig. 4l), obpyriform (8.7%; Fig. 4i-k), ellipsoid (4.1 %; Fig. 4m), elongated-ovoid (3.9 %; Fig. 4g-h, r-s), obovoid (1.8 %), broad-ovoid (1.5; Fig. 4c), mouse-shaped (1.7%; Fig. 4f, n) and other distorted shapes (8.7 %; Fig. 4o-q). Sporangia with special features such as curved apices (over all isolates 13.6 %; Fig. 4f, n-o, q), lateral attachment of the sporangiophore (10.3 %; Fig. 4e), intercalary insertion (7.7 %; Fig. 4o), hyphal swellings, sometimes catenulate, on the sporangiophore (2.4 %; Fig. 4t), a short hyphal projection (1.2 %; Fig. 4f), vacuoles (Fig. 4g, j, m-n, p-q) and a widening of the sporangiophore towards the base of the sporangium (Fig. 4l) were observed in all isolates. Sporangia in older water cultures usually germinated directly. Zoospores were discharged through exit pores of 8.2 ± 1.8 μm (Fig. 4r-s). Sporangial dimensions of 15 isolates of *P. acerina* averaged 52.0 ± 13 x 32.8 ± 7.7 μm (overall range 20.3 - 105.7 x 11.1 - 51.3 μm) with a the length/breadth (l/b) ratio of 1.6 ± 0.3 μm (overall range 1.2 – 2.6; range of isolate means 1.4 – 2.0).

Phytophthora acerina is homothallic with paragynous antheridia. Oogonia were readily produced in single culture on V8A. Oogonia of *P. acerina* were borne terminally or were laterally sessile (Fig. 5d, g, l), had smooth walls and were usually globose to slightly subglobose (Fig. 5a-l). Elongated oogonia (over all isolates 8.6 %; Fig. 5h), slightly elongated oogonia (2.7 %; Fig. 5i) and excentric oogonia (3.9 %; Fig. 5b, d, k, n) were present in all isolates. In some isolates of *P. acerina* oogonia walls turned golden-yellow to golden-brown during ageing. The oogonia of 14 isolates of *P. acerina* showed a mean diameter of 32.0 ± 4.4 μm (overall range 19.2 – 45.5 μm; range of isolate means 28.1 – 36.6 μm). Oospores of *P. acerina* were usually globose, but could be subglobose in elongated oogonia. Oospore diameters averaged 28.4 ± 3.9 μm (overall range 15.9 – 39.3 μm; range of isolate means 24.6 – 32.4 μm). The mean proportion of aplerotic oospores (Fig. 5b-f, h) over all *P. acerina* isolates was 69.6% (40 – 96 %). In most isolates of *P. acerina* an unusually high proportion of oogonia aborted before (Fig. 5i-j) or immediately after the formation of the oosporewall (Fig. 5l-n). The mean abortion rate of 15 isolates was 38.5 % with isolate means ranging from 10 - 99 %. Oospores had a medium wall thickness with a mean wall diameter of 2.0 ± 0.38 μm (overall range 0.77 – 3.36 ; range of isolate means 1.8 – 2.4) and a mean oosporewall index of 0.37 ± 0.05 (overall range 0.16 – 0.63; range of isolate means 0.35 – 0.42). Antheridia of *P. acerina* were exclusively paragynous (Fig. 5a-c, e-g, i), globose to obovoid, club-shaped or irregular, sometimes with a finger-like projection (2% over all the isolates), and usually attached close to the oogonial stalk. They measured 12.8 ± 3.4 x 9.5 ± 1.6 μm.

In ageing cultures (3-6 weeks old) *P. acerina* isolates produced globose to subglobose, appressoria-like and coralloid hyphal swellings (Fig. 6a-c) and abundant hyphal aggregations (Fig. 6d-p). The hyphal aggregations started with multiple lateral branching of short sections along the main hyphae (Fig. 6f, h-i, l) or by multiple branching at the end of main hyphae (Fig. 6j-k, o) or of short lateral hyphae (Fig. 6e, g-h). Subsequent twisting and dense intermingling (Fig. 6d) of these irregular,

coralloid lateral hyphae and swellings resulted in dense stromata-like structures with diameters of 10-150 µm.

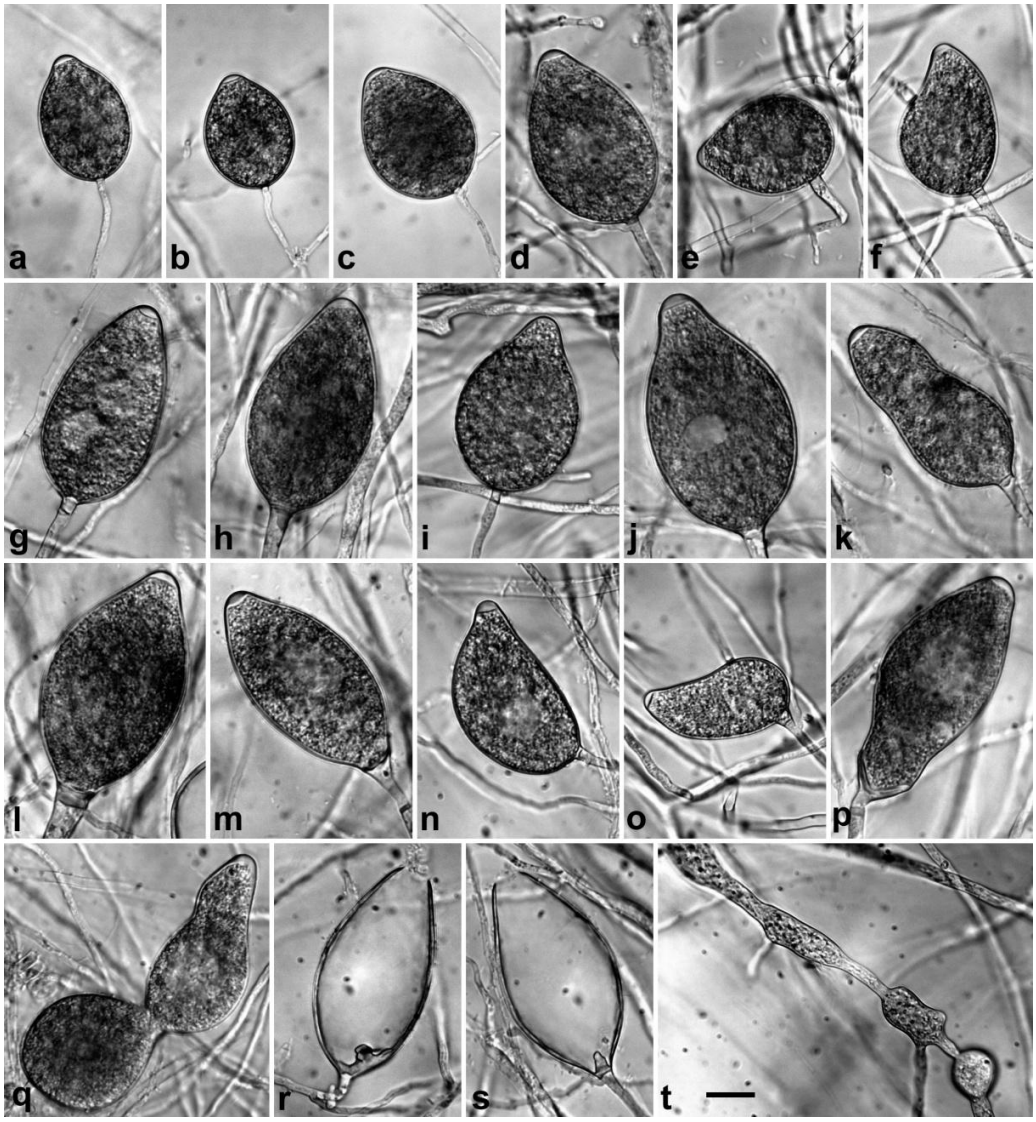


Figure 4 Semipapillate sporangia of *Phytophthora acerina* on V8 agar after 24–36 h flooding with non-sterile pond water; a-d. ovoid; e. ovoid, laterally inserted; f. mouse-shaped, intercalary inserted; g.elongated-ovoid with vacuole; h. elongated-ovoid; i-k. obpyriform, j. with vacuole; l. limoniform, sporangiophore widening towards base of sporangium; m. ellipsoid with vacuole; n. mouse-shaped with vacuole; o. distorted, intercalary inserted; p-q. distorted with vacuoles; r-s. elongated-ovoid, empty sporangia after release of zoospores, with conspicuous basal plugs; t. irregular catenulate hyphal swellings. — Scale bar = 10 µm, applies to a–t.

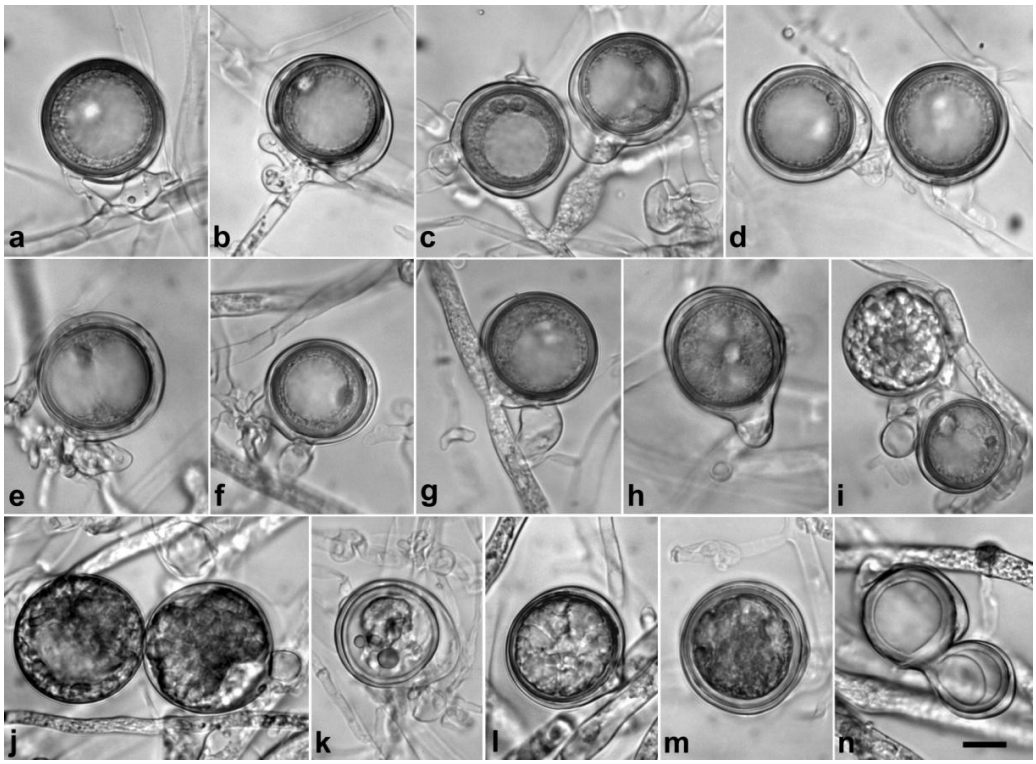


Figure 5 Oogonia of *Phytophthora acerina* formed in solid V8 agar. a-h. Mature viable oogonia with oospores containing ooplasts, and paragynous antheridia; a. plerotic oospore and paragynous antheridium with finger-like projection; b. elongated oogonium with aplerotic oospore and paragynous antheridium with finger-like projection; c. aplerotic oospores, the left one with two pellucid bodies (nuclei), the right one with two ooplasts; d. elongated oogonium with aplerotic oospore (left) and sessile oogonium with plerotic oospore (right); e. aplerotic oospore with two ooplasts; f. aplerotic oospore; g. sessile oogonium with almost plerotic oospore; h. elongated oogonium with tapering base and oospore with several small globules instead of one large ooplast; i. oogonium that aborted before oospore formation (top) and viable oogonium (bottom); j. two oogonia that aborted before oospore formation; k. excentric oogonium with aplerotic aborted oospore and paragynous antheridium; l. sessile oogonium with plerotic aborted oospore; m. oogonium with plerotic, thick-walled aborted oospore; n. 'double oogonium' with two extremely thick-walled aborted oospores. — Scale bar = 10 μm, applies to a-n.

Colony morphology, growth rates and cardinal temperature

Isolates of *P. acerina* formed appressed to submerged colonies with a uniform to faintly stellate growth pattern on MEA and rosaceous to petaloid colonies with moderate aerial mycelium on half strength PDA (Fig. 7). On V8A colony morphology was more variable ranging from chrysanthemum to faintly petaloid and stellate patterns, and from limited to wooly aerial mycelium. Diameters of primary hyphae of *P. acerina* averaged 5.1 ± 1.2 μm and varied from 2.6 to 8.7 μm. All five *P. acerina* isolates tested had identical cardinal temperatures and similar radial growth rates at all temperatures (Fig. 8). The maximum growth temperature for *P. acerina* was 32 °C. All isolates were not able to grow at 35 °C and did not start re-growth when plates previously incubated for 5 d at 35 °C where transferred to 18-20 °C.

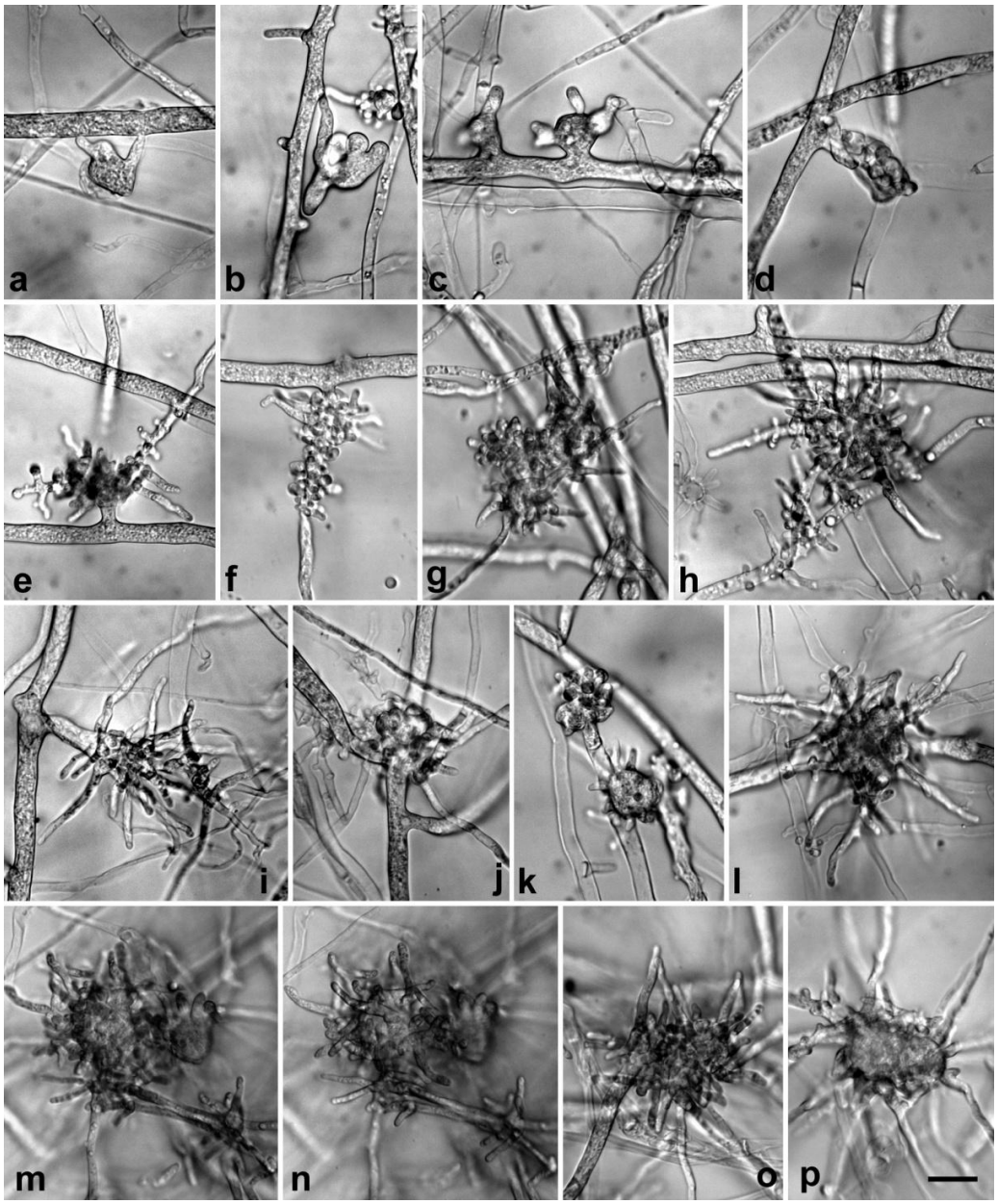


Figure 6 Vegetative structures formed by *Phytophthora acerina* in solid V8-agar; a-b. appressoria-like lateral hyphal swellings; c. lateral globose swelling with radiating short hyphae; d. two lateral hyphae twisting around each other; e. multiple branching at the end of a short lateral hypha; f. multiple branching along a lateral hypha; g-h. hyphal aggregations resulting from multiple successive branching and thickening of lateral hyphae; i. multiple branching along and at the end of a lateral hypha; j-k. multiple branching at the end of main hyphae; n-p. dense hyphal aggregations resulting from multiple successive branching, twisting, intermingling and thickening of lateral hyphae. — Scale bar = 10 µm, applies to a-p.

Optimum temperature for growth was 25 °C with radial growth rates ranging from 7.5 – 7.9 mm d^{-1}. At 20 °C *P. acerina* showed growth rates of 6.5 ± 0.2 mm d^{-1} on V8A and 4.3 ± 0.2 mm d^{-1} on PDA.

Under-bark inoculation test

All five isolates of *P. acerina* were pathogenic to one-year-old twigs from mature trees of both *A. pseudoplatanus* and *F. sylvatica* (Fig. 9).

After 22 d lesions lengths on *A. pseudoplatanus* measured on average 9.9 ± 0.3 cm (overall range 6.9 – 11.3 cm) while lesions lengths on *F. sylvatica* averaged 4.6 ± 0.2 cm (overall range 3.1 – 7.8 cm). This difference in aggressiveness to both tree species was statistically significant ($P < 0.001$).

Notes

Phytophthora acerina differs from all other known taxa of the '*P. citricola* complex' by the abundant production of dense stromata-like hyphal aggregations. In addition, no other taxon except of *P.* taxon 'emzansi' showed such a high oogonial abortion rate (Table 3). Further differences between *P. acerina* and other taxa of the '*P. citricola* complex' are listed below.

In *P. acerina* the proportion of sporangia with curved apices (15.6 %) or lateral attachment of the sporangiophore (10.3 %) was higher than in *P. citricola* s. str. (12 % and 6 %, respectively), *P. pini* (11 % and 6 %, respectively) and *P. multivora* (1.7 % and 9.3 %, respectively) but lower than in *P. plurivora* (17.6 % and 16.4 %, respectively) (Jung & Burgess 2009). The mean dimensions and the l/b ratio of *P. acerina* sporangia were significantly higher than those of *P. plurivora* ($p < 0.001$) even though the ranges overlapped widely. In contrast, dimensions of *P. acerina* sporangia were significantly smaller than in *P. pini* ($p < 0.05$) but l/b ratios of both species did not differ significantly. The mean width dimensions and the l/b ratio of *P. acerina* were significantly higher than those of *P. citricola* s.str. ($p < 0.001$) even if the ranges overlapped abundantly.

Oogonia of *P. acerina* were sometimes laterally attached and could even be sessile, a feature not observed in other taxa of the '*P. citricola* complex'. Oogonia and oospores of *P. acerina* were on average significantly larger than those of *P. plurivora* and *P. multivora* ($p < 0.001$), *P. citricola* s.str. ($p < 0.001$ and $p<0.05$, respectively) and *P. pini* ($p < 0.01$ and $p < 0.05$, respectively). With 69.6% the mean proportion of aplerotic oospores in *P. acerina* was higher than in all the other taxa (43 – 47 %). The oosporewall index of *P. acerina* was significantly lower than in *P. multivora* (0.52; $p < 0,001$) but significantly higher than in all other taxa ($p < 0,001$; Table 2).

Unlike all the other taxa from the '*P. citricola* complex' *P. acerina* isolates produced variable colonies on V8A with some isolates forming chrysanthemum patterns and limited aerial mycelium as in all other taxa while other isolates of *P. acerina* formed faintly petaloid to stellate patterns with wooly mycelium (Fig. 5). On PDA the rosaceous to petaloid colonies clearly discriminated *P. acerina* from *P. plurivora* (chrysanthemum), *P. citricola* (striate) and *P. multivora* (uniform). Similar to *P. plurivora*, *P. citricola* s.str., *P. citricola* E and *P. multivora, P. acerina* had a growth optimum on V8A at 25°C whereas the optimum temperature was 22.5 °C for *P. capensis* and *P.* t. 'emzansi' and 30 °C for *P. pini* (Jung & Burgess, 2009; Bezuidenhout *et al.* 2010). With 32.5 °C the maximum temperature for growth in *P. acerina* was markedly higher than in *P. capensis* and *P.* t. 'emzansi' (27.5 °C; Bezuidenhout *et al.* 2010). At 30 °C *P. acerina* had a markedly slower growth rate (0.9 ± 0.04 mm d^{-1}) than *P. pini* (9.2 mm d^{-1}; Jung & Burgess, 2009).

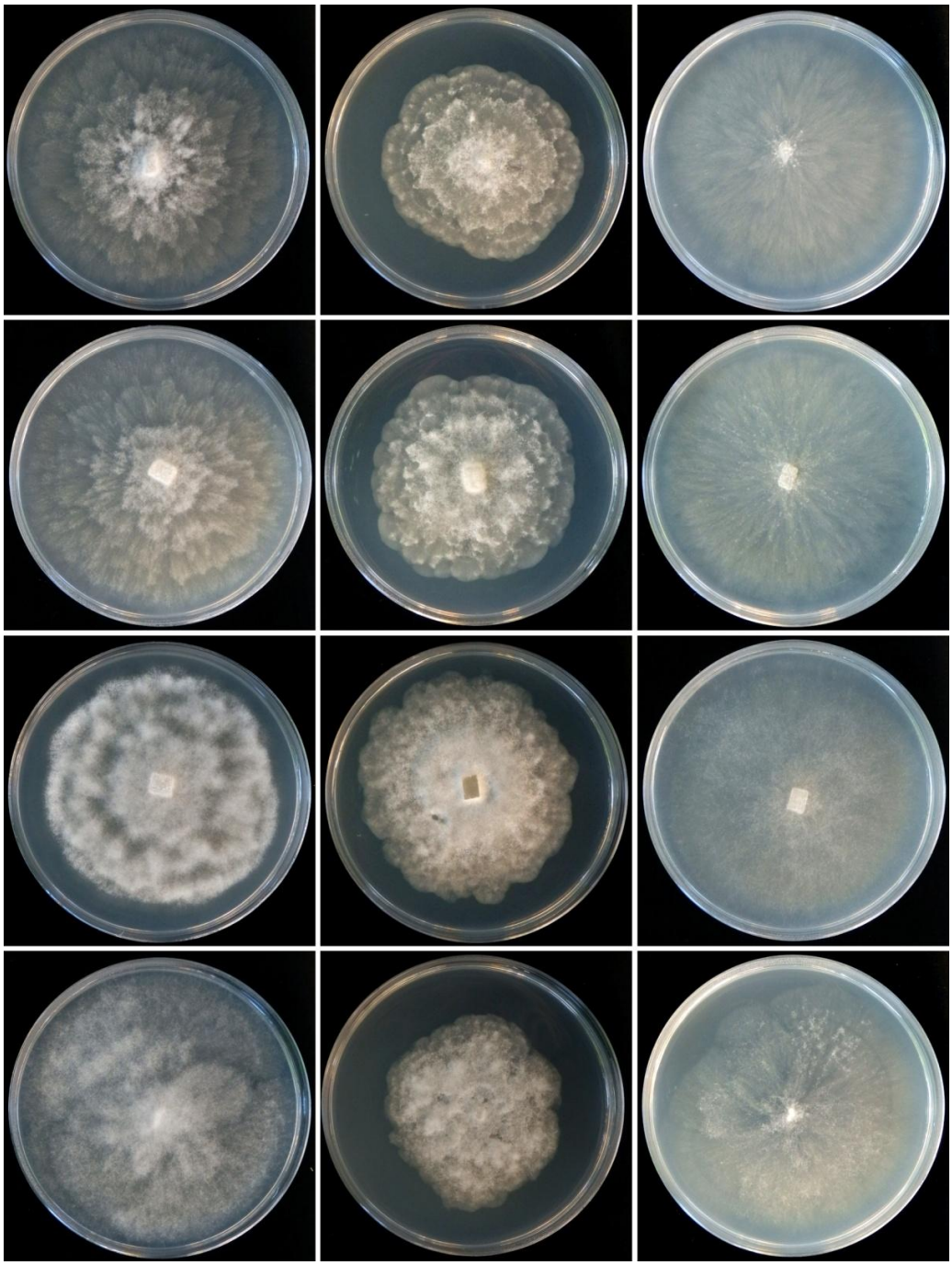


Figure 7 Colony morphology of *Phytophthora acerina* isolates B077, B063, B080 and B035 (from top to bottom) after 7 d growth at 20 °C on V8-agar, potato-dextrose agar and malt extract agar (from left to right).

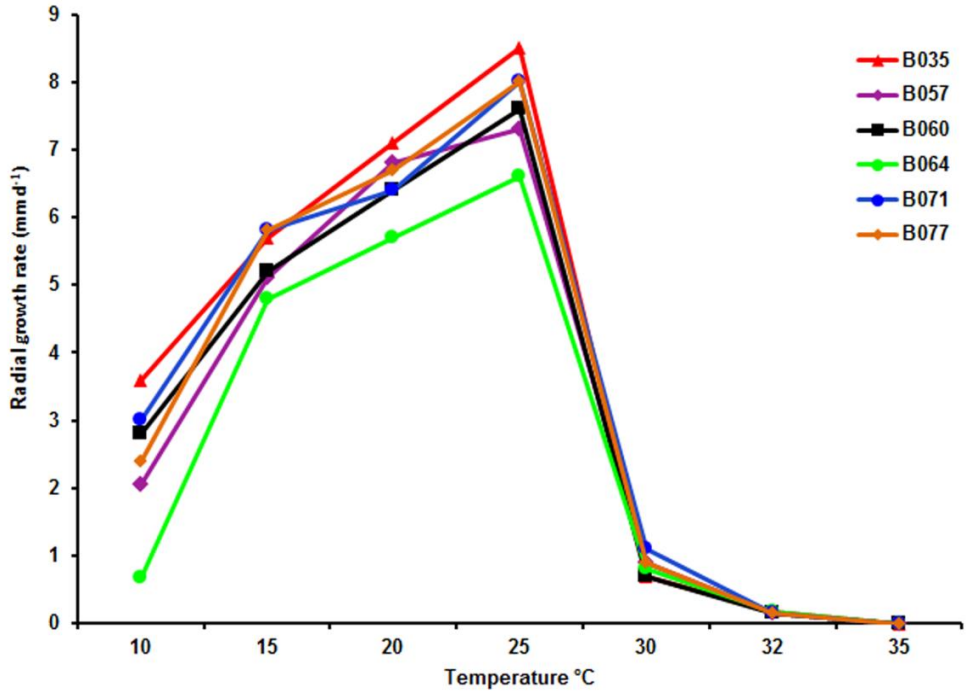


Figure 8 Radial growth rates of *Phytophthora acerina* (means and standard errors calculated from five isolates) on V8-agar at different temperatures.

Figure 9 Lesions caused by *Phytophthora acerina* in the underbark inoculation trial after 21 days: greyish to greenish lesions on twigs of *Acer pseudoplatanus* (left side) and orange-brown lesions on twigs of *Fagus sylvatica* (right side).

Table 3 Morphological characters and dimensions (µm) and temperature-growth relations of *Phytophthora acerina*, *P. capensis*, *P. citricola* s.str., *P. multivora*, *P. pini*, *P. plurivora* and *P.* taxon 'emzansi'.

	P. acerina	*P. capensis*	*P. citricola* s.str.	P. multivora	*P. pini* [1]	*P. pini*	*P. plurivora*	*P.* t. 'emzansi'
No. of isolates investigated	15 [2]	3 [3]	2 [4]	6 [5]	2 [4]	4 [6]	7 [4]	2 [3]
Sporangia								
lxb mean	52 ± 13 x 32.8 ± 7.7	39.1 ± 6 x 24 ± 3.3	52 ± 7.9 x 29.9 ± 5.1	51.0 ± 10.4 x 30.0 ± 5.1	53.7 ± 6.5 x 33.8 ± 3.9	47.4 x 31.5	47.4 ± 7.7 x 33.5 ± 5.9	46.9 ± 8.6 x 27.4 ± 5.7
Range of isolate means	42.5 - 61.6 x 26.8 - 38.3		50.9–52 x 29.9	44.2–62.1 x 26.2–34.2	51.2–56.2 x 33.5–34.1		39.6–52.3 x 28.9–38.8	
Total range	20.3 - 105.7 x 11.1 - 51.3	27.5 - 50 x 17.5 - 32.5	36–75 x 21–40	36–58 x 13–33	39–70 x 20–42.1	31.5 - 75.3 x 22.9 - 38.9	27.5–80.5 x 16.7–69.6	35 - 67.5 x 17 - 47.5
l/b ratio	1.6 ± 0.26	1.6 - 1.7	1.73 ± 0.28	1.7 ± 0.22	1.6 ± 0.16	1.52	1.43 ± 0.19	1.8 - 1.9
Oogonia								
Mean diam	32.0 ± 4.4	24 ± 2.5	30.0 ± 3.0	26.5 ± 1.9	31.2 ± 2.6	30.3	28.5 ± 3.3	30.7 ± 3.1
diam range	19.2 - 45.5	20 - 27.5	16.7–35.9	19–37	21.3–36	22.2 - 41.4	15–37.5	25 - 37.5
Range of isolate means	28.1 - 36.3		29.7–30.3	25.5–27.8	30.9–31.4		27.5–29.9	
Oospores								
aplerotic oospores	69.6 % (40 - 96 %)		44% (32–56%)	45% (36–52%)	43% (38–48%)		44.3% (22–62%)	47%
mean diam	28.4 ± 3.9	22.7 ± 2	27.1 ± 2.8	23.6 ± 1.8	27.7 ± 2.3	26	25.9 ± 3.1	27.9 ± 2.6
diam range	15.9 - 39.3	20 - 27.5	15.3–30.9	17.3–33.1	18.4–33.2	19.6 - 34.2	14–35.8	22.5 - 32.5
wall diam	2.0 ± 0.4	2.7	1.68 ± 0.35	2.6 ± 0.5	1.8 ± 0.36	1.7	1.45 ± 0.35	< 2.5

oospore wall index	0.38 ± 0.09	0.56 [8]	0.33 ± 0.05	0.52 ± 0.07	0.34 ± 0.05		0.3 ± 0.06	0.45 [8]
Antheridia								
lxb mean	12.8 ± 3.4 x 9.48 ± 1.6	9 ± 1.8 x 9.1 ± 1.7	12.8 ± 2.7 x 8.2 ± 1.7	12.9 ± 1.9 x 8.7 ± 1.3	12.2 ± 2.1 x 9.0 ± 1.6	12.6 x 11.1	11.1 ± 4.4 x 8.4 ± 3.1	14.5 ± 2.1 x 14 ± 1.4
lxb range	5.4 - 28.5 x 5.1 - 15.2	5 -12.5	7.5–18.5 x 5.4–14.4	8–20 x 5–14	7.7–16.9 x 6.1–12.6	10.2 - 15 x 10.2 - 12.9	7–21 x 5.3–16	10 - 20 x 12.5 - 17.5
Abortion rate	38.5 % (10 - 99 %)							42 - 46 % [9]
Hyphal aggregations	+	-	-	-	-	-	-	-
Maximum temperature (°C)	32	27.5	32	32	32	35	32	27.5
Optimum temperature (°C)	25	22.5	25	25	30	25	25	20
Growth rate on V8A at optimum (mm/d)	7.75 ± 0.19		6.9 ± 0.1	6.5 ± 0.02	9.2 ± 0.74		8.1 ± 0.18	
Growth rate at 20°C (mm/d)								
V8A	6.52 ± 0.20	6.6 [7]	6.2 ± 0.04	4.8 ± 0.6	6.3 ± 0.23		6.3 ± 0.1	5 [7]
PDA	4.28 ± 0,16		2.0 ± 0.2	3.3 ± 0	6.5 ± 0.42		3.2 ± 0.2	

[1] Designated as *P. citricola* I in Jung and Burgess (2009). [2] 5 of the 15 isolates were included in the growth tests.
Data from: [3] Bezuidenhout et al. (2010); [4] Jung and Burgess (2009); [5] Scott et al. (2009); [6] Hong et al. (2011).
[7] Growth rate on CA at 20°C. [8] Values calculated from data in Bezuidenhout et al. (2010). [9] Aborted plus immature oospores.

Discussion

In the first comprehensive phylogenetic analysis of the genus *Phytophthora* the major ITS Clade 2 contained seven species of which only two were homothallic with paragynous antheridia, *P. citricola* and *P. inflata* (Cooke *et al.* 2000). This clade has since expanded considerably including in 2012 25 species and informally designated taxa (Kroon *et al.* 2012; Hong *et al.* 2009, 2011; Bezuidenhout *et al.* 2010; Rea *et al.* 2010; Jung & Burgess, 2009; Abad *et al.* 2008; Maseko *et al.* 2007; Reeser *et al.* 2007; Aragaki & Uchida 2001). A molecular re-evaluation demonstrated that the original *P. inflata* of Caroselli & Tucker (1949) is a lost species and that isolates designated in recent years as *P. inflata* were conspecific with the newly described *P. plurivora* (Jung & Burgess, 2009). The morphospecies *P. citricola* (Sawada, 1927) turned out as a complex of morphologically similar and phylogenetically closely related taxa comprising *P. citricola* s. str., the four newly described species *P. capensis*, *P. multivora*, *P. pini* and *P. plurivora*, and the three informally designated taxa *P. citricola* III, *P. citricola* E and *P.* taxon 'emzansi' forming the '*P. citricola* complex' in Clade 2a and the two more distantly related new species *P. mengei* and *P. elongata* belonging to Clade 2c (Hong *et al.* 2009, 2011; Bezuidenhout *et al.* 2010; Rea *et al.* 2010; Jung & Burgess, 2009).

Phylogenetic analysis of the ITS, *cox*1 and β-tubulin gene regions as well as detailed morphological and physiological comparisons with all described species and designated taxa from the *P. citricola* complex demonstrate that *P. acerina* is unique and forms a separate cluster within the complex with *P. plurivora* being its closest known relative.

All 15 genetically analyzed isolates of *P. acerina* shared identical ITS, *cox*1 and β-tubulin sequences. At GenBank ITS sequences from four isolates designated as *P. citricola* can be found that are similar (but not completely homologous) to *P. acerina*. The isolates TARI 23044 isolated in 2003 from *Prunus persica* in Taiwan by Ann *et al.*, and P10366 from the World *Phytophthora* Collection (WPC:10366A631), submitted by Coffey *et al.* without information on host, geographic origin and date of isolation, share identical ITS sequences (GU111596.1 and GU259257.1, respectively). *Phytophthora acerina* differs from these two isolates by 1 bp showing an R instead of a G at position 564. The other two strains (CH95PHE28 and CH95PHE31), isolated from *Eustoma grandiflorum* in Japan by Villa *et al.* (2006), also share identical ITS sequences (AB217676.1 and AB217677.1) and differ from *P. acerina* in 2 bp showing an A instead of a G at position 564 and having an insertion of a C at position 752. The presence of A and G in positions where *P. acerina* shows an R is interesting because the existence of this polymorphism result genetically supported. In the ITS region *P. acerina* differs from *P. citricola sensu stricto* by 4 bp (ex-type IMI021173) and 5 bp (CH98U121C), respectively; from *P. pini* by 4 bp (CIT-US1) and 5 bp (ex-type CBS 181.25), respectively; from both *P. citricola* E (83-141) and *P. citricola* III (1E1) by 4 bp; from *P. plurivora* by 5 bp (ex-type CBS 124087) and 6 bp (PLU7), respectively; from *P. capensis* (P1822) and *P.* taxon emzansi (STE-U6269) by 8 and 11 bp, respectively; and from *P. multivora* (ex-type CBS 124095 and isolate Citri-P1817) by 10 bp. Analysis of the *cox*1 sequence data clearly separated *P. acerina* from all other taxa in the '*P. citricola* complex', with differences to other taxa ranging from 9-10 bp (*P. plurivora*) and 14 bp (*P. citricola* s.str.) up to 30-33 bp (*P. multivora*). In the β-tubulin gene region, the taxa from the '*P. citricola* complex' generally show only small differences to each other (Jung and Burgess 2009). *Phytophthora acerina* differs from *P. plurivora* by 1 to 3 bp, from *P. citricola* s. str. by 2-3 bp and from *P. pini* by 4 to 8 bp.

Though *P. acerina* shows morphological and physiological affinities with the other members of the '*P. citricola* complex' it is endowed with a number of distinguishing features that clearly discriminate it from related species. Besides many smaller differences to individual taxa which are listed in the notes the following features make *P. acerina* the most distinct taxon of the '*P. citricola* complex': the high abortion rate of 40-96 % of the oospores, a characteristic only found in the two known isolates of *P.* taxon 'emzansi' from South Africa (Bezuidenhout *et al.* 2010); a very high proportion of aplerotic oospores; globose to subglobose, appressoria-like and coralloid hyphal swellings and dense stromata-like hyphal aggregations produced in ageing cultures. Such aggregations are not produced by any other taxon of the '*P. citricola* complex' but are well known from several species in ITS Clade 6, namely *P. gregata*, a species with a comparably high oospore abortion rate, and the self-sterile *P. litoralis* (Jung *et al.* 2011). The hyphal aggregations of *P. acerina* resembled stromata formed by *P. ramorum*, a species with a partial incompatibility between isolates of the A1 and A2 mating types (Brasier & Kirk 2004), beneath the cuticle of infected leaves (Moralejo *et al*. 2006), and it is likely that they play a role as survival structures in the lifecycle of *P. acerina*. The high abortion rate and the formation of vegetative survival structures suggest that *P. acerina* probably evolved under environmental conditions that did not require oospores as resting structures so that natural selection was silenced and harmful mutations leading to distortions of the reproduction system could accumulate. Such conditions are found in aquatic habitats where the possibility of a continuos multiplication via sporangia and zoospores favours the evolution of sterile species with non-dormant resting structures such as chlamydospores or hyphal aggregations (Jung *et al*. 2011). However, this is not supported by the failed attempts to isolate *P. acerina* from water bodies in the infested Boscoincittà park. A more likely explanation is that *P. acerina* evolved in a mild, continuously humid climate in stable ecosystems with a high abundance of individual species (most likely including *Acer* spp.) and low species diversity. Under such conditions the two major functions of oospores as dormant long-term resting structures for survival of deep frost or extreme droughts and as source of new genetic variation to enable rapid adaptation to genotype changes in diverse host populations are not essential for the success of a co-evolved pathogen. In its natural habitat *P. acerina* most likely thrives as a mild nibbler of fine roots or leaves in undisturbed healthy ecosystems as has been demonstrated for *P. himalsilva* in Nepal, *P. citricola*, *P. heveae* and *P. katsurae* in Hainan and *P. cinnamomi* in Taiwan and Papua New Guinea (Webber *et al.* 2012; Vettraino *et al.* 2010; Zeng *et al.* 2008; Ko *et al.* 1978; Arentz & Simpson 1986).

The genetic uniformity of all 15 sequenced isolates, the high aggressiveness to two common forest tree species of Europe and the fact that none of the sequences submitted to GenBank from *P. citricola*-like isolates recovered during extensive surveys of thousands of nurseries, horticultural plantations and planted and seminatural forest stands in Europe is identical with *P. acerina* strongly support the hypothesis that of a recent clonal introduction of *P. acerina* to Europe and Northern Italy. This raises the question on the possible centre of origin of *P. acerina*. There are several indirect evidences indicating an origin of *P. acerina* in Eastern Asia. First, several of the closest relatives of *P. acerina* are native to Asia: Three of the four closest related isolates come from Japan (CH95PHE28 and CH95PHE31) and Taiwan (TARI 23044), respectively; *Phytophthora citricola* s. str. is without any doubt endemic in Eastern Asia (Jung & Burgess 2009; Zeng *et al.* 2009); the finding of a new taxon close to *P. plurivora* in remote, undisturbed healthy forests in Nepal (Vettraino *et al.* 2010) and several recent entries of *P. plurivora* isolates from forest soils and streams in Northwestern Yunnan, China, at GenBank (JQ730711.1, JQ730714.1, JQ730715.1 and JQ730716.1; Huai, *et al.* unpublished)

support the hypothesis of Jung & Burgess (2009) of an Asean origin of *P. plurivora*. Second, the high aggressiveness of *P. acerina* to *A. pseudoplatanus* also speaks for an Asean origin as it is most likely the result of a co-evolutionary arms race with other *Acer* species, and the majority of the 128 known *Acer* spp. are native to Asia (http://en.wikipedia.org/wiki/Acer_(genus)).

With the exception of *P. citricola* s. str. and *P. citricola* E which seem to be relatively specific to *Citrus* spp. and *Rubus idaeus*, respectively, most taxa from the '*P. citricola* complex' are able to cause root rot, bark cankers and less frequently shoot dieback on multiple hosts from different genera and families. In particular *P. multivora* and *P. plurivora* have wide host ranges with 16 species in seven dicotyledonous families and 45 species in 16 dicotyledonous and 4 coniferous families, respectively, listed in their original descriptions (Scott et al. 2009; Jung & Burgess 2009). Many new host species have been reported since. *Phytophthora plurivora* is strongly involved in the decline and dieback of forest and amenity stands of *Quercus* spp., *F. sylvatica*, *Acer* spp., *Alnus* spp., *Betula* spp., *J. regia* and *Tilia* spp. across Europe and is also causing root rot on *Fraxinus excelsior* (Orlikowski *et al.* 2011; Jung & Burgess, 2009; Jung *et al.* 1996, 2000, 2009; Brown & Brasier, 2007; Jung & Blaschke, 2004; Balci & Halmschlager 2003; Vettraino *et al.* 2002, 2005). Together with *P. cambivora*, *P. plurivora* is the most aggressive and most common causal agent of root rot, bark cankers and mortality of *F. sylvatica* trees in Europe (Jung *et al*. 2005, 2012b; Jung, 2009; Jung & Burgess, 2009). In the USA *P. pini* and less frequently also *P. plurivora* are causing on a large scale similar disease symptoms in planted *F. sylvatica* trees (Weiland *et al.* 2010; Jung *et al.* 2005). The recent arrival and spread of *P. pini* in the European nursery trade (Lilja *et al.* 2011; Jung & Burgess 2009) will most likely exacerbate the situation of beech decline in Europe. Both, *P. plurivora* and *P. pini* caused extensive bark lesions on young and mature trees of *F. sylvatica* (Weiland *et al.* 2010; Jung & Nechwatal 2008; Jung *et al.* 2005; Brasier & Jung 2003; Jung & Blaschke 2006) and extensive root losses and mortality of young *F. sylvatica* trees in soil infestation tests (Weiland *et al.* 2010; Jung *et al.* 2003; Fleischmann *et al.* 2002). Several common *Acer* species from Europe and North America are known hosts of species from the '*P. citricola* complex'. In a study that pre-dated molecular identification tools, Drilias *et al.* (1982) confirmed *P. citricola* s.l. as causal agent of a collar rot epidemic of *Acer sacharum* in the USA. Later *P. plurivora* and *P. citricola* III were isolated from declining *A. sacharum* trees in Canada and the USA, respectively (Hong *et al.* 2011; Jung & Burgess 2009). *Phytophthora plurivora* was also isolated from rhizosphere soil, collar rots and aerial bark cankers of *A. platanoides* and *A. pseudoplatanus* in Germany and the UK and from rhizosphere soil of declining *Acer campestre* trees in Germany (Jung & Burgess 2009; Jung et al. 2009; Brown & Brasier 2007), and from collar rot lesions of *Acer saccharinum* in Germany (T. Jung, unpublished). The pathogenicity of *P. plurivora* to *A. platanoides* and *A. saccharinum* was confirmed by soil infestation and underbark inoculation tests, respectively (Orlikowski *et al.* 2011; Jung & Burgess 2009). In the pathogenicity test of the present study, *P. acerina* was more virulent on its host species *A. pseudoplatanus* than on *F. sylvatica* although it also caused considerable bark lesions on the latter species. Therefore, *P. acerina* might pose an additional serious threat to maple and beech stands in Italy and the whole Europe if it cannot be contained in the forest stands of the Boscoincittà park near Milan and if it will not immediately be eradicated wherever it will turn up in nurseries and plantations in the future. More pathogenicity tests are urgently required to determine the potential host ranges of *P. acerina*, *P. pini*, *P. multivora*, *P. citricola* III and the yet not introduced *P. capensis* and *P.* taxon 'emzansi' among the native European tree and shrub species and the most important exotic tree species used in forest and horticultural plantations.

After *P. plurivora*, which has been introduced a long time ago and has become well established in seminatural ecosystems, plantings and nurseries across Europe, and the relatively recently arrived *P. multivora*, *P. pini*, *P. citricola* III and *P. citricola* E, *P. acerina* is the sixth species from the '*P. citricola* complex' that has been introduced to Europe. Between 1977 and 2012 large-scale *Phytophthora* surveys were conducted by 32 research groups in 21 European countries in 1620 stands of 603 forest, advanced tree, horticultural and ornamental nurseries and in 2353 forest, riparian, amenity, landscape and ornamental plantings and horticultural plantations (Jung *et al.* 2012b). Over all countries and nursery types, 79.4% of the stands in 91.5% of the nurseries and 63.6 % of the plantings were found infested by a total of 48 different species and designated taxa of *Phytophthora*. At least 32 taxa are considered exotic invasives in Europe, and beyond any doubt their most likely pathway into Europe and between and within countries of Europe was the import of exotic plants for planting and the intense European nursery trade. Fundamental changes in the international plant distribution chain, eg. increased complexity, increasingly blurred roles and distinctions of firms, specialisation of firms resulting in the flow of individual plants through several nurseries; more efficient and rapid transportation systems, packaging and shipping technologies; e-commerce; and changed consumer demands and in particular the demand for 'instant landscapes' have been and are still causing an exponential increase in the international trade in plants for planting and have extended the range of plant origin, availability and viability (Dehnen-Schmutz *et al.* 2010; Drew *et al.* 2010; Brasier, 2008). The fact that not a single one of these 32 exotic *Phytophthora* taxa has been intercepted at the ports of entry and the fact that the spread of the quarantine organisms *P. ramorum*, *P. kernoviae* and *P. lateralis* within the climatically favourable atlantic regions of Europe could not be halted despite of strict quarantine regulations demonstrate major failure of plant biosecurity in Europe. Often suppressed by fungicides or fungistatic chemicals, soilborne pathogens and in particular *Phytophthora* species, can travel as passive hitchhikers in roots and adhering soil particles of both host and non-host plants. Most of the ca 150 currently known species and designated taxa of *Phytophthora* were unknown to science before they turned up in other continents as invasive aggressive pathogens of native plants or plantation crops and their origins remain cryptic (Hansen *et al*. 2012; Jung *et al.* 2012a; Brasier, 2008; Hansen, 2008; Erwin & Ribeiro 1996). Apparently, in Europe and elsewhere the species-by-species regulation approach based on visual inspections for symptoms of listed quarantine organisms has largely failed as the exponentially increasing numbers of new invasive forest pathogens, and in particular oomycete pathogens, during the last four decades demonstrate (Santini *et al.* 2012). Partial controls along a pathway most likely cause complete failure in preventing introductions of both regulated and unregulated pathogens (Brasier, 2008) and eradication or even containment of introduced *Phytophthora* species seems impossible regarding the current allocation of human and financial ressources to plant biosecurity. A pathway regulation approach based on pathway risk analyses and recent scientific knowledge about pathogen biology, intense inspection regimes performed by a sufficient number of skilled staff, and the regular use of modern molecular detection tools and protocols at the ports of entry are urgently required to minimise the risks of further introductions of both known and unknown potential pathogens to Europe.

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Chapter III

The impact of *Phytophthora Acerina* on *Acer pseudoplatanus* stands in a park of Northern Italy

The impact of *Phytophthora Acerina* on *Acer pseudoplatanus* stands in a park of Northern Italy

Beatrice Ginetti, Alessandro Ragazzi and Salvatore Moricca

Dipartimento di Biotecnologie agrarie - Sezione di Protezione delle Piante, Università degli Studi di Firenze, Piazzale delle Cascine 28, 50144-Firenze, Italy
Corresponding author: S. Moricca; E–mail: salvatore.moricca@unifi.it; phone: ++390553288349; fax: ++390553288273

Abstract

This article reports on the researches that have led to the identification of *Phytophthora acerina* as the agent responsible for the extensive dieback and death of *Acer pseudoplatanus* trees observed at the Boscoincittà park, Milan. Plants were inspected for disease symptoms and assayed *in situ* by an immunodiagnostic method (lateral flow test). Those that were given a positive verdict were sampled. Isolations in the laboratory from tree tissue, soil and water samples enabled to detect several taxa of *Phytophthora*: *P. acerina, P. gonapodyides*, *P. inundata*, *P. lacustris*, *P.* taxon PgChlamydo and *P.* taxon walnut, plus a few hybrids between *P. lacustris* and *P.* taxon PgChlamydo. Infection tests confirmed *P. acerina* as the causative agent. *A. pseudoplatanus* trees appeared to be also strongly affected by environmental constraints, in particular drought stress. It is hypothesized that the low tolerance of *A. pseudoplatanus* to adverse abiotic factors is to be ascribed to the destruction of the root system of trees by *P. acerina*.

Keywords: *Phytophthora acerina*, *Acer pseudoplatanus* dieback, artificial inoculation tests

Introduction

The European rural landscape has suffered a major change over the last century, especially in its second half. Starting from the end of the II World War, the transformation of society changed the socio-economic conditions of many Western European citizens, with a growing push toward industrialization that caused the 'marginalisation' of many agricultural lands (DLG, 2005).

Numerous lands, once cultivated or subjected to grazing of domestic animals, were no longer exploited and resulted overgrown by shrubs or trees (MacDonald *et al*., 2000). This trend was particularly marked in suburb areas, where such lands were incorporated in the urban agglomeration and replaced by buildings, road networks and other human infrastructures, while a few lucky ones were converted into green spaces accessible by the urbanités for multiple purposes.

The Boscoincittà park is one of the few cases of former agricultural land, located in the immediate vicinity of a large metropolis, which had the good fortune to be converted into an urban green area. Located in the western suburbs of the city of Milan, this public park, owned by the city, was founded in 1974 on the initiative of the nature conservation organisation "Italia Nostra". With an extension of roughly 110 hectares of woods, clearings, paths and waterways, it represents the first example of urban afforestation in the country, designed to provide a range of benefits to the population.

The artificial forest, mostly a mix of broadleaf species, over the last 10 years, began to show a progressive decline and an increasing mortality rate. Sycamore maple (*Acer pseudoplatanus*), one of

the most represented tree species, resulted severely affected, with hundreds of individuals that were brought to death in a couple of seasons. A number of specific and nonspecific symptoms were observed on declining trees. Among the nonspecific symptoms, the most frequent were microphyllia, discoloration of the foliage, leaf abscission, crown transparency, stunted growth, branch dieback. In addition to these uncertain injuries, that might be ascribed to a variety of biotic or abiotic factors, some very specific symptoms, like crown dieback accompanied by production of tarry exudates on the stem, necrotic patches on the bark, flame-shaped underbark discolourations, necroses at the level of phloem and the underlying xylem strongly suggested a possible *Phytophthora* attack. The observed symptoms closely resembled the symptomology induced by *Phytophthora ramorum* and *Phytophthora citricola* on *Acer pseudoplatanus* in UK (Brown & Brasier, 2007).

To test the hypothesis that it could be one of the above host-*Phytophthora* associations, alerted by the risk posed by the possible occurrence of *P. ramorum*, and concerned about the quarantine measures that were to be urgently implemented if the presence of this pathogen had been confirmed, an integrated approach, based on pre-screening ELISA tests (diagnostic kits), coupled with traditional (analyses of macro- and micro-morphology; infection tests) and molecular (e.g. DNA-based) identification, was carried out to gain insight into the etiology of the decline observed.

The study revealed the widespread occurrence of a new species of *Phytophthora*, *P. acerina* sp. nov. (Ginetti *et al*., 2013, submitted). The present paper reports in details investigations that lead us to determine: a) the massive presence of *P. acerina* in the study area; b) its tight association with declining *Acer pseudoplatanus* trees; and c) the unequivocal involvement of this new taxon in the dieback and death of *Acer pseudoplatanus* trees.

Material and Methods

Study site

Boscoincittà park (lat. 45°29’06’’N, long. 9°05’32’’E) extends over a plain area (altitude 134 m a.s.l.) in the province of Milan, Lombardy, Northern Italy, and represents one of the largest parks fruited by citizens and schools (for a number of educational activities) in the country.

The site, occupied today by plantation forestry, was exploited for agricultural crops of cereals and grasses until 1974, when the processing to public green areas destination began. The conversion from an agricultural setting to a forested area was a gradual process and it was pursued over the years thanks to the work of volunteers. The co-occurrence at the time of livestock (mostly dairy cattles) in some plots required cultures that would also ensure the production of forage.

Soil analyses revealed the first 20 cm to be composed of gravel, sand and fine gravel; immediately below the layer of topsoil subsists clayey ground mixed with gravel and sand, occasionally outcropping on the surface. Uninterrupted layers of clay are present at 40, 60 and 100 cm of depth, while sporadic layers of conglomerates and sandstones occur around 90-96 cm.

The tree species planted were either indigenous taxa considered adapted to the climate and the soil of the area or exotic species chosen for aesthetic/ornamental purposes. The dominant species were sycamore maple (*Acer pseudoplatanus*), field maple (*Acer campestre*), English oak (*Quercus robur*), red oak (*Q. rubra*), elm (*Ulmus* spp), poplar (*Populus* spp), European ash (*Fraxinus excelsior*), hornbeam (*Carpinus* spp), black locust (*Robinia pseudoacacia*), alder (*Alnus* spp), Persian walnut (*Juglans regia*), willow (*Salix* spp). Numerous native shrub species were also used.

The planting material constituted of 1-2 year-old seedlings routinely produced for afforestation by several public nurseries (forest services), kindly donated free of charge. The diverse source of seedlings, the repeated donations, as well as the very nature of the forest (arisen with the main contribution of volunteer citizens) make it nowadays impossible to trace the origin of the planting material. "Social gardens" were also created in 1988 to further involve citizens in the care of green.
The park is rich in water. It is crossed by a number and waterways and embodies water sources and a wet zone with scattered small lakes and ponds. At the end of the eighties, a new artificial lake was created in the park with the aim of improving its microclimate and encourage the development of aquatic and terrestrial flora and fauna. The area is equipped with facilities like boardwalks over the water bodies, benches and tables.

Elisa test

Phytophthora-specific lateral flow kits (Pocket Diagnostics) were used as predictors of *Phytophthora* infection in the field. This preliminary screening served as an aid in the selection of plants to be sampled for subsequent investigations in the laboratory. The positivity of symptomatic plants to infection by the oomycete was tested on 3-8 small pieces (roughly 5 x 10 mm) of necrotic phloem and other tissue fragments and debris of varying shape. This material was added to the commercial buffer and agitated for about 1 minute. A few drops of the resulting suspension were taken with a plastic pipette and poured in a hole present in the upper part of the diagnostic device, to allow drops to reach by absorption the underlying membrane. The result was evident in a couple of minutes, with two blue lines, in correspondence with the “T” (test) and the “C” (control) letters, indicating the positivity of the immunoassay test.

Sampling and isolation from plant tissue

Trees positive to the lateral flow test were sampled during spring (May-June 2010 and 2012) and winter (December 2010 - February 2011) seasons. All trees exhibited the characteristic symptoms of *Phytophthora* infection: production of tarry exudates on the basal portion of the stem (in some cases up to 2 m in heigth), wave-shaped necrotic lesions at the cambium level with evident green-browning or dark streaks.
The outer part of the bark was removed with the use of a hatchet or a billhook. Tissue pieces of various shapes and dimensions (panels, slivers, scales and debris) were taken from the exposed symptomatic wood areas by means of the above billhook or with the aid of boxcutters and scalpels. Sampled tissue portions were inserted into high density polyethylene bags and stored in a few hours at 4°C until use.
Isolation was also attempted from the fine roots of randomly chosen *A. pseudoplatanus* trees. Roots samples were escavated from the soil near symptomatic trees, coarsely freed from soil particles and stored as above.
Samples were processed no later than 24 hours from collection in the field, with isolations that were carried out in the laboratory under sterile conditions in a laminar flow cabinet.
The isolations were attempted on the following nutrient media, known by the literature for their effectiveness in the growth of these microorganisms (quite refractory to *in vitro* culture): 1) selective medium with V8 tomato juice (Campbell), with the addition of antibiotics such as Pimaricin, Ampicillin, Rifampicin, Pentachloronitrobenzene (PCNB), Nystatin and Hymexazol

(PARPNH) (Tsao, 1983); 2) Acidified Potato Dextrose Agar (APDA) (Reilly *et al*. 1998); and 3) Corn Meal Agar (CMA) (Streito *et al*. 2002).

Large wood panels were dipped in 75% ethanol for 30 s, in 4% sodium hypochlorite for 1min, and again in 75% alcohol for 15 s, with a final rinsing in sterile water for 3 min. They were then surface-dried with a paper towel.

Root samples were washed and rinsed in running tap water for six hours, dried on filter paper and then furtherly sterilized as the wood panels were.

Isolations were pursued by putting 4-5 wood slivers, taken as such or excised from larger pieces of wood, or small portions (3-5 mm) of necrotic fine roots, on 9-cm-diam plastic Petri dishes, each containing 20 ml of one of the above media. The Petri dishes were then incubated at 20 °C in the dark.

Almost all sampled tissues were from declining individuals (32) of *Acer pseudoplatanus* scattered in various parts of the study area that had resulted positive to *Phytophthora*-specific lateral flow test in the field (Fig. 1). A few other individuals (6 in total) of the other broadleaf species that grew intermixed with *A. pseudoplatanus* in the area and showed clear decline symptoms were sampled: they were 2 English oak and Persian walnut trees and 1 individual each of red oak and hornbeam.

Soil sampling and apple baiting from the soil

Soil samples were taken either under the canopy of sampled trees (roughly at 1,5 m from the base of the stem) either on the bank of the main stream that crosses the park (Fig. 1). Soil portions of about 250 g were taken at an average depth of 15 cm, placed into high density polyethylene bags and stored at 4°C until isolation. A selective bait (apple) was used for the isolation of the *Phytophthora* spp. from collected soil samples. Four holes (1-cm diam, 2-cm-depth) equidistant to one another (on the 4 cardinal points) were made with a sterile scalpel on each apple fruit following its surface sterilization with 95% ethyl alcohol. Soil was placed inside these cavities till entirely filled up, then it was wet with sterile deionized water to induce the germination of *Phytophthora* oospores. Apples so processed were finally wrapped with transparent film in order to hold soil and to isolate fruit infection courts from the external environment. Baits were incubated at 18 °C for 5-7 days, then they were unwrapped under a sterile laminar flow cabinet. Small pieces of fruit pulp (approximately 2,5 mm^3 in size) were picked up at the interface between the diseased area and the healthy apple tissue and placed on selective medium V8A-PARPNH. Growing colonies were transferred after 24-48h onto PDA, in order to obtain mono-hyphal colonies, then on nutrient Frozen Pea Medium-FPM and V8agar and incubated again for 3-5 days. Mycelium agar squares were submerged in filtered pond water in order to induce asexual reproductive structures production (zoosporangia).

Sampling and isolation from streams and small lakes

In December 2010 baits (apples) were placed at four points along the main stream that crosses the park (Fig. 1). Lots of 3-5 apples were placed first into jute bags and then into small metal cages created on purpose to prevent them being eaten by rodents and other aquatic animals which are abundant in the park. Cages were then immersed in the water. Baits were removed seven days after installation and transported to the laboratory, where isolations were promptly made according to the above described for soil samples protocol (V8A-PARPNH, PDA, FPM, V8A and pond water). On V8A-PARPNH typical, whitish mycelial intials were visible against the light after a few hours.

A new survey was conducted again in February 2011 with the same procedure to ascertain the presence of oomycetes in the other ponds and water bodies scattered within the area.

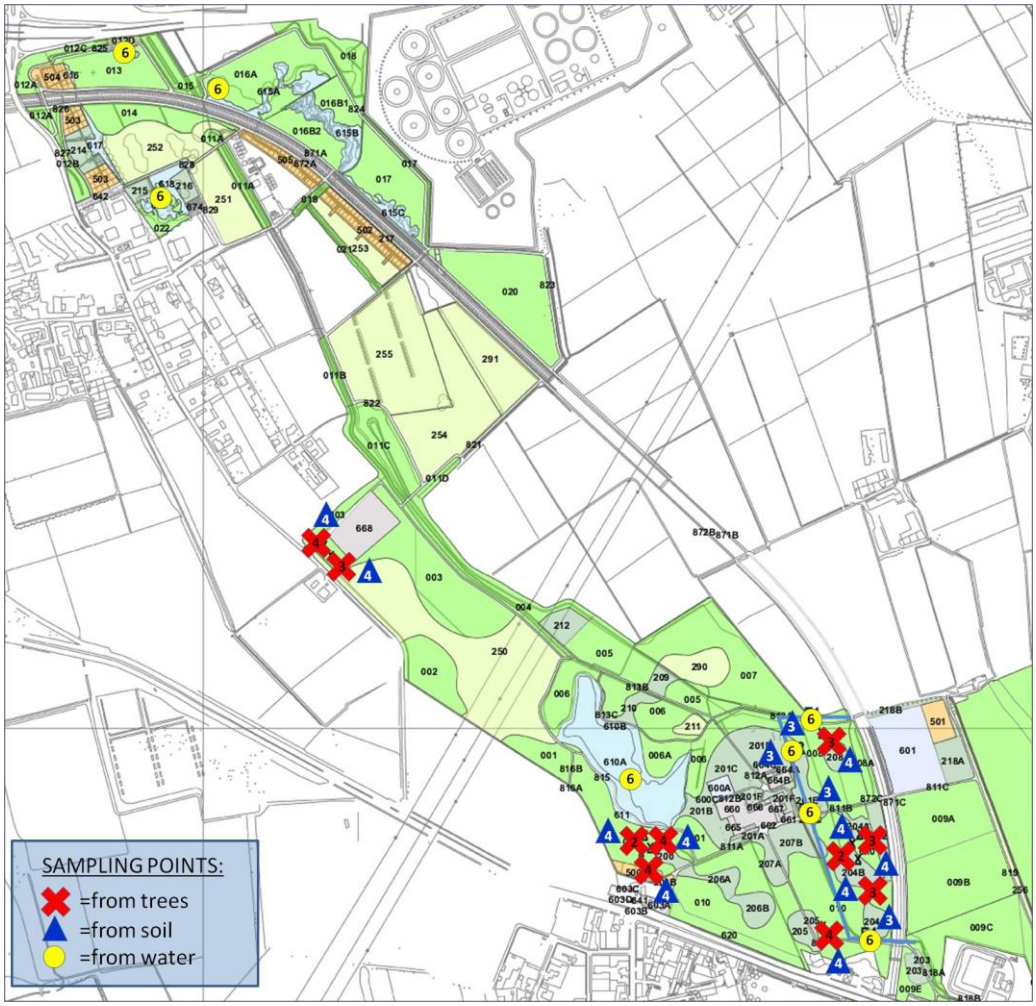

Figure 5 Map of Boscoincittà park showing the sampling points from various natural substrates. Numbers within icons indicate the number of individual trees, or soil specimens, or water specimens sampled.

Isolate identification

Following isolation on selective V8A-PARPNH medium, *Phytophthora* isolates were transferred on PDA in 9-cm-diam Petri dishes and incubated for 4 days at 20°C. Hyphal tips were aseptically taken from these colonies under a sterile laminar flow cabinet using a stereoscope to obtain mono-hyphal subcultures. Colony morphologies were investigated on various media (V8-agar, MEA, CMA and PDA). To induce sexual and asexual reproductive structures, colonies were transferred onto nutrient V8A substrate. Morphology and size of sporangia, antheridium type (amphigynous or paragynous), sizes of oogonia, oospores and wall index, growth rates under a range of temperatures (10, 15, 20, 25, 30, 32, 35, 37, 39 °C), were employed for the traditional screening and identification of isolates.

Isolates were also analysed by means of molecular methods. The ITS region, including the ITS1 and ITS2 spacers and the internal 5.8S gene of the rRNA operon, a portion of the mitochondrial *cox*1 gene region and a portion of the ß-tubulin nuclear gene region were PCR-amplified.
The ITS region was then subjected to digestion with MspI and AluI restriction enzymes; the *cox*1 gene region was digested with RsaI.
Amplicons of the above DNA regions of the isolates which showed variation in macro- and micro-morphological characteristics or which revealed different restriction endonuclease cleavage site data, were also sequenced (Table 1). Sequences were then BLASTed in GenBank to individuate the best scores with the closest taxonomically identified sequences. Sequence data were also used for making phylogenetic inferences.
The approach led to identification of several taxa of *Phytophthora*. Among these, *P. acerina* (Ginetti *et al*, submitted) was used in the subsequent artificial inoculation trials since it was the only species associated with declining *A. pseudoplatanus* trees.

Infection tests
Five isolates of *P. acerina* were employed in the inculation trials: B035, B060, B064, B077 and B071. Colonies were recovered from stock cultures (carrot agar-bearing mycelium squares in deionized, sterilized water) and grown in the dark at 20°C for 7 days. Approximately 5 mm diam mycelial plugs, taken from the margin of actively growing colonies, served as the inoculum. Inoculations were from genetically identical plant material (one single individual per tree species) collected at an experimental field at CeSpeVI (Centro Sperimentale per il Vivaismo – Pistoia, Tuscany). One-year-old twigs (diam approx. 5-10 mm) from trees of *Acer pseudoplatanus*, *Fagus sylvatica*, *Fraxinus excelsior*, *Juglans regia*, *Quercus robur* and *Q. rubra* were collected in April, at the beginning of the growing season. Leaves were detached and twigs were cut to a length of about 13 cm.
A portion of about 0.5 cm of bark was raised aseptically with a razor blade and a V8A disc taken from the margin of a freshly growing culture was placed over the wound. Inoculated twigs were covered with the removed bark portion, covered with cotton wool soaked in sterile water and wrapped with aluminum foil. Fifty replicates per tree species (10 per isolate) were inoculated with the oomycete. As controls, 10 twigs were inoculated with sterile V8A agar discs, and the wounds protected as above. Twigs were placed over two layers of wet filter paper in autoclaved 18-cm-diam glass Petri dishes. Dishes were sealed with parafilm and incubated for 3 weeks in the dark at 20°C. Inoculated twigs were inspected for the presence of lesions after 21 days. The length of the lesions was measured after peeling off the bark with a scalpel and the lesions of the infected and control twigs were photographed. Portions of discoloured xylem surrounding the lesions were randomly excised from inoculated twigs for reisolation and identification of the microorganism in order to fulfill Koch's postulates. Small (0.3 - 0.5 cm^2) pieces of tissue were cut from the edge of the lesions, plated on selective V8-PARPNH-agar, and incubated at 20 °C in darkness.

Table 1. Sequence-characterized isolates of *Phytophthora* recovered in the Boscoincittà park

Collection nos.	Identity	Substrate	Host	Collection date	GenBank Accession No.		
					ITS	Cox1	ß-tubulin
B057	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951285	KC156134	KC201283
B080	*P. acerina*	Soil	*A. pseudoplatanus*	Dec 2010	JX951291	KC156140	KC201289
B035	*P. acerina*	Tissue	*A. pseudoplatanus*	Jun 2010	JX951282	KC156131	KC201281
B053	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951283	KC156132	KC201282
B054	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951292	KC156141	KC201290
B055	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951293	KC156142	KC201291
B056	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951284	KC156133	
B058	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951286	KC156135	KC201284
B060	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951287	KC156136	KC201285
B062	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951294	KC156143	KC201292
B063	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951295	KC156144	KC201293
B064	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951288	KC156137	KC201286

B071	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951289	KC156138	KC201287
B077	*P. acerina*	Tissue	*A. pseudoplatanus*	Dec 2010	JX951290	KC156139	KC201288
B081	*P. acerina*	Soil	*A. pseudoplatanus*	Dec 2010	JX951296	KC156145	KC201294
B079	*P. gonapodyides*	Soil	Apple bait	Dec 2010	KC291561	KC291593	
B098	*P. gonapodyides*	Water	Apple bait	Dec 2010	KC291562		
B155	*P. inundata*	Water	Apple bait	Feb 2011	KC201295	KC238311	
B189	*P. inundata*	Water	Apple bait	Feb 2011	KC201296	KC238312	
B126	*P. lacustris*	Water	Apple bait	Dec 2010	KC291582	KC291611	
B135	*P. lacustris*	Water	Apple bait	Feb 2011	KC291573		
B137	*P. lacustris*	Water	Apple bait	Feb 2011	KC291566	KC291597	
B138	*P. lacustris*	Water	Apple bait	Feb 2011	KC291563	KC291594	
B140	*P. lacustris*	Water	Apple bait	Feb 2011	KC291578	KC291607	
B143	*P. lacustris*	Water	Apple bait	Feb 2011	KC291572	KC291603	
B146	*P. lacustris*	Water	Apple bait	Feb 2011	KC291579	KC291608	
B147	*P. lacustris*	Water	Apple bait	Feb 2011	KC291581	KC291610	
B153	*P. lacustris*	Water	Apple bait	Feb 2011	KC291583		
B175	*P. lacustris*	Water	Apple bait	Feb 2011	KC291574	KC291604	
B178	*P. lacustris*	Water	Apple bait	Feb 2011	KC291571	KC291602	
B185	*P. lacustris*	Water	Apple bait	Feb 2011	KC291567	KC291598	
B191	*P. lacustris*	Water	Apple bait	Feb 2011	KC291564	KC291595	
B192	*P. lacustris*	Water	Apple bait	Feb 2011	KC291580	KC291609	
B193	*P. lacustris*	Water	Apple bait	Feb 2011	KC291570	KC291601	
B196	*P. lacustris*	Water	Apple bait	Feb 2011	KC291569	KC291600	
B199	*P. lacustris*	Water	Apple bait	Feb 2011	KC291575	KC291605	
B201	*P. lacustris*	Water	Apple bait	Feb 2011	KC291565	KC291596	

B206	*P. lacustris*	Water	Apple bait	Feb 2011	KC291568	KC291599
B212	*P. lacustris*	Water	Apple bait	Feb 2011	KC291577	KC291606
B215	*P. lacustris*	Water	Apple bait	Feb 2011	KC291576	
B089	*P. lacustris*-PgChlamydo	Water	Apple bait	Dec 2010	KC291551	KC291585
B101	*P. lacustris*-PgChlamydo	Water	Apple bait	Dec 2010	KC291552	KC291586
B106	*P. lacustris*-PgChlamydo	Water	Apple bait	Dec 2010	KC291553	KC291587
B163	*P. lacustris*-PgChlamydo	Water	Apple bait	Feb 2011	KC291554	KC291592
B087	*P.* taxon PgChlamydo	Water	Apple bait	Dec 2010	KC291556	
B107	*P.* taxon PgChlamydo	Water	Apple bait	Dec 2010	KC291557	KC291588
B086	*P.* taxon PgChlamydo	Water	Apple bait	Dec 2010	KC291555	
B115	*P.* taxon PgChlamydo	Water	Apple bait	Dec 2010	KC291558	KC291589
B116	*P.* taxon PgChlamydo	Water	Apple bait	Dec 2010	KC291559	KC291590
B117	*P.* taxon PgChlamydo	Water	Apple bait	Dec 2010	KC291560	KC291591
B164	*P.* taxon walnut	Water	Apple bait	Feb 2011	KC291550	KC291584

Data analysis

Statistical analyses were carried out using STATA11 (Stata Statistical software, College Station, Texas, USA). Values were tested for homogeneity of variances between independent data. In case of homogeneity a two-sided t-test was applied. If the hypothesis of homoscedasticity was violated, a modified t-test for heterogeneous variances was used.

Results

All symptomatic *A. pseudoplatanus* trees gave a 100% positivity to *Phytophthora* infection when tested in the field with the immunodiagnostic kits (Pocket Diagnostics), confirming the reliability of the test.

A total of 206 *Phytophthora* isolates were recovered from the various natural substrates (tree tissue, soil, water). The combined conventional/molecular approach was very successful, since it led to the unequivocal detection and identification of several members of the genus *Phytophthora*: *P. acerina* (Ginetti *et al*., submitted), *P. gonapodyides*, *P. inundata*, *P. lacustris*; two taxa to date only partially described but not formally named: *P.* taxon PgChlamydo and *P.* taxon walnut (both new for Italy); some hybrids between *P. lacustris* and *P.* taxon PgChlamydo (Ginetti *et al*., submitted). A subsample of these microorganisms, selected because of their variation in macro- and micro-morphology in pure culture, substrate of origin (plaut tissue, soil or water), or DNA restriction profiles, was sequence-characterized and is reported in Table 1.

A total of 78 isolates were obtained from the 32 *A. pseudoplatanus* trees sampled. No isolate of *Phytophthora* was obtained from the other broadleaf tree species that were sampled because they exhibited symptoms of decline and grew in the vicinity of *A. pseudoplatanus* trees.

P. acerina was very widespread in the Boscoincittà park, being found on several *A. pseudoplatanus* trees that were quite distant from each other (Fig.1). The oomycete was isolated at a very high frequency from infected *A. pseudoplatanus* tissue (63% of positive isolations from xylem surfaces), confirming its massive occurrence in the study area and its tight association with this tree species. In fact, except for the isolates B080 and B081 of *P. acerina* recovered from the soil (sampled under the canopy of *Acer pseudoplatanus* individuals) and for two other isolates that came from root samples (taken at the base of symptomatic *A. pseudoplatanus* trees), all the rest of isolates from this species were from infected stem tissue. No isolate of *P. acerina* came from water bodies, from which were obtained, on the contrary, all the other *Phytophthora* species (Table 1). *P. gonapodyides* was the only species to be detected, besides than from the water, also from the soil. The rest of taxa were all from the water.

The taxonomic positioning of *P. acerina* is no doubt in the '*Phytophthora citricola* complex'. However, this newly described taxon is also somewhat different from the other members of the complex. It sequences showed high similarity to sequences of some other taxa clustering in the ITS Clade 2 such as *P. citricola*, *Phytophthora plurivora* and *Phytophthora pini*. Phylogenetic analyses demonstrated that *P. acerina* forms a separate cluster within the '*P. citricola* complex' (Ginetti *et al.* submitted).

P. acerina presented several morphological distinguishing features. It was homotallic and presented, after about 4-5 days of growth on V8A, spherical oogonia with smooth surface and paragynous antheridia. The oogonia had a diameter varying from 19.2 – 45.5 μm (average 32.0 ± 4.4 μm). Oospores displayed a high abortion rate (69,6%). Sporangia were persistent, typically borne

terminally on unbranched sporangiophores. In all isolates sporangial shapes showed a wide variation, including ovoid, limoniform, obpyriform, ellipsoid, elongated-ovoid, obovoid, broad-ovoid, mouse-shaped and other distorted shapes. Sporangial dimensions of *P. acerina* averaged 52.0 ± 13 x 32.8 ± 7.7 μm (overall range 20.3 - 105.7 x 11.1 - 51.3 μm) with a the length/breadth (l/b) ratio of 1.6 ± 0.3 μm (overall range 1.2 – 2.6; range of isolate means 1.4 – 2.0). Dense stromata-like hyphal aggregations were another characteristic of this species.

The same morphological and molecular approaches were carried out also for the identification of the rest of the species isolated from the soil and the water (Ginetti *et al*., submitted). Details of the identity, host of origin, substrate, isolation technique, and GenBank accession numbers of the *Phytophthora* species that were investigated in this study are shown in Table 1.

Inoculation test revealed that *P. acerina* was highly virulent to *A. pseudoplatanus*, mildly virulent to *F. sylvatica* and nonpathogenic to the other tree species artificially infected (Fig. 2).

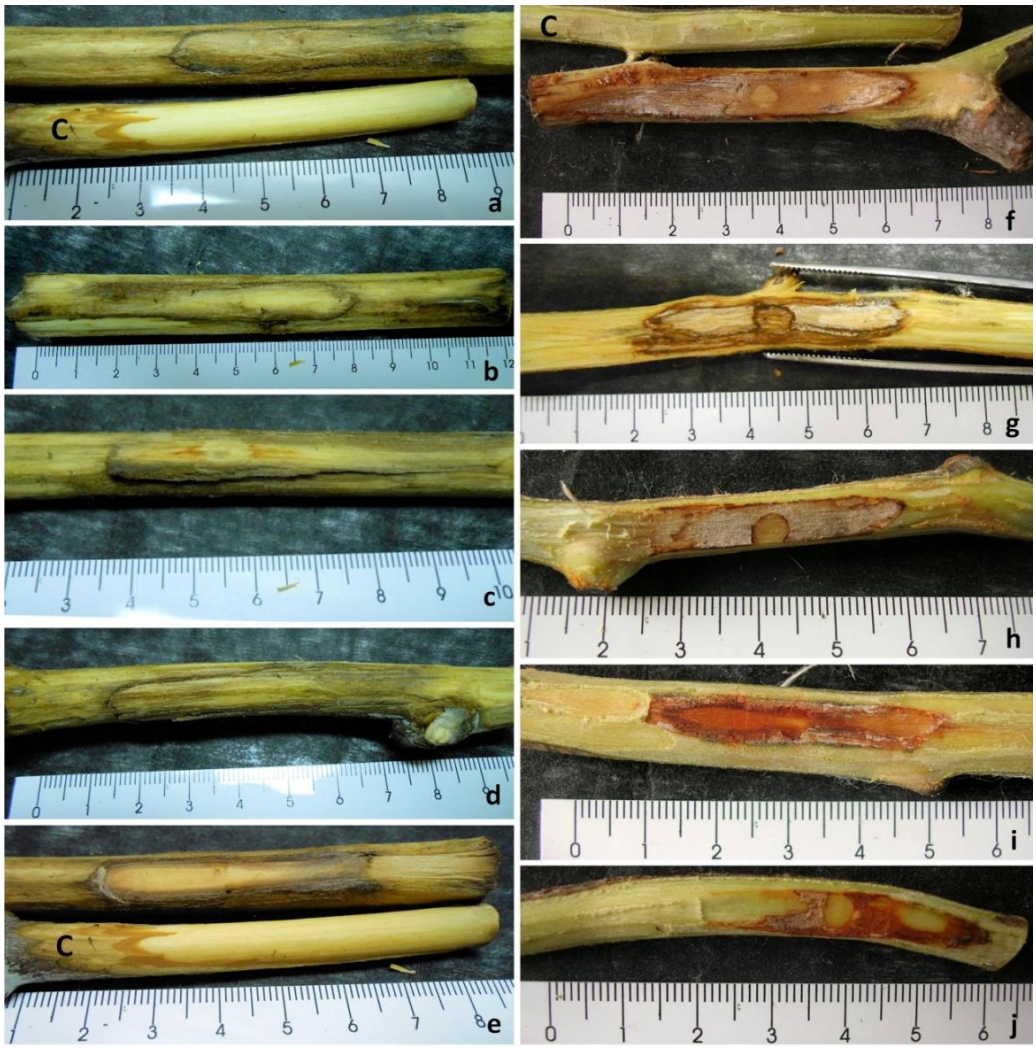

Figure 2 a-j. Lesions caused by *Phytophthora acerina* in infection tests after 21 days: greyish to greenish lesions *Acer pseudoplatanus* twigs (figs. a-e); and orange-brown lesions on *Fagus sylvatica* twigs (figs. f-j). C = Controls.

Average lesion lengths differed significantly among *A. pseudoplatanus* and *F. sylvatica* and between these two species and their respective controls ($P < 0{,}001$). *A. pseudoplatanus* and *F. sylvatica* presented also lesion lengths much larger and significantly different from those of the rest of inoculated tree species and of their controls (Fig. 3). In fact, inoculation trials gave on *Acer pseudoplatanus* and *Fagus sylvatica*, after 3 weeks of incubation at 20 °C in the dark, the following results (data on lesion length induced by the five isolates tested were combined on the basis of statistical analyses and homogeneity of the variances): average lesion lengths on *Acer pseudoplatanus* 9,9 ± 0,3 cm (overall range 6,9 – 11,3); average lesion lengths on *Fagus sylvatica* 4,6 ± 0,22 cm (overall range 3,1 – 7,8). Lesion lengths on the rest of tree species were significantly much lower, with no significant difference among them. A high variation in average lesion sizes turned thus out among some tree species, ranging in length from a few millimeters (2.4 mm) in *Fraxinus excelsior*, the species which resulted the less susceptible, to almost 1 decimeter (9.9 cm) in *A. pseudoplatanus*, the most prone to infection by this oomycete (Fig. 3). Reisolation of *P. acerina* from twig lesions was successful from all the twig specimens from which it was attempted.

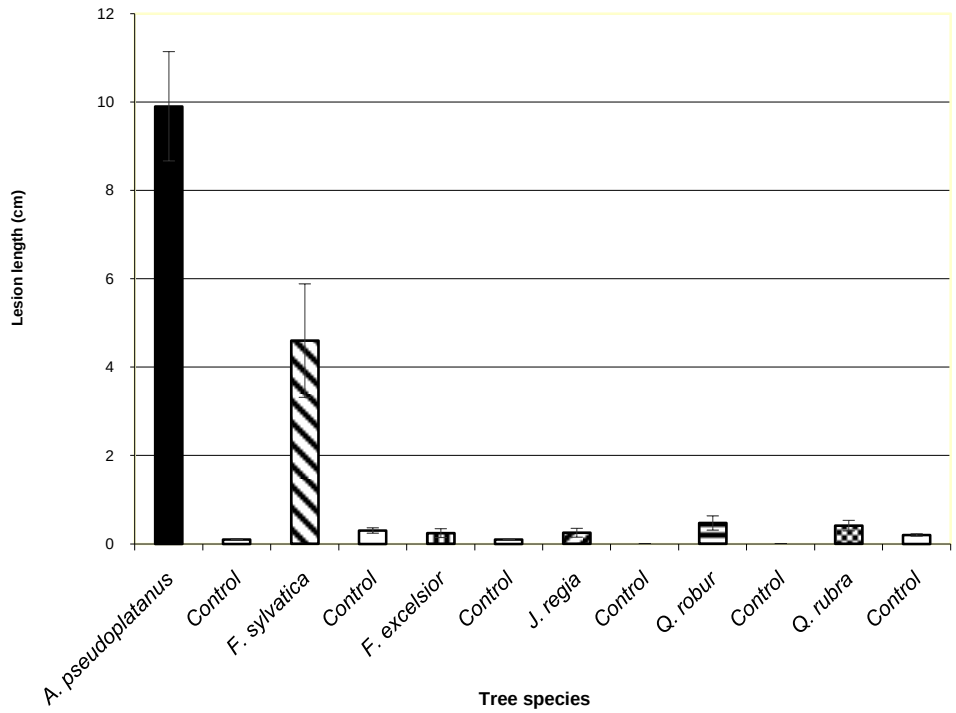

Figure 3 Mean length of underbark lesions induced by *Phytophthora acerina* isolates on twigs of *Acer pseudoplatanus*, *Fagus sylvatica*, *Fraxinus excelsior*, *Juglans regia*, *Quercus robur* and *Q. rubra* at 21 days from inoculation. Bars are standard errors of the mean.

Discussion

A serious dieback of *Acer pseudoplatanus* caused by the newly described species *Phytophthora acerina* in a plantation forest in northern Italy is reported in this paper. Observations carried out in

the investigated stands revealed that the about two-thirds of the *A. pseudoplatanus* trees growing in the Boscoincittà park showed clear symptoms of decline and a number of them was infected by this oomycete pathogen. Trees with symptoms showed wilting branches, craking and loosening of the bark, crown thinning, aggregation of foliage in clusters, epicormic shoots and a generalized crown dieback (Figs. 4a-k). Half of these trees came to death within a year (Figs. 4l-m). If one adds to this the consideration that a fraction of approximately 10% of apparently asymptomatic plants was from time to time added, over a period of approximately 5-6 months (the time lag between the winter and spring-summer samplings), to plants with evident symptoms, it can be concluded that disease incidence and severity was very high. The high rate of positive isolation of the pathogen, on the other hand, confirmed that the inoculum load of the pathogen was massive in the examined area.

Stem symptoms included collar root, production of orange-brown exudates in the basal part of the stem, bleeding cankers, in correspondence of which the bark turned discoloured and necrotic. The phloem beneath bark lesions revealed vast, flame-shaped or irregular necroses, with the underlying cambium and xylem surfaces that exhibited a gradation of discolorations or stains spanning from ochre to faint brown to intense brown, and from pale green to brilliant green. Chromatic alterations changed after exposure to the air, turning to dark brown, green or black. Under bark lesions appeared in various forms, from discoloured areas to strips, pits and island lesions (Fig. 5 a-r).

The high frequency of isolation of *P. acerina* from *A. pseudoplatanus* in the field, and its tight association to infected tissue of this species, provided overwhelming evidence that the oomycete is a primary pathogen of *A. pseudoplatanus* and it can be considered the unique oomycete responsible for the extensive dieback of this tree species in the Boscoincittà park. On the other hand, the other *Phytophthora* species were never isolated from *A. pseudoplatanus*. It can thus be deduced that *A. pseudoplatanus* is a non-host for these microorganisms. On the contrary, the host range of *P. acerina* appeared to be restricted to this tree species. Such assumption is reinforced by the outcome of artificial inoculation tests. The extensive and rapid colonization of *A. pseudoplatanus* by *P. acerina* in artificial inoculation trials, and the reduced (on *Fagus sylvatica*) or neglibible effect of its inoculation on the other tree species, are a proof of the aggressiveness and pathogenic role of the oomycete on *A. pseudoplatanus*, which appeared to be its preferential or exclusive host.

Being *P. acerina* a new pathogen, there are no data available in the literature that can be analyzed in order to disentangle its possible pathways of introduction or to compare its ecology and role across taxa and ecosystems. On the other hand, the origin itself of the Boscoincittà park (arisen on ex-agricultural lands) surrounds the issue in an aura of uncertainty. Portions of the area now covered by the forest were in the past flooded annually for rice cultivation (Paola Pirelli, personal communication). Several other crop species (mainly cereals) were grown in other plots. Volunteer participation of citizens to the establishment of the forest caused a continuous movement of planting material, soil, work tools, and men from one plot to another. All these aspects are knowledge gaps that hamper anamnestic investigations aimed at understanding the possible migrational routes or introduction pathways of the oomycete.

Many *Phytophthora* species have a worldwide distribution and parasitize several host species. The other *Phytophthora* species and isolates found in the present study were not an exception to this rule. *Phytophthora gonapodyides* was retrieved from debris in water in the UK; from soil particles close to *Q. robur* in Germany and to oak species in France; from the soil of dying vegetation in Tasmania, from *Ilex* sp. roots in the UK (Brasier *et al.* 2003) and from *Salix* roots in the UK (Cooke *et*

al. 2000). *P. lacustris* was isolated from root debris of *Alnus* in ponds in Denmark (Brasier *et al.* 2003); from roots and soil of *Alnus glutinosa* in Hungary; from soil of *Phragmites australis* in Germany and from *Prunus roots* in Italy, from sump soil in Australia, from storage water in New Zealand and from river soil in the USA (Nechwatal *et al.* 2012). *P.* taxon walnut was isolated from *Juglans hindsii* in the USA (Brasier *et al*. 2003). *P.* taxon PgChlamydo was detected from *Prunus* roots in the UK (Brasier *et al*. 2003); from soil in native forests in Australia (Burgess *et al*. 2009) and from water in Oregon (Reeser *et al*. 2011). *P. inundata* was isolated from *Vitis* in South America, from roots of *Aesculus* and *Salix* in the UK, from *Olea* roots in Spain and from *Malus* sp. in USA (Brasier *et al*. 2003), from soil of both *Banksia attenuata* and *Xanthorroea preissii* in Australia (Jung *et al*., 2011,) while in Italy it was reported from both young and mature olive trees in Sicily (Cacciola *et al*., 2005). In case of lacking detailed data on ways and rates of pathogen introduction , an examination of ecosystem traits and of the environmental factors in the area under study is fundamental for ascertaining factors triggering the disease outbreak. This assessment can help in predicting future trends and identifying possible management options (Colautti *et al*., 2006). The decline of forest tree species is a phenomenon known for decades. Various members of the genus *Phytophthora* have often been called into question in various contexts as a possible cause. In many cases, these microorganisms were identified as the primary cause of the decline, while in other cases they were numbered among the contributory factors. Environmental constraints, above all drought, especially prolonged drought extended for more growing seasons (in recent times more and more often associated with Global Warming), have often been called into question as factors predisposing the trees to the attack of pathogens. This has long been recognized as the classic paradigm of forest pathology for explaining decline phenomena occurring especially in the Mediterranean climates (Moricca *et al*., 2012). Such explanation, however, cannot be applied to all contexts. The area investigated in the present study is extraordinarily rich in water (Fig. 6). A dense network of ditches and canals irrigates much of its surface, so that even in periods of extended drought, it is unlikely that the plants are to suffer from water shortages. Nevertheless, many *A. pseudoplatanus* trees specifically analyzed in their tissues for possible symptoms of water shortage showed indeed to suffer from water scarcity. The most plausible explanation therefore is that the plants suffered from water deficit not because this element was absent in the ground, but because they were unable to absorb water, having most of the feeder roots been destroyed by the parasitic action of *Phytophthora* sp. (Jung *et al*., 1996). The destruction of the root system was on the other hand verified by direct observation of the state of the roots of *A. pseudoplatanus* trees, inspected at different locations within the area under examination (Fig. 7).

It is also true that the anomalies of the climate of the last decades have destabilized the host-parasite interaction, weakening the plant, impairing its physiology and making it more prone to infection by plant pathogens (Moricca and Ragazzi, 2008). The soil pathogens, such as a number of pathogenic species of *Phytophthora*, are particularly favoured by debilitation of the plant, being able to attack and colonize it with greater ease (Moreira and Martins, 2005).

The climate changes that have occurred in recent decades in the Mediterranean basin have caused an increase of the mean annual temperatures, of the frequency of extreme events, such as excess rainfall in the winter months and of the drought periods during summer time (Giorgi & Lionello, 2008). Under these conditions forest trees undergo severe physiological stress that limit their vigour and predispose them to aggressive and progressive colonization by plant parasites (Brasier, 1996).

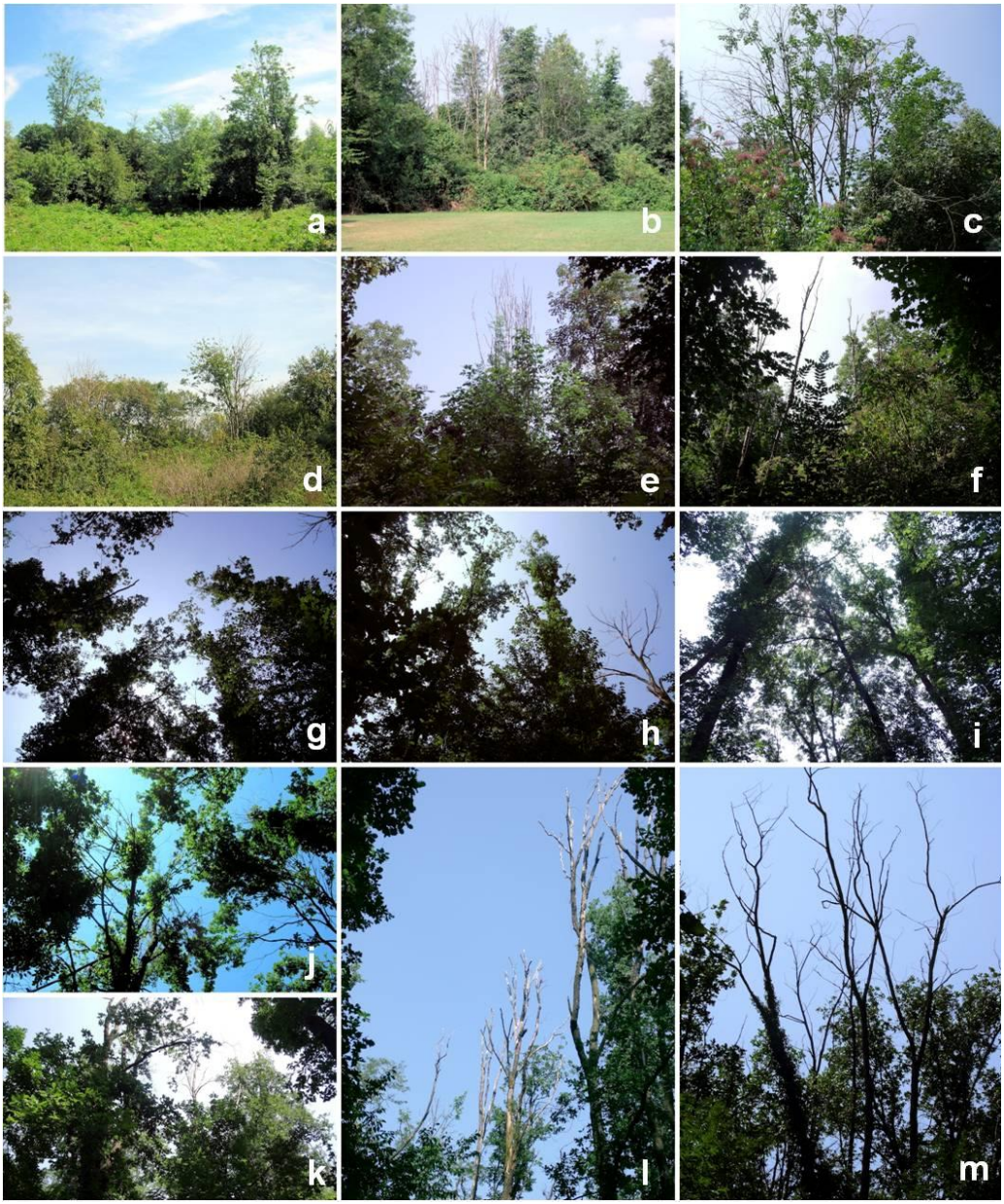

Figure 4 a-m. Extensive dieback of *A. pseudoplatanus* trees in the Boscoincittà park. Trees with evident simptoms of decline (figs. a-f); high transparency of upper crown portions (figs. e-k); sprout and foliage aggregation in clusters (figs. g-k); and severe branch dieback and death of adult *A. pseudoplatanus* trees (figs. l-m).


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Figure 5 a-r. *A. pseudoplatanus* trees showing extensive aerial stem bleeding cankers as a consequence of infection by *Phytophthora acerina*. Phloem cankers and necroses (figs. a-b); bark discoloration and necroses with production of orange-brown tarry exudates on the bark (figs. c-g); xylem staining and bleeding cankers showing color variegation, spanning from faint brown to intense brown, to green-red, to green-dark, to brilliant green (figs. h-r).

On the other hand, it is a known fact that the mild, wet winters, accompanied by relatively high temperatures and rainfall events, promote plant infection by *Phytophthora* zoospores during the winter that induce a progressive destruction of the root system (Jung *et al*., 1996).
This study provides circumstantial evidence that the oomycete *P. acerina* is the agent responsible for the extensive dieback of *A. pseudoplatanus* observed at Boscoincittà park. Further research is necessary to analyse into details some aspects of the epidemiology of the disease, in order to obtain information that may facilitate plant health protocols against this harmful pathogen.

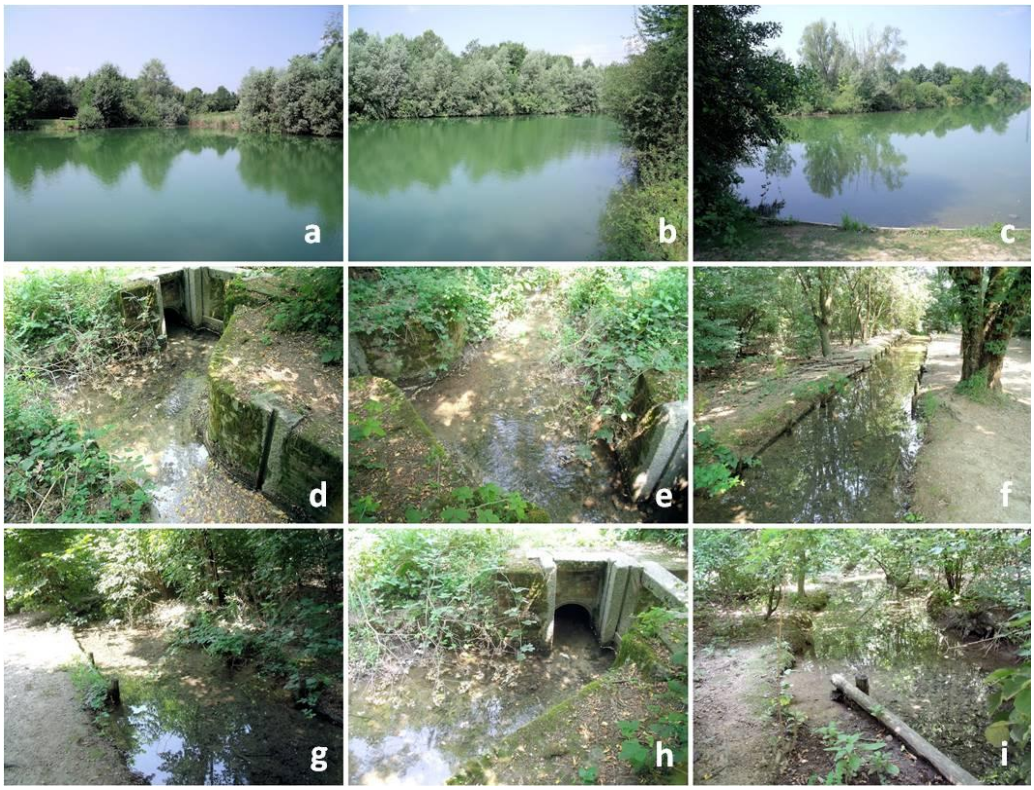

Figure 6 a-i. Lakes, ponds, water bodies (fig a-c), ditches and streams (fig.d-l) forming a network of watercourses within the Boscoincittà park.

Figure 7 a-b. Portions of the root system of a declining, mature *A. pseudoplatanus* tree infected by *P. acerina*, showing necroses of several fine roots (fig. a); the destruction of most of the hairy roots and complete absence of mycorrizhal roots are clearly noticeable (fig. b).

Acknowledgments

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Chapter IV

Multiple *Phytophthora* species from ITS Clade 6 recovered from semi-natural ecosystems and streams

Multiple *Phytophthora* species from ITS Clade 6 recovered from semi-natural ecosystems and streams

B. Ginetti[1], J. Squires[2], D. Cooke[2], A. Ragazzi[1], S. Moricca[1]

[1]University of Florence, Agricultural and Biotechnology Department, Section of Plant Protection, Cascine Square 28 -50144, Florence, Italy.
[2]The James Hutton Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK.

Abstract

Six *Phytophthora* species belonging to the ITS Clade 6 and a possible hybrid species between *P. lacustris* and *P*. taxon Pg Chlamydo, were identified in the Boscoincittà park in the North of Italy and in a plantation near Grosseto (GR) in Tuscany. Isolates were sampled both from water and from soil by baiting with fruits (apples) and subsequent isolation on selective media. Species were identified by RFLPs analysis and sequencing on the nuclear ITS and mitochondrial *cox*1 regions and by morphological characterization. Colony morphology was described on 4 different substrates and growth rates were investigated at several temperatures. The assemblage of species found in the North of Italy was quite similar to the ones found in several other/places in the world (such as North America, Europe and Australia), suggesting an ongoing adaptation of these *Phytophthora* species to a saprophytic lifestyle in forest streams and riparian ecosystems. In Tuscany, just a species from the ITS Clade 6 was detected from soil in a 10 year old *Pinus pinea* plantation. All the species were artificially inoculated on the stem of various tree species to test their pathogenecity and to fulfill Koch postulates. Species previously only partially described but not formally named, taxa new for Italy and Europe, and a presumed hybrid species were found.

Introduction

The genus *Phytophthora* includes aquatic plant pathogens that are notorious threats for agricultural crops, nurseries, plantations and natural and artificial forests. These pathogens are diploid organisms that produce biflagellate zoospores which need free water to be produced and to move into the environment. Many species produce oospores capable of surviving for long periods, giving a chance of survival to the species during adverse times and representing a source of genetic variability for the progeny. Despite the wide knowledge on renowned *Phytophthora* species that represented and still represent a plague on plant species (e.g. *P. infestans*, *P. ramorum* etc.), little is known about a number of *Phytophthora* species which survive in streams and soils. These species are adapted to an almost saprophytic lifestyle but can be potentially pathogenic, representing a risk to vegetation especially during stressful periods. *Phytophthora* species are in fact relatively abundant in the soil of more or less healthy forests, but they are usually poorly characterized and their ecology is essentially unknown (Reeser *et al*., 2011). Cooke *et al*. (2000) subdivided the *Phytophthora* genus into eight main lineages or clades on the basis of the phylogenetic analysis of 50 described *Phytophthora* species. These eight Clades could be shared into two major groups: the first one with Clades 1-5, which mainly comprises *Phytophthora* species with papillate caducous sporangia and provided with mechanisms of aerial dispersal; and the second with Clades (6-8) mostly characterized by non papillate species closely related to soil habitat. Originally Clade 6 included just three species: *P. gonapodyides*, *P. humicola* and P. *megasperma* (Erwin & Ribeiro,

1996), whilst now more than 20 species (most of which not yet formally described) have been included in it. Several *Phytophthora* species belonging to the ITS Clade 6 were found to live as assemblages in riparian ecosystems in Europe (Brasier *et al*., 2003a), in Oregon and Alaska (Reeser *et al*., 2011) and in Australia (Jung *et al*., 2011). Thanks to the advent of molecular tools in the identification of *Phytophthora* species many new not described taxa were identified within this ITS Clade. On the basis of the ITS data, Clade 6 has been divided into three sub-clades; sub-clade I with *P. humicola*, *P. inundata*, *P. rosacearum* and several other undescribed taxa, *P.* taxon walnut included; sub-clade II contains *P. gonapodyides*, *P. megasperma* and the latest described *P. lacustris* (Nechwatal *et al*., 2012); sub-clade III just contains *P.* taxon asparagi. Most of the species belonging to the ITS Clade 6 are usually isolated from riparian and streams ecosystems and generally they don't represent a threat in agriculture and horticulture stands. In any case the ecological function of most of these taxa in natural ecosystems is not clear (Jung *et al*., 2011). Brasier *et al*., (2003 a,b) assumed that several members of this ITS Clade changed to a saprophytic lifestyle, a conjecture supported by their, often dominant, presence in most of the water bodies monitored. Despite this, some of these species can be opportunistic and even aggressive to plants (Brown & Brasier, 2007; Duran *et al*., 2008; Jung, 2009). Indeed *P. gonapodydes* and *P. inundata* sporadically caused root and collar rot and aerial cankers in Europe, especially during very humid periods, on *Fagus sylvatica*, *Quercus robur*, *Alnus glutinosa* (*P. gonapodyides*; Jung *et al*., 1996; Jung & Blaschke 2004; Brown and Brasier 2007) and on *Aesculus, Salix* and olive trees (*P. inundata*; Sanchez-Hernandez *et al*., 2001; Brasier *et al*., 2003b). Furthermore, inoculation trials conducted with *P. lacustris*, *P. gonapodydes* and *P. megasperma* demonstrated their capability of inducing significant fine root damages to flooded *Alnus glutinosa* and *Prunus persica* seedlings; weak to moderate aggressiveness of *P. lacustris* on wounded stems or twigs of *Alnus*, *Prunus* and *Salix* species was established too (Nechwatal *et al*., 2012).

In June and December 2010 and in February 2011, during surveys in the Boscoincittà park in the North of Italy (Milan, 45° 27' N 09° 11' E), isolates of *P.gonapodydes*, *P. inundata*, *P. lacustris*, *P*. taxon Pg Chlamydo, *P*. taxon walnut and a possible hybrid between *P. lacustris* and *P.* taxon Pg Chlamydo were baited with apple fruits from several water bodies in the park. Furthermore, a *P. humicola* isolate was isolated from soil of symptomatic *Pinus pinea*, in July 2010, in a plantation in Tuscany, in central Italy (Grosseto, 42° 39' N, 11° 06' E) (Ginetti *et al*., 2012).
Species were identified by their morphological characters, with observation and description of their reproductive structures (asexual and sexual ones) formed on V8A and on V8A plugs in pond water. The morphology of the colonies was observed on four different substrates (V8A, MEA, CMA, PDA; according to Jung *et al.,* 2011) and the colony growth rates were tested on V8A at different temperatures. Isolates were subjected to molecular investigations such as RFLPs analysis and the sequencing of the ITS rDNA and the mitochondrial *cox*1 regions.
With the aim of better understanding the ecology and the potential pathogenecity of these species, under-bark inoculation trials were made for all the species using one-year-old twigs collected in the field from single mature trees of *Acer pseudoplatanus* and *F. sylvatica*. *P. humicola* was also inoculated on its own host *Pinus pinea*.

Material and methods

Sampling and *Phytophthora* isolation

Soil portions of about 250 g were taken near the canal that crosses the park at a depth of approximately 15 cm. *Phytophthora* isolation from soil samples was performed using a selective bait (apple) as a substrate, one for each soil sample collected in the field. The surface of the fruit was carefully cleaned and disinfected with 95% ethanol. Four holes (1- cm-diam) equidistant from each other (on the four cardinal points) and with a depth of about 2 cm were made on the circumference of each apple fruit with the use of a sterile scalpel. Each cavity was completely filled with soil. Holes were subsequently wet with sterile deionized water, in order to induce the germination of oospores possibly occurring in the soil. Each apple fruit was finally covered with a transparent film in order to both contain the soil and isolate the inoculated fruit from the external environment. Baits were incubated for about 5-7 days at a temperature of 18 °C, then opened under a sterile hood cabin. Portions of fruit at the interface between the diseased area and the healthy tissue were placed on selective medium V8A-PARPNH. The isolates obtained were subsequently transferred and cultured on nutrient medium V8-agar for 3-5 days. Portions of mycelium were finally placed in pond water in order to induce the production of reproductive structures such as sporangia.

In December 2010 four baits (apples) were placed along the entire length of a canal that crosses the Boscoincittà park (Milan). These were first inserted into jute bags, then placed into small cages (to prevent their predation by rodents or other small animals) and then immersed in the water (E1, E2, E3, E4). A new survey was conducted during February 2011, in order to verify the presence of oomycetes in the other lakes that are abundant in the park. The new samplings were carried out in four ponds called: Laghetto, Giardino d'Acqua, Aree Nuove e Chiusa Madre (Fig. 1).

Baits were removed and transported to the laboratory after 7 days. Isolations were promptly carried out according to the protocol described above for soil samples (V8A-PARPNH, FPM, pond water).

DNA isolation and amplification

Phytophthora isolates obtained were subsequently transferred on Potato Dextrose Agar (39 g of PDA and 5 g of agar in 1 liter of deionized water) and cultivated at a temperature of 22 °C for 1 week. The mycelium was collected in sterile 1.5 ml Eppendorf, gently scratching the surface of the colony with the aid of a sterile scalpel and placed in the freezer (-20 °C) for at least 12 hours, up to complete freezing. DNA was extracted following the protocol recommended by the extraction GenElute^{TM} plant Genomic DNA Miniprep Kit (Sigma Aldrich) and stored at -20 °C.

The ITS region (Internal Transcribed Spacer) of the ribosomal DNA was amplified using the primers ITS-6 (5' GAA GGT GAA GTC GTA ACA AGG 3 ') (Cooke *et al*., 2000) and ITS-4 (5 'TCC TCC GCT TAT TGA TAT GC 3') (White *et al*., 1990); the amplification protocol applied is reported in table 1. Amplification occurred by using the following program: step (1) initial denaturing for 3 min at 95 °C; step (2) denaturing for 30 sec at 95 °C; step (3) annealing for 30 sec at 55 °C; step (4) extension for 1 min at 72 °C; step (5) final extension for 5 min at 72 °C. The steps 2-4 were repeated 35 times.

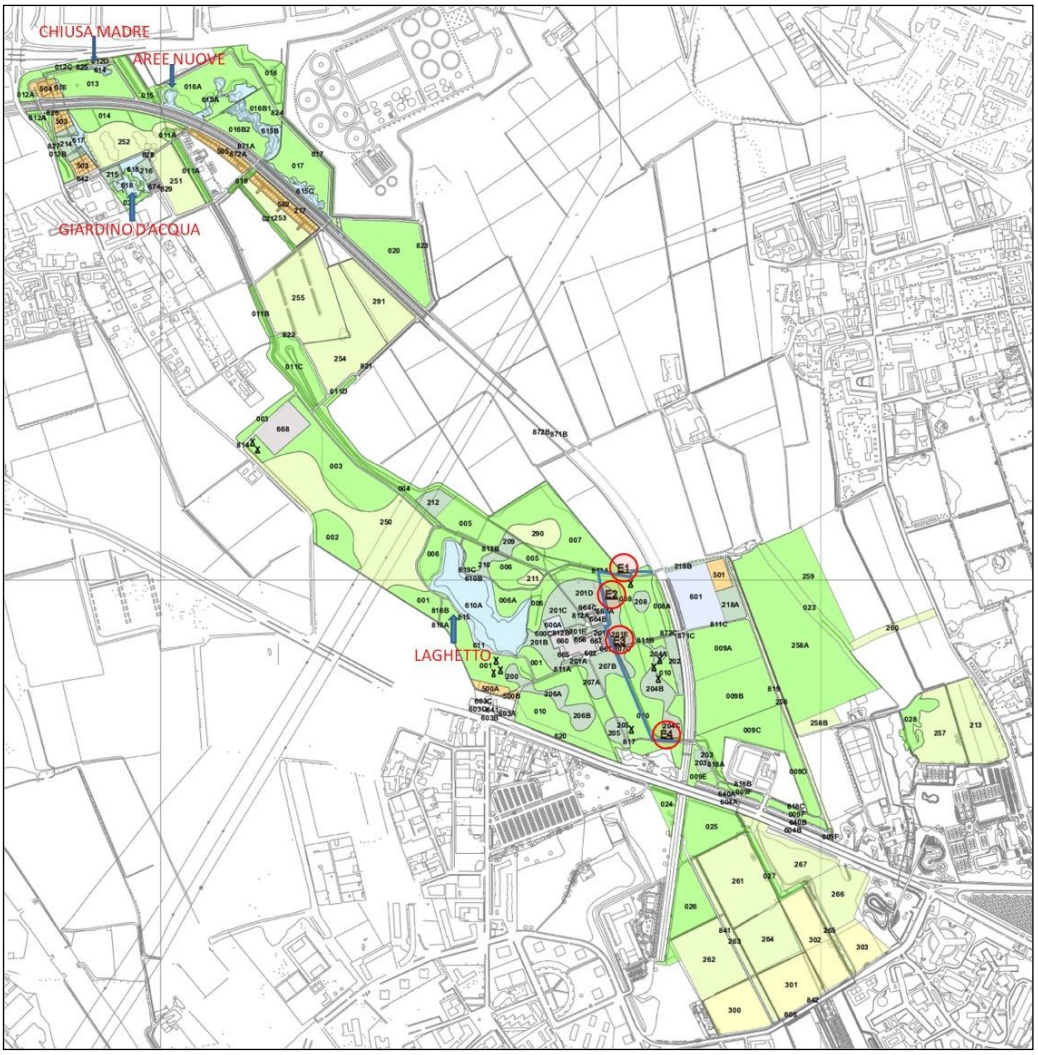

Figure 6 Map of the "Boscoincittà" park showing the various isolation points into water bodies Chiusa Madre, Aree Nuove, Giardino d'Acqua and Laghetto (in red written) and the four isolation points (E1-E2-E3-E4) along the canal (red circles).

The mitochondrial region of the *cox*1 gene was amplified with primers OomCoxILevup (MGA TGG CTT TTT 5'TCA WCW TCA AC 3') and Fm85mod (5 'RRH WAC KTG DAT RAT ACT ACC AAA 3'), as reported by Martin & Tooley (2003). The amplification protocol is shown in table 2. The following amplification program was used: step (1) initial denaturing for 2 min at 95 °C; step (2) denaturing for 1 min at 95 °C; step (3) annealing for 1 min at 55 °C; step (4) extension for 1 min at 72 °C; step (5) final extension for 10 min at 72 °C. The steps 2-4 were repeated 35 times.

Table 1 ITS amplification protocol

	Final concentration	Mix per sample
Sterilized H_2O	_	17,155 μL
Buffer 10x	1x	2,5 μL
$MgCl_2$ 25 mM	2,0 mM	2,0 μL
dNTPs 10 mM	0,25 mM	0,625 μL
Primer forward ITS6 20 μM	0,25 μM	0,31 μL
Primer reverse ITS4 20μM	0,25 μM	0,31μL
Taq DNA polimerasi 5 U/μL	0,02 U/μL	0,1 μL

(FINAL VOLUME 25 μL)

Table 2 *cox*1 amplification protocol

	Final concentration	Mix per sample
Sterilized H_2O	_	23,1 μL
Flexi Buffer 5x	1x	10 μL
dNTPs 10 mM	100 μM	0,5 μL
BSA 10 mg/ml	50 μg/ml	5 μL
$MgCl_2$ 25 mM	5,0 mM	10 μL
Primer forward OomCoxILevup 100 μM	1,0 μM	0,5 μL
Primer reverse Fm85mod 100 μM	1,0 μM	0,5μL
Taq (GoTaq 5 U/μL)	0,04 U/μL	0,4 μL

(FINAL VOLUME 50 μL)

Restriction fragment lenght polymorphysms (RFLPs) analysis and sequencing

ITS amplicons were digested separately with Alu I and Msp I restriction enzymes. Alu I digestion was made with 2.8 μL of sterile H_2O, 1 μL of supplied Buffer, 0.1 μL of BSA, 0.2 μL of restriction enzyme and 5.9 μL of amplicon; samples were finally incubated for 2 h at 37 °C. Msp I digestion was carried out with 1 μL of Buffer, 1 μL of restriction enzyme and 8 μL of PCR product; obtained samples were incubated at 37 °C for 1 h.

*Cox*1 amplicons were subjected to digestion with Rsa I mixing 12.3 μL of sterile H_2O, 2 μL of Buffer, 0.2 μL of BSA, 0.5 μL of restriction enzyme and 5 μL of amplicon; obtained samples were incubated at 37 °C for at least 4 h.

ITS fragments were electrophoresed in a 2% agarose gel for 30 min at 120V with a 100 bp marker; *cox*1 fragments were subjected to electrophoresis in 3% agarose gel for 30 min at 130 V and an additional 15 min at 150 V, with a 100 bp marker.

Digestion bands were analyzed and measured by comparison to the ladder.

Isolates selected for subsequent sequencing were chosen on the basis of: 1) difference in restriction profiles; 2) the morphology of colonies (on V8A cultures); 3)the source material and 4) differential geographic origin of some isolates.

Samples were purified with ExoSAP and subjected to sequencing.

Phylogenetic analysis

Phytophthora isolates obtained in this work were compared with the closely related species (ITS clade 6; Jung *et al*., 2011) and other *Phtyophthora* species representative of other ITS clades as outgroups. Less sequences were available for *cox* 1 region and for this reason the dataset for this region is smaller.

The obtained sequences were inspected, analyzed and corrected with the program Sequencer 4.9 and then aligned with Clustal X2. Manual adjustments were made visually by inserting gaps where necessary in BioEdit Sequence Alignment Editor. The aligned sequences were subsequently processed with the program Topali 2.5 for the construction of phylogenetic Neighbour Joining trees.

Morphology of asexual and sexual structures

Sporangia, hyphal swelling and chlamydospores of selected isolates for species were observed and measured on V8-agar. Sporangia were produced by flooding 10 x 10 mm agar squares taken from the margin of growing 3-5 old day colonies completely submerged into sterilized deionized water in 9 cm Petri dishes after incubated at 20 °C in natural daylight. Sterilized water was replaced with not sterilized filtered pond water after 6 and 24 h. After 24-36 h dimensions and characteristic of 30 mature sporangia and exit pores were determined at x400 magnification (ZEISS, West Germany).

Colony morphology and growth rate at different temperatures

Colony growth patterns were described from 7 day old cultures grown at 20°C in the dark on V8-agar (V8A), malt extract agar (MEA), corn meal agar (CMA) and potato dextrose agar (PDA). Colony morphologies were described according to Erwin and Ribeiro's (1996) terminology.

For investigating temperature-growth relationships, representative isolates were subcultured onto V8-agar plates and incubated for 24h at 20 °C to stimulate the onset of growth (Hall, 1993, Jung *et al*., 2011). Then three replicate plates for each isolate were transferred and incubated at 10, 15, 20, 25, 30, 32, 35, 37 and 39 °C, with the aim of reaching limit values for maximum temperature. Radial growth was recorded daily (every 24h) for 5 days along two lines intersecting the center of the inoculum at angles of 90° and the mean growth rates (mm per day) were calculated.

Under-bark inoculation trials

This test was made using one-year-old twigs (diameter approx. 5-10 mm) collected in the field from single mature trees of *Acer pseudoplatanus* and *Fagus sylvatica* in March and May, shortly after bud burst; leaves were removed and the twigs were cut into lenghts of about 12 cm

A bark portion was removed aseptically with a razor blade and a V8-agar disc (0.5 cm diameter), taken from the margin of freshly growing cultures of selected *Phytophthora* species, was placed on the wound. The inoculum was covered by the removed bark portion and autoclaved wet cotton and sealed with a tape (parafilm) and aluminium foil. Controls received only sterile V8A discs. Ten twigs were inoculated per isolate or control. The ten twigs per isolate were placed in autoclaved glass Petri dishes containing two layers of moist filter paper. Plates were sealed with parafilm and incubated for 3 weeks at 20 °C in the dark. Lesion lengths were assessed 21 days after removing the outer bark. Random reisolations were made using selective V8-PARPNH-agar to confirm *Phytophthora* as the causal agent of the lesions.

Statistical analysis

Statistical analyses were carried out using STATA11 (Stata Statistical software, College Station, Texas, USA) to determine if differences between analyzed Phytophthoras were statistically significant. Regarding sporangia comparisons, due to the number of at least 30 measurements for every isolate, data showed a normal distribution, allowing the use of parametric tests. Data were tested for homogeneity of variances between independent data. In case of homogeneity a two-sided t-test was applied. If the hypothesis of homoskedasticity was violated, a modified t-test for heterogeneous variances was used. Lesions measurements resulted from the inoculation trials (about 10 measurements per isolates) were on the contrary processed with the rank sum test (a non parametric test for independent data).

Results

Sampling and *Phytophthora* isolation

The isolates obtained were 119; 117 from water bodies and 2 from soil. One isolate of *P. humicola* was isolated from soil of infected *Pinus pinea* in a plantation in Tuscany (Grosseto). One isolate of *P. gonapodyides* was isolated from soil near a symptomatic *Acer pseudoplatanus* in the Boscoincittà park, Milan, while another one was baited from the canal that crosses the park at the site E2. Two isolates of *P. inundata* were obtained from the water bodies Chiusa Madre and Giardino d'acqua and one isolate of *P.* taxon walnut was isolated from the water at Chiusa Madre. *P. lacustris* was isolated from all the investigated water bodies, in particular: five isolates from E1; seven isolates each from E2 and E3; 11 isolates each from E4 and from Chiusa Madre; 16 isolates each from Aree Nuove, Giardino d'acqua and Laghetto; in total 89 isolates of this recently described species were obtained. *P.* taxon PgChlamydo was obtained from water at four different sites; one isolate each from E1 and E4; four isolates from E2 and two isolates from Chiusa Madre. Sixteen species which seemed to be possible hybrids between *P. lacustris* and *P.* taxon PgChlamydo were isolated from all water bodies except from Laghetto, precisely: one isolate each from E1, E4 and Giardino d'acqua; two isolates each from E3 and Chiusa Madre; five isolates from E2 and finally four isolates from Aree Nuove. The number of isolates per species that were isolated from the various water bodies are summarized in Table 3.

Table 3 Number of isolates of *Phytophthora* species recovered from water bodies in the Boscoincittà park (Milan).

Isolation site	**E1**	**E2**	**E3**	**E4**	**Aree nuove**	**Chiusa Madre**	**Giardino d'acqua**	**Laghetto**
***Phytophthora* species**								
P. gonapodyides	-	1	-	-	-	-	-	-
P. inundata	-	-	-	-	-	1	1	-
***P.* taxon walnut**	-	-	-	-	-	1		-
P. lacustris	5	7	7	11	16	11	16	16
***P.* taxon PgChlamydo**	1	5	-	1	-	2	-	-
Hybrids	1	4	2	1	4	2	1	-

Phylogenetic analysis

Identity, host, location, isolation information and GenBank accession numbers for Clade 6 *Phytophthora* isolates used in this study are shown in Table 4.

For the sequencing of the ITS region and *cox*1 gene respectively 37 and 32 *Phytophthora* isolates were selected, by considering 1) all the different profiles on the gel obtained by digestion with restriction enzymes; 2) the morphology of the colonies on V8-agar cultures; 3) the source of the isolates ; and 4) the geographic origin.

Phylogenetic analyses results are shown in Neighbor-Joining tree calculated with 500 bootstrap runs (Fig 2-5). Excluding outgroups, the aligned dataset for ITS (82 sequences) and *cox*1 (68 sequences) consisted of 838 and 701 characters, respectively. The number of phylogenetic informative sites of the nucleotide alignment data was of 173 (20,64%) characters for the ITS and 529 (75,46%) nucleotides for the *cox*1.

Regarding the ITS sequences, isolate B033 clustered with *P. humicola* (AF541902) while isolates B155 and B189 clustered with *P. inundata* (AF541912). Isolate B164 resulted belonging to the *P.* taxon walnut group with two isolates of the same species (AF541910 and DQ512952). Twenty-four isolates (B089, B101, B106, B126, B135, B137, B138, B140, B143, B146, B147, B153, B175, B178, B185, B191, B192, B193, B196, B199, B201, B206, B212, and B215) grouped with several *P. lacustris* (JF907579, AF541909, AF266793, HQ01956, AY762973, HM004219). Isolates B086, B087, B107, B115, B116, B117 and B163 resulted grouped with two isolates of *P.* taxon PgChlamydo (AF541900 and AF541902). The two isolates B079 and B098 clustered with several *P. gonapodyides* (AF541892, AF541890, JF912516, GU993893, AF541888 and JF912517) (Fig. 2). Phylogenetic relationships obtained with the cox1 gene sequence analysis confirmed the results above produced with the rDNA ITS, with the exception of four isolates: B089, B101, B106 and B163 (Fig. 3). Indeed, the first three isolates (B089, B101 and B106) clustered with all the *P. lacustris* isolates on the basis of the ITS, but seemed to be more closely related to the *P.* taxon PgChlamydo, together with three other isolates deposited as *Phytophthora* sp. in GenBank (EF468468, EF468470, EF468469). On the contrary, the fourth isolate (B163) presented the ITS sequence closely related to the *P.* taxon PgChlamydo group, while its cox1 sequence clustered with all the other *P. lacustris* (Fig. 4-5).

Table 4 Identity, host, location, isolation information and GenBank accession numbers for Clade 6 Phytophthora isolates used in this study.

Reference collection no. [1]	Other collection no.	Identity	Substrate	Host	Location	Isolated by	Date	GenBank Accession No.	
								ITS	cox1
VHS173150		*P. fluvialis*	Water	nd	Australia, WA, Badgingarra	nd	nd	EU593261	JF01440
VHS21998 CBS127951		*P. gibbosa*	Soil	*Acacia pycnantha*	Australia, WA, Scott River	VHS	2009	HQ012933	HQ012846
	B079	*P. gonapodyides*	Soil	Apple bait	Milan, IT	B Ginetti	2010	KC291561	KC291593
	B098	*P. gonapodyides*	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291562	
IMI389727	P897	*P. gonapodyides*	Soil	Native Forest	Australia, TAS, Pine Lake	K Shanahan	1996	AF541888	
CBS544.67	P149	*P. gonapodyides*	Lake water	nd	Blelham Tarn, Cumberland, UK	MW Dick	nd	AF541892	
IMI389729	P501	*P. gonapodyides*	Roots	*Ilex* sp.	Alice Holt, Surrey, UK	T Reffold & CM Brasier	1985	AF541890	
	H-14/02	*P. gonapodyides*	Roots	*Alnus glutinosa*	Ócsa, HU	JB	2002	JF912516	JF742604
	UKN-BuKN 1b	*P. gonapodyides*	Soil	*Fagus sylvatica*	Konstanz, DE	JN	2003	JF912517	JF742605
	NY393	*P. gonapodyides*	nd	*Malus sylvestris*	USA, New york	nd	nd		AY129175
MUCC761		*P. gonapodyides*	Water	*Eucalyptus obliqua* forest	Australia, VIC, Toolangi North	WA Dunstan	2008		HQ012850
IMI345174		*P. gonapodyides*	nd	nd	UK	nd	2010	GU993893	
IMI389769	P515	*P. gonapodyides*	Water	Debris	Cheshire, UK	E Perrot	nd	AF541891	
VHS21962 CBS127952		*P. gregata*	Soil	*Patersonia* sp.	Australia, WA, Busselton	VHS	2009	HQ012942	HQ012858
VHS21992		*P. gregata*	Soil	Native Forest	Australia, WA, Scott River	VHS	2009		HQ012859
IMI389745	P1049	*P. gregata*	Roots	Raspberry	Victoria, Australia	G McGregor	1996	AF541904	
MUCC760	SPLA107	*P. gregata*	Soil	Pasture	Australia, VIC, Devlins Bridge	WA Dunstan	2008		HQ012855
CBS129249	B033	*P. humicola*	Soil	*Pinus pinea*	Tuscany, IT	B Ginetti	2010	JQ757060	

IMI389748	P856	*P. humicola*	Soil	*Citrus* sp.	Changhua, Taiwan	P Ann & WH Ko	1986	AF266792	
	B155	*P. inundata*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC201295	KC238311
	B189	*P. inundata*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC201296	KC238312
IMI389750	P210	*P. inundata*	Roots	*Aesculus hippocastanum*	Buckinghamshire, Claydon, UK	CM Brasier	1970	AF541912	
VHS16836		*P. inundata*	Soil	*Xanthorrhoea preissii*	Australia, WA, Boyup Brook	VHS	2007		HQ012860
VHS19081		*P. inundata*	Soil	*Banksia attenuata*	Australia, WA, Bold park	VHS	2008		HQ012861
SCPR989	P14	*P. lacustris*	River soil	nd	Allegan County, Michigan, USA	D Fulbright	2000	JF07579	JF896563
	B126	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291582	KC291611
	B135	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291573	
	B137	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291566	KC291597
	B138	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291563	KC291594
	B140	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291578	KC291607
	B143	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291572	KC291603
	B146	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291579	KC291608
	B147	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291581	KC291610
	B153	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291583	
	B175	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291574	KC291604
	B178	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291571	KC291602
	B185	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291567	KC291598
	B191	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291564	KC291595
	B192	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291580	KC291609
	B193	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291570	KC291601
	B196	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291569	KC291600
	B199	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291575	KC291605
	B201	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291565	KC291596
	B206	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291568	KC291599
	B212	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291577	KC291606
	B215	*P. lacustris*	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291576	

	HSA 1959	*P. lacustris*	Sump soil	nd	Australia, Welshpool	R Hart	1994	HQ012956	HQ012880
	UKN-Ph1	*P. lacustris*	Soil	*Phragmites australis*	Konstanz, DE	JN	2003	AY762973	
IMI389725, WPC-P10337	P245	*P. lacustris*	Roots	*Salix matsudana*	Bexley Heath UK	CB	1972	AF266793	
	P878	*P. lacustris*	Debris	*Alnus* sp.	Odense DK	K Thinggaard	1995	AF541909	
	WA21-091603	*P. lacustris*	stream bait	nd	Curry County, Oregon, USA	nd	nd	HM004219	
	PESCO RC	*P. lacustris*	Roots	*Prunus* hybrid	Calabria IT	SC	1999		JF896562
	B089	*P. lacustris*-PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291551	KC291585
	B101	*P. lacustris*-PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291552	KC291586
	B106	*P. lacustris*-PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291553	KC291587
	B163	*P. lacustris*-PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291554	KC291592
VHS20763 CBS127953		*P. litoralis*	Soil	*Banksia* sp.	Australia, WA, ravensthorpe	VHS	2008	HQ012948	HQ012866
MUCC763		*P. litoralis*	Water	Stream baiting	Australia, WA, Borden	D Hüberli	2008		HQ012863
IMI13317		*P. megasperma*	nd	nd	nd	nd	nd	AF266794	
ATCC28765	P250	*P. megasperma*	Soil	Around *Populus* sp.	Norfolk, UK	CM Brasier & RG Strouts	1984	AF541895	
IMI389738	P1058	*P. megasperma*	Water	nd	Amance Forest, Lorraine, FR	EM Hansen & C Delatour	1998	AF541894	
	P1	*P. megasperma*	baited soil	Fraser fir	Missaukee County, MI, USA	nd	nd	AY995339	

DDS3599		*P. megasperma*	nd	*Xanthorrhoea platyphylla*	Australia, WA, Fitzgerald River NP	nd	nd	EU593258	
DDS3432		*P. megasperma*	Soil	*Banksia* sp.	Australia, WA, North Dinninup	VHS	1992		HQ012867
VHS17183		*P. megasperma*	Soil	*X. platyphylla*	Australia, WA, Esperance	VHS	2007		HQ012868
	695T	*P. megasperma*	nd	nd	nd	nd	nd		L04457.1
CMW26667		*P. pinifolia*	Needles	*Pinus radiata*	Chile, Arauco, Llico plantation	A Durán	2007	EU725805	
CMW26668		*P. pinifolia*	Needles	*Pinus radiata*	Chile, Arauco, Llico plantation	A Durán	2007	EU725806	
IMI389749	P462	*P. rosacearum*	nd	*Malus* sp.	California, Sonoma County, USA	SM Mirchetich	1979	AF541911	
	UQ2141	*P.* taxon asparagi	nd	*Asparagus* sp.	nd	nd	nd	AF266795	
VHS17175		*P.* taxon asparagi	Soil	*Banksia media*	Australia, WA, Esperance	VHS	2007	EU301167	
IMI389747	P1054	*P.* taxon forestsoil	Soil	Native Forest	Alsace, Illwald forest, FR	EM Hansen	1998	AF541908	
MUCC768	DH02	*P.* taxon humicola-like	Water	Stream baiting	Australia, WA, Esperance	D Hüberli	2008		HQ012883
MUCC769	DH03	*P.* taxon humicola-like	Water	Stream baiting	Australia, WA, Esperance	D Hüberli	2008		HQ012884
	UASWS0321	*P.* taxon hungarica	Soil	*Alnus glutinosa*	Poland, Adamowizna	L Belbahri	2006	EF522144	
MUCC770		*P.* taxon kwongan	Soil	*Hibbertia* sp.	Australia, Cooljarloo WA	WA Dunstan	2008	HQ012953	HQ012876
IMI389733	P1055	*P.* taxon oaksoil	Soil	*Quercus robur*	Alsace, Illwald forest FR	EM Hansen	1998	AF541906	
MUCC765	SLPA166	*P.* taxon paludosa	Water	Pond baiting	Australia, VIC, Sugarloaf Reservoir Reserve	WA Dunstan	2008	HQ012953	HQ012876
VHS14801		*P.* taxon personii	Soil	*Grevillea mccutcheonii*	Australia, WA, busselton	VHS	2005	EU301169	HQ012877

	B087	*P.* taxon PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291556	
	B107	*P.* taxon PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291557	KC291588
	B086	*P.* taxon PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291555	
	B115	*P.* taxon PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291558	KC291589
	B116	*P.* taxon PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291559	KC291590
	B117	*P.* taxon PgChlamydo	Water	Apple bait	Milan, IT	B Ginetti	2010	KC291560	KC291591
IMI389730	P236	*P.* taxon PgChlamydo	Roots	*Prunus* sp.	Cheltenham, UK	CM Brasier	1982	AF541900	
IMI389731	P510	*P.* taxon PgChlamydo	Roots	*Pseudotsuga* sp.	Walley, Vancouver, British Columbia	PB Hamm & EM Hansen	1984	AF541902	
DDS3753		*P.* taxon PgChlamydo	Soil	Native Forest	Australia, WA, Manjimup	VHS	1995		HQ012878
		P. taxon PgChlamydo	Soil	Native Forest	Australia, WA, Manjimup	VHS	1999		HQ012879
IMI389746	P1050	*P.* taxon raspberry	Roots	*Rubus idaeus*	Sweden, Scania	CHB Olsson	1994	AF541905	
	RAS1	*P.* taxon raspberry	Soil	*Betula pendula*	Germany, Bavaria, Neuburg	T Jung	2006		HQ012888
	P1044	*P.* taxon riversoil	Soil	Riparian vegetation	Worcestershire, Riverbank UK	J Delcan	1997	AF541907	
HSA2530		*P.* taxon rosacearum-like	Water	Baiting	Australia, WA Cooljarloo	R Hart	1998	HQ012963	HQ012887
DDS2909		*P.* taxon rosacearum-like	Soil	*Pinus radiata*	Australia, Albany WA	MJC Stukely	1989		HQ012882
	B164	*P.* taxon walnut	Water	Apple bait	Milan, IT	B Ginetti	2011	KC291550	KC291584
IMI389735	P532	*P.* taxon walnut	nd	*Juglans hindsii*	USA, California, Merced County	SM Mircetich	1988	AF541910	

	P532	*P.* taxon walnut	nd	*Prunus avium*	Switzerland	nd	nd	DQ512952	
VHS13530 CBS127954		*P. thermophila*	Soil	*Eucalyptus marginata*	Australia, WA, Dwellingup	VHS	2004	EU301155	HQ012872
VHS7474		*P. thermophila*	Soil	Native Forest	Australia, WA, Manjimup	VHS	2000		HQ012871
	H-6/02	*Phytophthora* sp.	nd	nd	Hungary	nd	nd		EF468468
	H-7/02	*Phytophthora* sp.	nd	nd	Hungary	nd	nd		EF468469
	H-7/02	*Phytophthora* sp.	nd	nd	Hungary	nd	nd		EF468470
	P10337	*Phytophthora* sp.	nd	nd	United Kingdom	nd	1972		HQ261455
CBS111346		*Phytophthora* sp. BOLD:AAO6255	nd	nd	South Korea, Suwon	nd	nd		HQ708402

[1] Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Uthecht, Netherlands; IMI = CABI Bioscience (International Mycological Institute), UK; VHS = Vegetation Health Service Collection, Department of environment and Conservation, Perth, Australia; DDS = earlier prefix of VHS collection; HAS = Hart, Simpson and Associates, in VHS collection; MUCC = Murdoch University Culture Collection; ATCC = American Type Culture Collection; SCRP = Scottish Crop Research Institute, UK; UKN = University of Kostanz, Germany; WPC = World *Phytophthora* Collection, USA. Nd = date unknown.

Figure 7 Neighbor-Joining tree based on rDNA ITS sequences showing phylogenetic relationships within *Phytophthora* ITS Clade 6. Numbers in blue represent bootstrap support for the nodes.

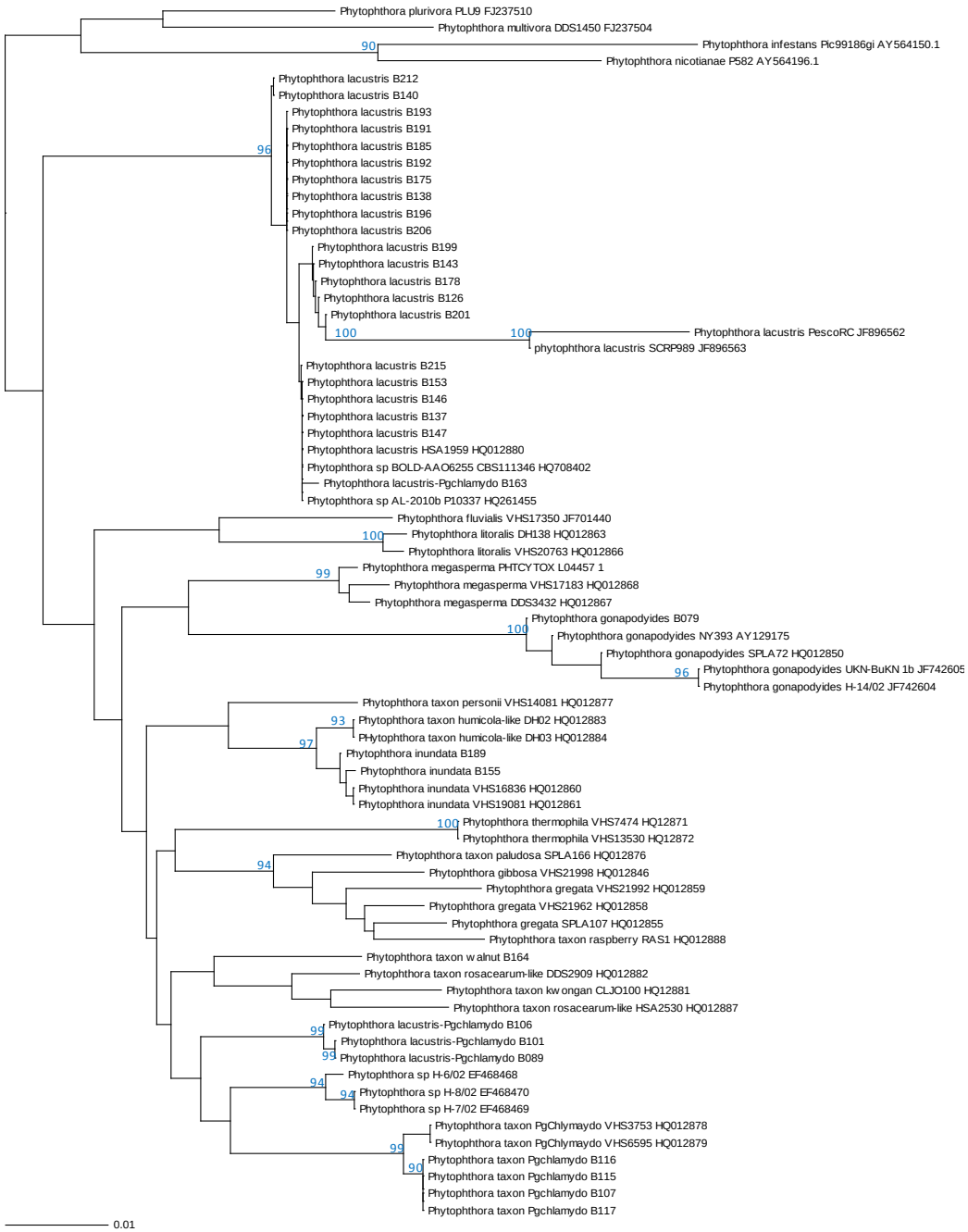

Figure 8 Neighbor-Joining tree based on mitochondrial gene *cox*1 sequencing showing phylogenetic relationships within *Phytophthora* ITS Clade 6. Numbers in blue represent bootstrap support for the nodes.

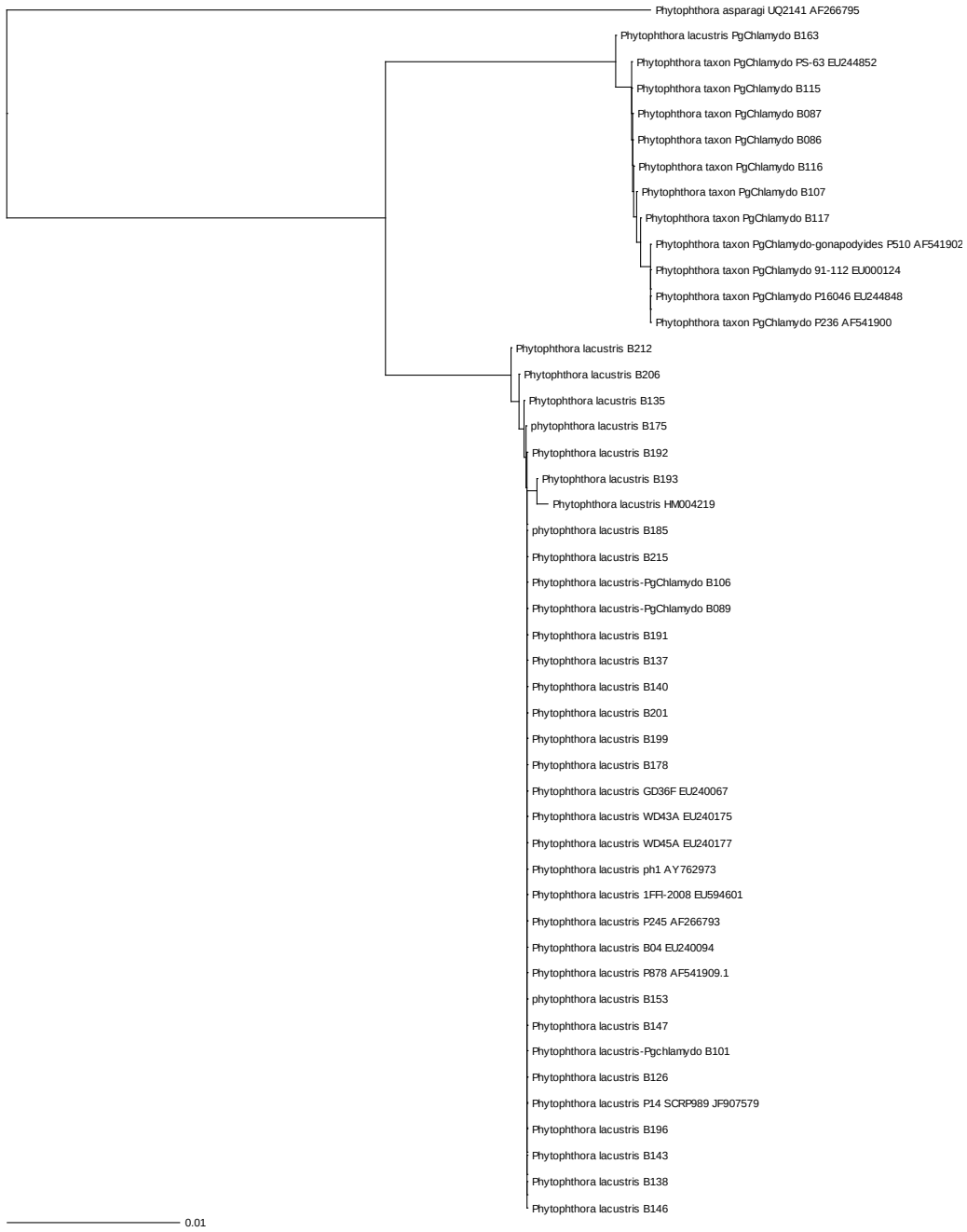

Figure 9 Neighbor-Joining tree based on rDNA ITS sequences showing phylogenetic relationships focusing on *P. lacustris, P.* taxon PgChlamydo and the possible hybrids between these two species (*Phytophthora lacustris*-PgChlamydo B089, B101, B106 and B163).

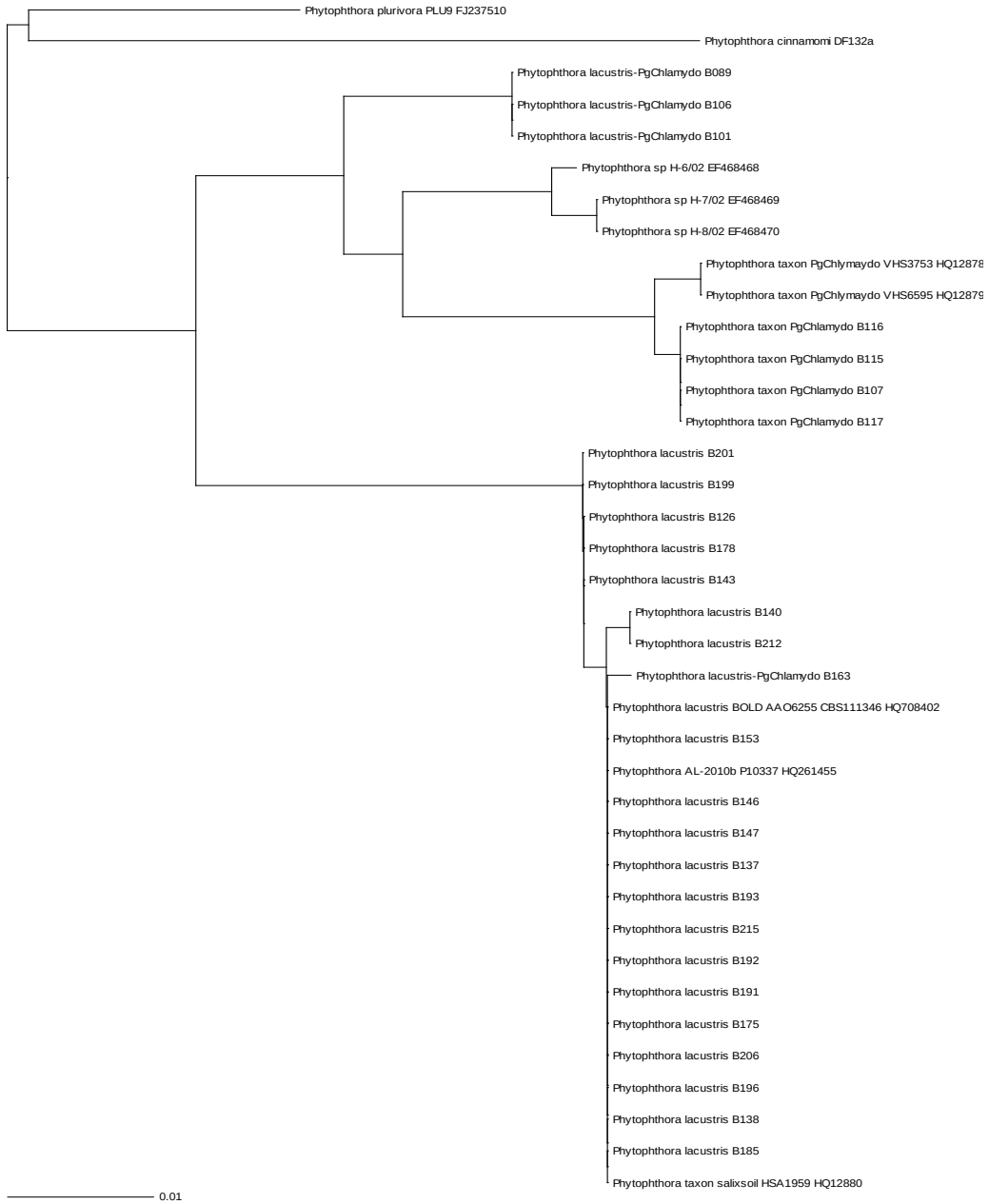

Figure 10 Neighbor-Joining tree based on mitochondrial gene *cox*1 sequencing showing phylogenetic relationships focusing on *P. lacustris, P.* taxon PgChlamydo and the possible hybrids between these two species (*Phytophthora lacustris*-PgChlamydo B089, B101, B106 and B163).

Colony morphology, growth rates and cardinal temperature

The morphology of the colony of selected isolates per each species is shown in Figure 6, 7 and 8. Colonies were photographed after 7 days of growth at 20 °C on V8-agar, malt extract agar (MEA), corn meal agar (CMA) and potato-dextrose agar (PDA).

In *P. humicola* (B033), hyphae radiated uniformly and were medium-fluffy on both V8-agar and MEA; the colony pattern was uniform and not fluffy on CMA and it grew showing broadly rounded sectors (faintly petal-like) densely fluffy on PDA (Fig. 6, 1st row).

P. inundata (B155, B189) presented a uniform colony pattern varying from moderate fluffy to scanty-fluffy on V8-agar; colonies were faintly petaloid or stellate and moderately cottony on MEA; they were uniform and not fluffy on CMA and showed faintly stellate and densely fluffy on PDA (Fig. 6, 2nd and 3rd rows).

In *P. gonapodyides* (B079, B098) hyphae grew faintly stellate or stellate and scanty-fluffy on V8-agar; they were faintly petaloid and scanty-fluffy on MEA; they were uniform and not fluffy on CMA and presented a fluffy rosaceous growth on PDA (Fig. 6, 4th and 5th rows).

P. taxon walnut (B164) showed a uniform to faintly stellate scanty-fluffy colony pattern on both V8-agar and MEA; colony was uniform and not cottony on CMA while it was faintly chrysanthemum and scanty-fluffy on PDA (Fig. 6, 6th row).

P. taxon PgChlamydo (B107, B115) presented a uniform to faintly stellate not fluffy colony pattern on V8-agar; a scanty-fluffy faintly petaloid pattern on MEA; colonies were uniform and not cottony on CMA and they were stellate and scanty-fluffy on PDA (Fig. 7, 1st and 2nd rows).

P. lacustris (B137, B191, B215) showed a chrysanthemum to stellate scanty-fluffy colony pattern on both V8-agar and MEA; colonies were uniform and not cottony on CMA and they were rosaceous to stellate and medium-densely fluffy on PDA (Fig. 7, 3rd, 4th and 5th rows).

Regarding the hybrid isolates between *P. lacustris* and *P.* taxon PgChlamydo, on V8-agar isolates B089, B101 and B106 presented a faintly stellate and scanty cottony pattern while isolates B121 and B163 were uniform to faintly stellate and not cottony (Fig. 8,1st column); on MEA all hybrid isolates showed a rosaceous-faintly stellate scanty-fluffy pattern (Fig. 8, 2nd column); on CMA isolates B089, B101, B106 and B163 showed a uniform and not cottony colony pattern while isolate B121 grew with a faintly petaloid and not cottony mycelium (Fig. 8, 3rd column); on PDA isolates B089, B101 and B106 were petaloid with a medium-densely fluffy mycelium, isolate B121 presented a petaloid-faintly stellate and medium-densely fluffy pattern while isolate B163 was chrysanthemum-petaloid and medium-densely fluffy (Fig. 8, 4th column).

Selected isolates per each species were chosen for investigating their radial growth rate at different temperatures (10, 15, 20, 25, 30, 32, 35, 39 °C) on V8-agar (Fig. 9). *P. humicola* isolate (B033) presented the optimum temperature for growth at 30 °C with radial growth rates ranging from 8.0 – 8.2 mm d^{-1} while the maximum growth temperature was 32 °C. This isolate was not able to grow at 35 °C and did not start re-growth when plates, previously incubated for 5 d at 35 °C, were transferred to 18-20 °C. Two isolates of *P. inundata* (B155, B189) showed the optimum temperature for growth at 30 °C with radial growth rates of 7.1 ± 0.14 mm d^{-1}. The maximum growth temperature was 35 °C. These isolates weren't able to grow at 37 °C but one of the two isolates (B155) started re-growth when plates, previously incubated for 5 d at 37 °C, were transferred to 18-20. B155 wasn't able to grow again, after 5 d at 39 °C, when incubated at room temperature again. Two isolates of *P. gonapodyides* (B079, B098) presented the optimum (3.9 ± 0.15 mm d^{-1}) and the maximum temperature at respectively 25 and 32 °C. Isolates weren't able to grow at 35 °C but the

isolate B079 started re- growth when transferred to 18-20. B079 isolate wasn't able to grow again, after 5 d at 37 °C, when incubated at room temperature again. *P.* taxon walnut (B164) presented the optimum temperature for growth at 30-32 °C with radial growth rates ranging from 5.6 – 5.8 mm d^{-1} while the maximum growth temperature was 37 °C. This isolate was not able to grow at 39 °C and did not start re-growth when plates were transferred to room temperature. Three isolates of *P. lacustris* (B137, B191, B215) showed the optimum temperature for growth at 30 °C (5.9 ± 0.2 mm d^{-1}) and the maximum at 35 °C; isolates weren't able to grow at 37 °C and did not start re-growth when plates where transferred to room temperature. Two isolates of *P.* taxon PgChlamydo (B107, B115) presented the optimum temperature at 30 °C (4.4 ± 0.1 mm d^{-1}) and the maximum at 32 °C; isolates weren't able to growth at 35 °C and did not start re-growth when plates where transferred to room temperature. Four hybrid isolates (B089, B106, B101, B121) had the optimum temperature at 30 °C (5.0 ± 0.14 mm d^{-1}) and the maximum at 35 °C; isolates weren't able to grow at 37 °C and did not start re-growth when plates where transferred to room temperature. Hybrid isolate B163 showed the optimum temperature at 30 °C (5.3 ± 0.15) and the maximum at 35 °C; this isolate wasn't able to grow at 37 °C and did not start re-growth when transferred to room temperature.

Figure 11 Colony morphology (from top to bottom) of *P. humicola* (1st row), *P. inundata* (2nd and 3rd rows), *P. gonapodyides* (4th and 5th rows) and *P.* taxon walnut (6th row) after 7 d growth at 20 °C on (from left to right) V8 agar, malt extract agar, corn meal agar and potato-dextrose agar.

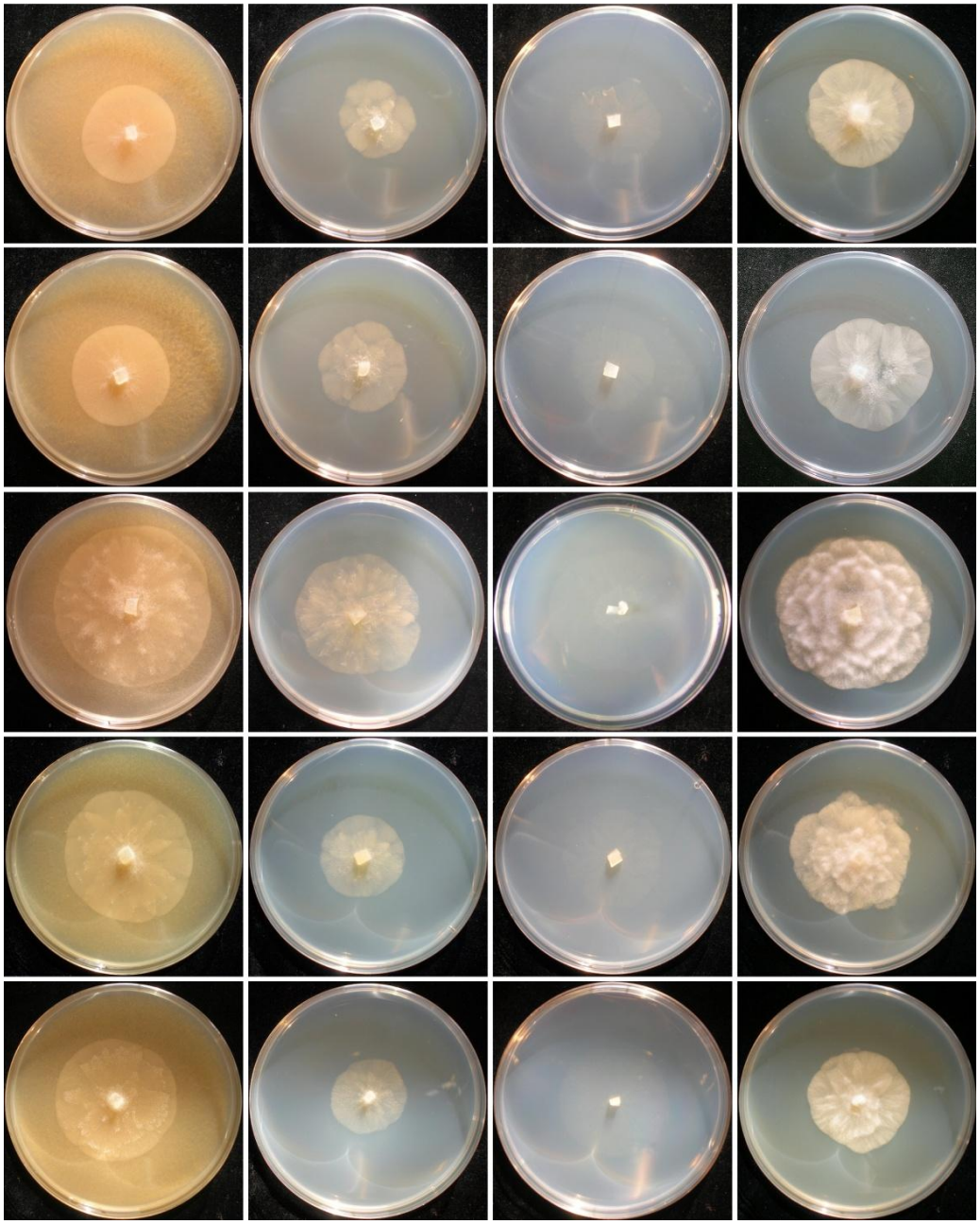

Figure 12 Colony morphology (from top to bottom) of *P.* taxon PgChlamydo (1st, 2nd rows) and *P. lacustris* (3rd, 4th and 5th rows) after 7 d growth at 20 °C on (from left to right) V8 agar, malt extract agar, corn meal agar and potato-dextrose agar.

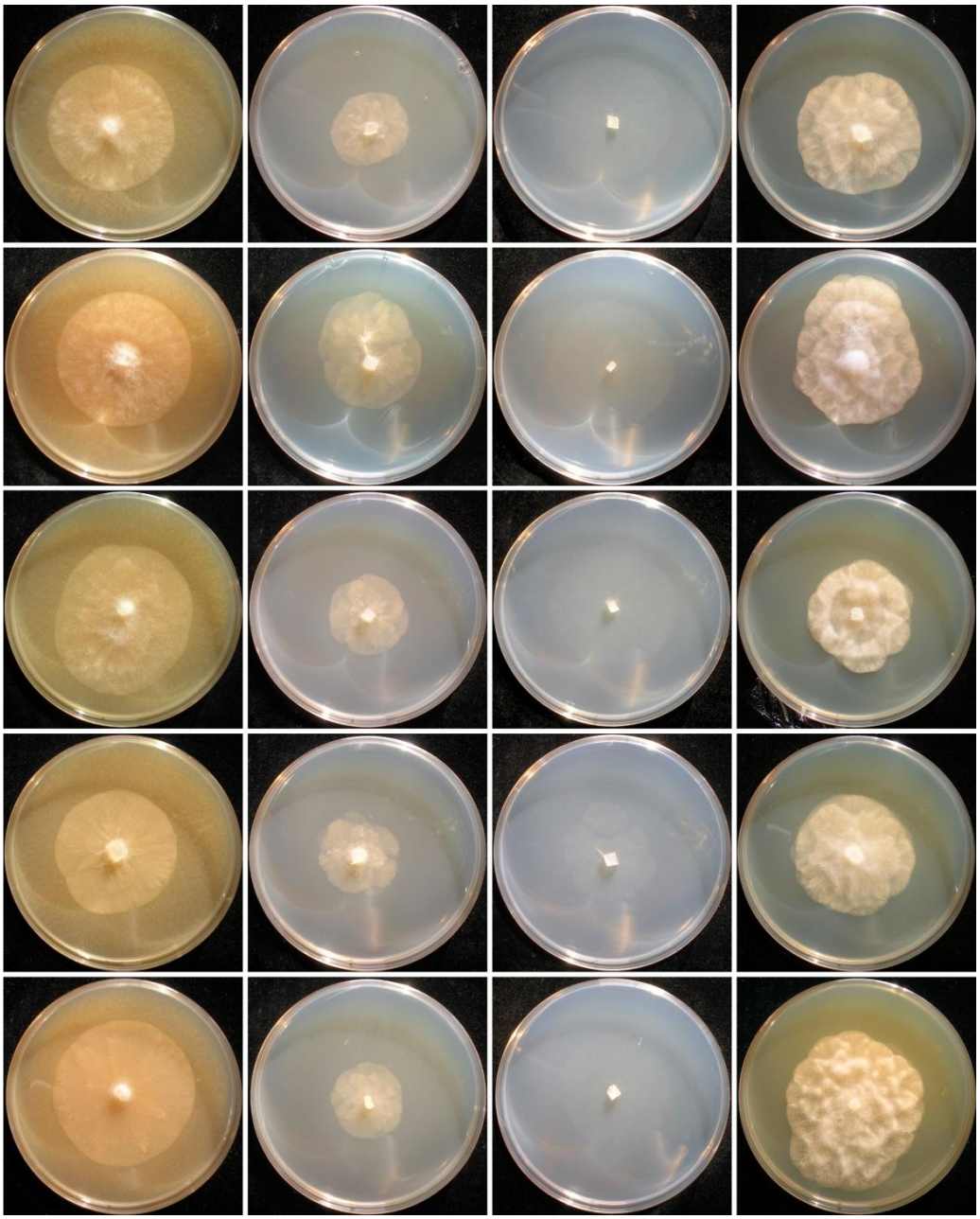

Figure 13 Colony morphology (from top to bottom) of isolate B089, B101, B106, B121 and B163 after 7 d growth at 20 °C on (from left to right) V8 agar, malt extract agar, corn meal agar and potato-dextrose agar.

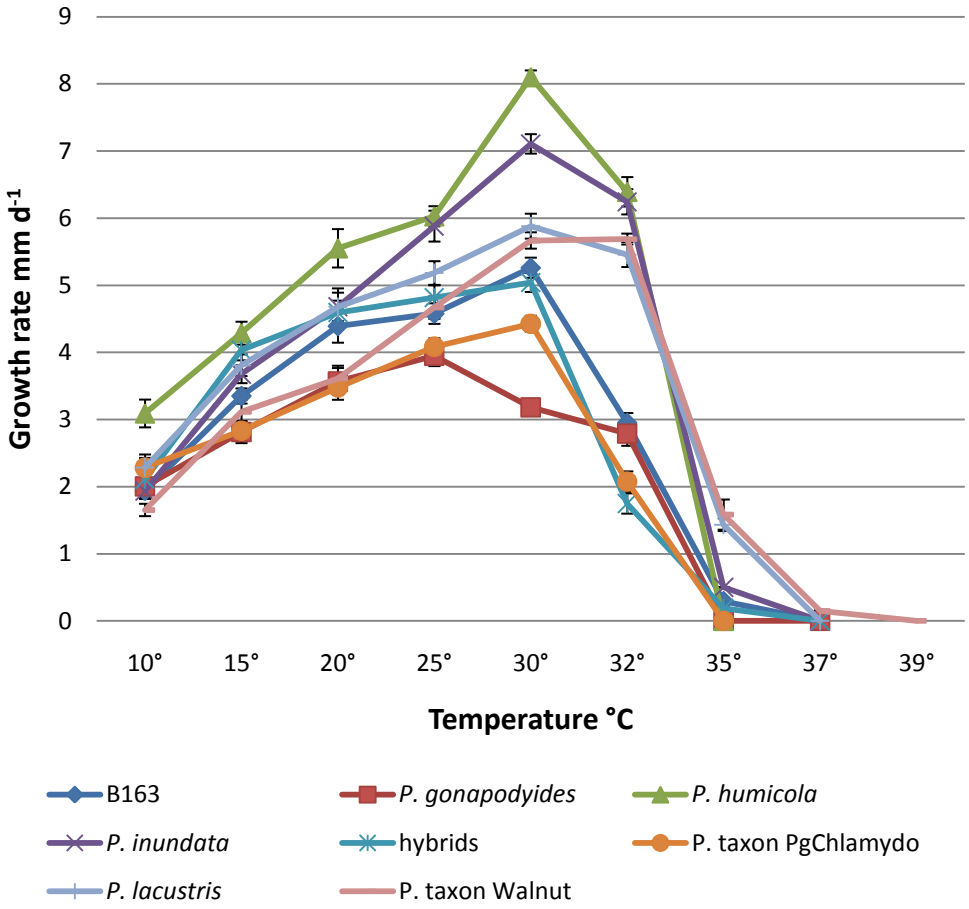

Figure 14 Mean radial growth rates of *Phytophthora humicola*, *P.* taxon walnut, B163 (one isolate each), *P. inundata*, *P. gonapodyides*, *P.* PgChlamydo (two isolates each), *P. lacustris* (three isolates) and hybrid Phytophthoras (four isolates) on V8-agar at different temperatures.

Morphology of asexual and sexual structures

Mature, non-papillate, persistent sporangia were produced by all the morphologically analyzed Phytophthoras flooding agar squares which were taken from the margin of 3-5 day old growing colonies completely submerged with pond water after incubation at 20 °C in natural daylight.

Morphology and sizes of sexual and asexual structures of all the analyzed species are showed in table 5. The morphology of sporangia observed in the clade 6 isolates of this study are showed in figure 10.

P. gonapodyides is self sterile in single culture but it's able to induce gametangial formation in an A2 type of another species (Brasier *et al*., 2003b). The isolate B079 presented both internal and external proliferation and the production of ovoid sporangia with sporadic presence of obpyriform or bipapillate sporangia (Fig. 10 a-d); no hyphal swellings were observed in this isolate but this feature could be present in some isolates of this species (Jung *et al*., 2011).

Table 5 Morphological characters, dimensions and temperature-growth relations of *P. gonapodyides, P. inundata*, *P. humicola*, *P.* taxon walnut , *P. lacustris*, *P.* taxon Pg Chlamydo and two hybrid species between *P. lacustris* and *P.* taxon Pg Chlamydo.

	P. gonapodyides	***P. inundata***	***P. humicola***	***P.*taxon walnut**	***P.lacustris***	***P.* taxon Pg Chlamydo**	**Hybrid B**	**Hybrid 2**
Sporangia	Ovoid, obpyriform, nonpapillate	Broad ovoid, ovoid, ovoid with pointed apex	Ovoid, broad ovoid and globose	Ovoid, ellipsoid	Ovoid, lymoniform	Ovoid, obpyriform	Ovoid	Ovoid
lxb mean (μm)	46.4 ± 6.9 x 32.5 ± 3.5	49.5 ± 7.6 x 40.4 ± 5.3	45.0 ± 9.1 x 36.1 ± 7.5	52.5 ± 9.6 x 32.9 ± 4.7	52.7 ± 5.9 x 34.0 ± 2.6	42.4 ± 6.2 x 29.9 ± 3.5	42.2 ± 5.3 x 27.8 ±2.3	47.4 ± 5.4 x 28.9 ± 2.3
Total range (μm)	33.1 – 66.6 x 23.1 – 37.4	37.7 – 65.7 x 30.4 – 54.4	27.6 – 58.7 x 19.5 – 51.9	30.8 – 67.0 x 22.5 – 42.8	41.2 – 64.3 x 29.3 – 39.2	30.0 – 56.1 x 22.5 – 38.0	29.9 – 54.6 x 23.0 – 34.4	38.2 – 61.7 x 24.4 – 34.0
l/b ratio	1.43 ± 0.17	1.23 ± 0.14	1.25 ± 0.09	1.6 ± 0.2	1.55 ± 0.15	1.42 ± 0.18	1.4 ± 0.14	1.6 ± 0.12
Total range (μm)	1.18 – 2.00	1.05 – 1.82	1.02 – 1.5	1.27 – 2.05	1.36 – 2.09	1.2 – 2.0	1.2 – 1.8	1.3 – 1.9
Exit pores								
Width	12.8 ± 2.15	14.8 ± 2.8	12.2 ± 2.4	11.1 ± 1.7	15.3 ± 2.4	11.74 ± 1.4	11.6 ± 2.5	10.6 ± 2.1
Total range (μm)	6.6 – 15.9	10.3 – 19.3	8.5 – 15.2	7.3 – 14.2	10.6 – 18.2	9.0 – 14.6	6.1 – 17.1	5.9 – 15.3
Proliferation	Int. ne, ext	Int. ne, ext	Int. ne, ext	Ext	Int. ne, ext	Int. ne, ext	Int. ne, ext	Int. ne, ext
Hyphal swellings	Not observed	+	+	+	+	+	+	+
Sexual system	Sterile, silent A1 [1]	Heterotallic[1]	Homotallic	Sterile[1]	SS silent A1[1]	SS silent A1[1]	sterile	sterile
Oogonia								
Mean diam (μm)	–	–	39.0 ± 3.2[2]	–	–	–	–	–
Total range (μm)	–	–	33.6 – 44.9[2]	–	–	–	–	–
Maximum temperature (°C)	32	35	32	35	37	32	35	35
Optimum temperature (°C)	25	30	30	30 - 32	30	30	30	30
Growth rate on V8A at optimum (mm/d)	3.9 ± 0.5	7.1 ± 1.0	8.1 ± 0.3	5.6 ± 0.8	6,4 ± 0.4	4.4 ± 0.4	5.3 ± 0.5	5.0 ± 0.5
Growth rate on V8A at 20°C (mm/d)	3.6 ± 0,7	4.7 ± 0.9	5.5 ± 1	3.6 ± 0.7	3.6 ± 0.7	3,5 ± 3.6	4.4 ± 0.9	4.6 ± 0.6

Abbreviatons: Int ne, internal nested proliferation; ext, external proliferation; SS silent A1, self steril but able to induce gametangial formation in an A2 type of another species (see Brasier *et al*., 2003b)

[1]Data from Jung *et al*., 2011

[2]Data from Ginetti *et al*., 2012

P. inundata is an heterothallic species; isolate B189 presented both internal and external proliferation and the production of broad-ovoid sporangia (Fig. 10 e-h); globose to subglobose hyphal swelling were observed in this isolate.
P. taxon walnut is a sterile species (Jung *et al*., 2011); isolate B164 showed external sporangial proliferation and the formation of ovoid sporangia with sporadic occurrence of elongated-ovoid or ellipsoid sporangia (Fig. 10 i-l); globose to subglobose hyphal swellings were detected in this isolate.
P. humicola is an homothallic species; isolate B033 presents oogonia with paragynous antheridia (anphygynous type sometimes occurs), mostly ovoid or broad-ovoid sporangia (Fig. 10 m-o) and both internal and external sporangial proliferation; globose to subglobose hyphal swellings were observed in this isolate.
P. lacustris is self sterile in single culture but it can act as 'silent A1', inducing gametangial formation in an A2 type of another *Phytophthora* species (Nechwatal *et al*., 2012). The isolate B137 presented both internal and external proliferation and the production of ovoid with pointed apex sporangia with sporadic presence of limoniform sporangia (Fig. 10 p-r); ellipsoid hyphal swellings were noticed in this isolate.
P. taxon PgChlamydo is self sterile in single culture but could be able to induce gametangial formation in an A2 type of another species (Brasier *et al*., 2003b). The isolate B107 presented both internal and external sporangial proliferation and the production of ovoid sporangia with sporadic formation of obpyriform sporangia (Fig. 10 s-v); ellipsoid and irregular hyphal swellings were noticed in this isolate. Chlamydospores were abundantly produced by this species (e.g. by isolates B107 and B115).
Phytophthora hybrids between *P. lacustris* and *P*. taxon PgChlamydo were sterile; all the analyzed isolates (B101, B106, B163) presented both internal and external sporangial proliferation and the production of ovoid sporangia (Fig. 10 w-e1). Hybrid isolate B101 presented sporangia length and an l:b ratio significantly different from *P.* taxon PgChlamydo B107 ($p < 0.01$ and $p < 0.001$ respectively); there was no difference between the two isolates in the sporangia width. B101 was statistically different from *P. lacustris* (isolate B137) in sporangia length, breadth and l:b ratio ($p < 0.001$, $p < 0.001$ and $p < 0.05$ respectively). Hybrid isolate B163 presented sporangia breadth and an l:b ratio significantly different from *P.* taxon PgChlamydo B107 ($p < 0.01$ and $p < 0.05$ respectively) while there was no difference between the two isolates in the sporangia length. Hybrid isolate B163 was statistically different from *P. lacustris* (isolate B137) in sporangia length and breadth ($p < 0.001$) but there wasn't a significant difference between the two isolates in the l:b ratio. The two hybrids B101 and B163 presented sporangia length and an l:b ratio significantly different ($p > 0.001$) between them but there was no difference between the two isolates in the sporangia width ($p = 0.07$). Globose to subglobose and ellipsoid hyphal swellings were observed in all hybrid isolates. Chlamydospores were observed on isolate B106.

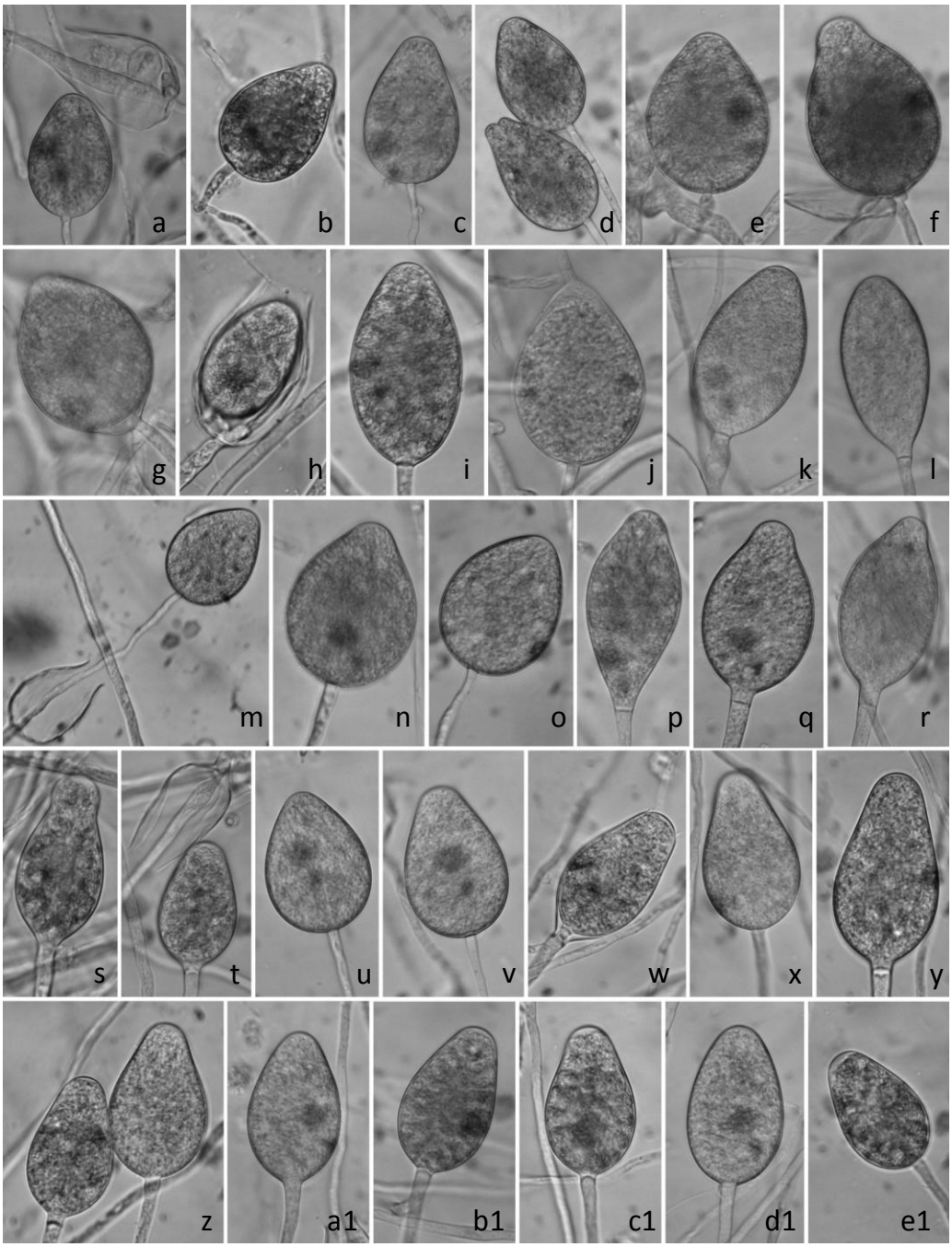

Figure 15 a-e1) Mature non papillate, persistent sporangia produced on V8-agar flooded with pond water: ovoid sporangia (a-d) and an empty sporangium with proliferation (a) of *P. gonapodyides*; ovoid sporangia (e-g) and an empty sporangium with internal proliferation (h) of *P. inundata*; ovoid and elongated-ovoid sporangia (i-l) of *P.* taxon walnut; ovoid sporangia (m-o) and an empty sporangium with external proliferation (m) of *P. humicola*; limoniform to ovoid sporangia (p-r) of *P. lacustris*; pyriform (s), ovoid (t-v) sporangia and an empty sporangium with proliferation (t) of *P.* taxon PgChlamydo; ovoid sporangia (w-e1) and an empty sporangium with internal proliferation (w) of hybrid Phytophthoras.

Under-bark inoculation test

Under-bark inoculation trials were conducted on one-year-old twigs of *Acer pseudoplatanus* and *Fagus sylvatica*. A V8-agar disc of *Phytophthora* was placed on a wound artificially created with a razor blade and the material was incubated at 20 °C for 3 weeks in the dark (Fig. 11).

P. humicola was pathogenic on *A. pseudoplatanus* (7 infected stems out of 10) and *F. sylvatica* (8 infected stems out of 10). After 22 d lesion lengths on *A. pseudoplatanus* measured on average 5.89 ± 1.6 cm (overall range 3.5 – 8.0 cm), while lesion lengths on *F. sylvatica* averaged 3.63 ± 0.3 cm (overall range 3.2 – 4.0 cm) (Fig. 11 a-b). This difference in aggressiveness to the two tree species was statistically significant ($P < 0.01$). *P. humicola* was pathogenic to its host *Pinus pinea* (5 infected stems out of 10) to which it caused lesions, after two weeks of incubation in the dark at 20°C, of 2,2 ± 0.34 cm (overall range 1.8 – 2.7 cm) (Ginetti *et al*., 2012).

P. gonapodyides resulted pathogenic to one-year-old twigs from mature trees of both *A. pseudoplatanus* (7 infected stems ou of 10) and *F. sylvatica* (6 infected stems out of 10). After 22 d lesion lengths on *A. pseudoplatanus* measured on average 4.87 ± 0.7 cm (overall range 4.4 – 6.2 cm) while lesion lengths on *F. sylvatica* averaged 3.15 ± 0.3 cm (overall range 2.7 – 3.6 cm) (Fig. 11 c-d). This difference in aggressiveness to both tree species was statistically significant ($P < 0.01$).

P. inundata isolates resulted pathogenic to one-year-old twigs from mature trees of both *A. pseudoplatanus* (16 infected stems out of 20) and *F. sylvatica* (15 infected stems out of 20). After 22 d lesions lengths on *A. pseudoplatanus* measured on average 5.69 ± 1.2 cm (overall range 3.9 – 8.5 cm) while lesions lengths on *F. sylvatica* averaged 3.56 ± 1.19 cm (overall range 1.1 – 6.0 cm) (Fig. 11 e-f). This difference in aggressiveness to both tree species was statistically significant ($P < 0.001$).

P. taxon walnut resulted pathogenic to one-year-old twigs from mature trees of both *A. pseudoplatanus* (10 infected stems out of 10) and *F. sylvatica* (10 infected stems out of 10). After 22 d lesions lengths on *A. pseudoplatanus* measured on average 6.07 ± 1.0 cm (overall range 4.0 – 7.3 cm) while lesions lengths on *F. sylvatica* averaged 3.92 ± 0.3 cm (overall range 3.5 – 4.4 cm) (Fig. 11 g-h). This difference in aggressiveness to both tree species was statistically significant ($P < 0.001$).

P. lacustris isolates resulted pathogenic to one-year-old twigs from mature trees of both *A. pseudoplatanus* (14 infected stems out of 20) and *F. sylvatica* (6 infected stems out of 10). After 22 d lesion lengths on *A. pseudoplatanus* measured on average 6.39 ± 1.1 cm (overall range 4.8 – 8.2 cm) while lesions lengths on *F. sylvatica* averaged 3.25 ± 0.76 cm (overall range 1.9 – 4.2 cm) (Fig. 11 i-j). This difference in aggressiveness to both tree species was statistically significant ($P < 0.001$).

P. taxon PgChlamydo resulted pathogenic to one-year-old twigs from mature trees of *F. sylvatica* (8 infected stems out of 10) but was not pathogenic to one-year-old twigs from mature trees of *A. pseudoplatanus*. After 22 d lesions lengths on *F. sylvatica* averaged 2.56 ± 0.5 cm (overall range 2.1 – 3.5 cm) (Fig. 11 k).

Regarding the tested hybrid species between *P. lacustris* and *P.* Pg Chlamydo, no taxon resulted pathogenic to inoculated stems of *Acer pseudoplatanus* but three isolates (B089, B106, B163) were able to induce formation of lesions on inoculated stems of *F. sylvatica*: B089 (6 infected stems out of 10) causing lesions of 3.2 ± 1.3 cm (overall range 1.6 – 5.3 cm); B106 (4 infected stems out of 10) causing lesions of 3.6 ± 0.9 cm (overall range 2.3 – 4.5 cm) and B163 (5 infected stems out of 10) causing lesions of 2.4 ± 0.7 cm (overall range 1.7 – 3.5 cm) (Fig. 11 l-n).

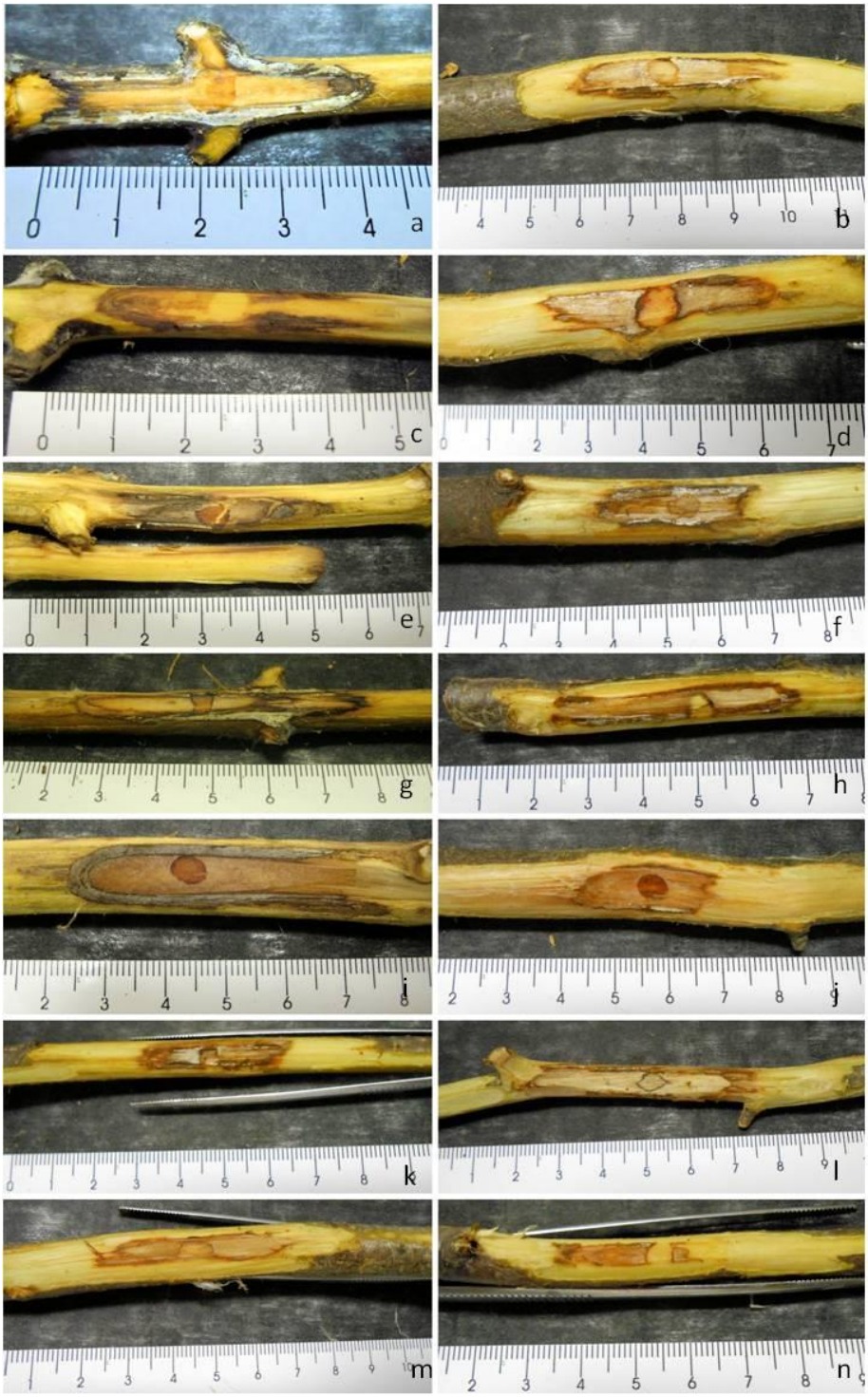

Figure 16 a-j: Twigs of *A. pseudoplatanus* (on the left) and *F. sylvatica* (on the right) inoculated with: *P. humicola* (a,b); *P. gonapodyides* (c,d); *P. inundata* (e,f); *P.* taxon walnut (g,h); *P.* lacustris (i,j); k-n: Twigs of *F. sylvatica* inoculated with *P.* taxon PgChlamydo (k) and hybrid species B089 (l); B106 (m) and B163 (n).

Discussion and conclusions

The aim of this work was to investigate the ecological and pathological role of some *Phytophthora* species belonging to Clade 6 in forestry and in semi-natural ecosystems, in particular in the planted forest in which they were isolated during the winter of 2010-2011. The major part of the isolates found in this area was isolated from water bodies, in particular from four locations along the artificial canal that crosses the Boscoincittà park (E1, E2, E3 and E4) and from four different little lakes: Laghetto (L), Giardino d'Acqua (GdA), Chiusa Madre (CM) and Aree Nuove (ANve) located further north of the canal but still within the park. All the obtained isolates were identified by sequencing of the ITS region, which is generally considered sufficient for identification of *Phytophthora* species. However, the sequencing of another region, such as the mitochondrial *cox*1, can add confidence and also new information to phylogenetic analysis. With the exception of four isolates (B089, B101, B106 and B163) the phylogenetic analysis based on the *cox*1 gene confirmed the one carried out on the ITS region (Fig. 2-3). On the contrary, isolated B089, B101 and B106 clustered with *P. lacustris* in the ITS phylogenetic tree and were related to *P.* taxon PgChlamydo in the cox1 phylogenetic tree. Exactly the opposite happened with the isolate B163 that clustered with *P.* taxon PgChlamydo in the ITS phylogenetic tree and was more similar to *P. lacustris* in the *cox*1 phylogenetic analysis (Fig. 4-5). This outcome suggests with a good chance that these four isolates are hybrids between *P. lacustris* and *P.* taxon PgChlamydo and that isolates B089, B101 and B106 inherited the maternal component from a local *P.* taxon PgChlamydo while isolate B163 inherited the same component from a *P. lacustris*.

Undoubtedly, water plays a fundamental role in the ecology of the genus *Phytophthora* but primary acts just as medium for zoospore displacement and also as reservoir for the inoculum and not as a natural habitat for their regular life cycle. *P. lacustris* was abundantly detected in all the investigated water bodies (Table 3) while *P.* taxon PgChlamydo was baited from several sites along the canal (E1, E2 and E4) and only in the CM pond. Species that seemed to be hybrids between these two species were isolated from all the locations where they resulted both present, but also from E3, GdA and ANve (in which *P*. taxon PgChamydo was absent). No doubt, the existence of hybrid species in the E3 placement despite a missing *P.* taxon PgChlamydo isolation could be easily explained by its presence in the rest of the canal. The existence of some hybrids in GdA and ANve, despite the absence of *P.* taxon Pgchlamydo, could be explained by several hypotheses: 1) *P.* taxon PgChlamydo lives in that ponds but we weren't able to isolate it; 2) *P.* taxon PgChlamydo was there in the past but it's no more there; 3) hybrid species were transferred by a vector from CM (where both parental species were present) to ANve and/or GdA. Few isolates of the other species like *P. gonapodyides*, *P. inundata* and *P.* taxon walnut were found to be widespread in the detected areas. In any case an assemblage of 2-5 of these species has been found in all the sites, apart from the Laghetto lake (in which just *P. lacustris* resulted to live). We don't know if these species interact with the other organisms in the ecosystem but we can assume that some of these species are able to mate together. Furthermore, sequences presented lots of polymorphisms and especially double peaks, also within the same species (*P. lacustris* and *P.* taxon PgChlamydo), suggesting occurrence of intra-taxon variation. The interpretation of these data, to understand the occurrence of possible interactions between the various individuals, appears to be however a challenge. We can just assume that the occurrence of polymorphisms and double peaks prove the existence of some forms of relationship among these individuals.

It is remarkable to note that, with respect to the major part of *Phytophthora* species, all the taxa detected in the North of Italy could be described as "high temperature tolerant" individuals; in fact most of them presented an optimum temperature for growth at 30 °C and a maximum temperature from 32 °C to 37 °C for the *P.* taxon walnut isolate (tabella 5, Figure 9). This suggests that the ancestors of the species belonging to Clade 6 were probably of tropical origin (Brasier *et al*., 2003a). Results derived by radial growth rates reinforced the hypothesis, based on the phylogenetic analysis, that isolates B089, B101, B106 and B163 are hybrid species; in fact their growth rates are within the range given from *P. lacustris* (higher in average) and the lower growth rate of *P.* taxon PgChlamydo. This fact is especially evident between 20 and 35 °C, range in which distances among curves tend to increase (Fig. 9).

It must be considered that species which have genetically changed towards a nearly saprophytic life style have maintained, in the mean time, this ancestor character, especially in cool-temperate or even arctic-alpine environments. This led us to think that such character is a physiological adaptation to some aspects of their ecology such as the ability to undertake litter breakdown or the capability to survive in the warm conditions that can be found at the margins of water bodies during summer, when the water level is very low (Nechwatal *et al*., 2012). In addition to tolerance to high temperatures, the taxa found in the North of Italy, as several other species within the Clade 6, exhibit an uncommon combination of properties such as their aptitude to colonize riparian habitats and being sexually sterile, partially infertile or inbreeding species. All these species were in fact unable to produce gametangia *in vitro*. It has been hypothesized that these species have probably evolved such lifestyle in order to better compete with other saprophytic microorganisms in the decomposition of plants, or plant parts, fallen inside water bodies (Brasier *et al*., 2003a,b). The almost total loss of the ability to reproduce sexually fits well with the saprophytic lifestyle in canals and small ponds. In these habitats the release of a large number of zoospores into the surrounding environment in short times is in fact a successful strategy. Furthermore, asexual reproduction is for the organism much less expensive in terms of energy than sexual reproduction. In this study, this hypothesis was confirmed by the wide presence of these *Phytophthora* species in the water bodies in the park. *Phytophthora* species such as *P. gonapodyides, P. lacustris* and *P.* taxon PgChlamydo are known in literature to be self-sterile but still able to induce in vitro gametangial formation with an A2 type of another species (Brasier *et al*., 2003b, Jung *et al*., 2011); this character could be a vestiges of their previous capability to implement sexual reproduction. Indeed, a prove of a possible ancient pathogenic lifestyle, rather than a saprophitic lifestyle, can also be found in results of the pathogenicity tests carried out on both *Acer pseudoplatanus* and *Fagus sylvatica* twigs. (Fig. 11) *P. gonapodyides*, *P. inundata*, *P.* taxon walnut and *P. lacustris* resulted pathogenic to both *Acer* and *Fagus* twigs. For all of them the pathogenity on *Acer* twigs was significantly higher than on *Fagus*. *P.* taxon PgChlamydo was on the contrary harmless to *Acer* (no lesions), but was able to cause necroses of about 3 cm on *F. sylvatica* twigs. Similarly, all the tested hybrids (B089, B106, B163) weren't able to induce lesions on maple, but produced necroses of about 2.5-3.5 cm lenght on beech. This suggests that the loss of pathogenicity on maple was probably inherited by the *P.* taxon PgChlamydo parent. *P. humicola* resulted dangerous to *Pinus pinea* twigs (Ginetti *et al*., 2012), being this species the symptomatic host from which it was isolated. In this study, *P. humicola* resulted aggressive to *Acer* and *Fagus* twigs too, showing its potential aggressiveness to several species.

These inoculation trials underline the potential pathogenity of the species belonging to the Clade 6, which are adapting to a saprophytic lifestyle, but which can anyway be harmful to plants, especially under biotic or abiotic stress conditions.

Three *Phytophthora* species detected in this work are reported for the first time in Italy. *P.* taxon walnut has been found in the USA (Jung *et al*., 2011), while *P.* taxon PgChlamydo was reported from the USA, Canada, Patagonia, Australia, South Africa, Europe (France and Serbia) (Burgess *et al*., 2012, Oh *et al*., 2012, Milenkovic *et al*., 2012, Velez *et al*., 2012, Jung *et al*., 2011, Brasier *et al*., 2003a), but never from Italy. *P. humicola* was reported from Asia (Taiwan) (Ko and Ann, 1985). *P. gonapodydes* was reported from the USA, Australia and New Zeland, and Europe (Nechwatal *et al*., 2012, Jung *et al*., 2011, Reeser *et al*., 2011,Vettraino *et al*., 2002). *P. inundata* was isolated from South America, Australia and Europe (Jung *et al*., 2011, Brasier *et al*., 2003b), while in Italy it was reported from Sicily (Cacciola *et al*., 2005). *P. lacustris* has been isolated in USA, in Australia and New Zeland, in Europe and in Italy, reported from Calabria and Emilia Romagna (Nechwatal *et al*., 2012). The high occurrence of *Phytophthora* species from all the above mentioned localities and especially the worldwide spread of these microorganisms point out the risks inherent with the global trade of plants. The process of globalization, with the increasing movement of goods, natural vectors and people, in a very short time, further exhacerbates the problem, which has to be also attributed, at least in part, to the failure of plant biosecurity measures adopted by several countries.

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Chapter V

Root Rot and Dieback of *Pinus pinea* caused by *Phytophthora humicola* in Tuscany, central Italy

Root Rot and Dieback of *Pinus pinea* caused by *Phytophthora humicola* in Tuscany, central Italy

B. Ginetti, A. Uccello, M. Bracalini, **A. Ragazzi**, Dipartimento di Biotecnologie Agrarie, Sezione Protezione delle Piante. Piazzale delle Cascine, 28, I-50144, Florence, Italy; **T. Jung,** Phytophthora Research and Consultancy, Thomastrasse 75, D-83098 Brannenburg, Germany; and **S. Moricca,** Dipartimento di Biotecnologie Agrarie, Sezione Protezione delle Piante. Piazzale delle Cascine, 28, I- 50144, Florence, Italy

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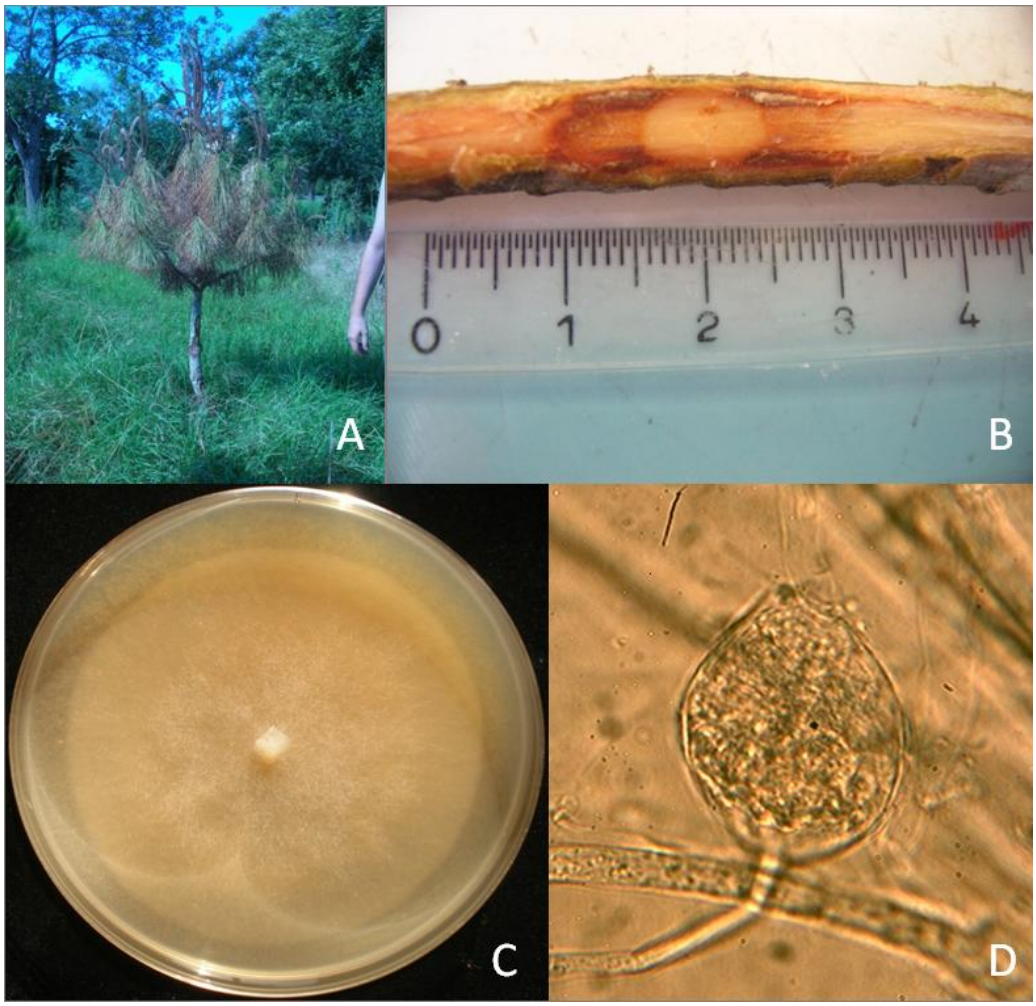
High mortality was noticed in a 10-year-old stand of *Pinus pinea* in the Alberese area (Grosseto, central Italy, elev. 40 m a.s.l., 42 º 39' 46" 24 N, 11º 06' 91" E) in July 2010. Aerial symptoms of trees included chlorosis, crown thinning, stunted growth, bark lesions at the stem base with resinous exudations and extensive necroses of the underlying xylem tissue. The woody roots of two uprooted trees exhibited bark necroses and a high proportion of the fine roots was destroyed. Soil around necrotic roots was baited using apple fruits (cv Gala). After one week incubation at 24ºC, typical firm fruit rot developed and small tissue samples were transferred to clarified V8-Agar amended with 5ml/l PARPNH and incubated at 24ºC. After 7 days, stellate to rosaceous, finely lobed cottony colonies arose that were transferred to FPM medium and incubated at 24ºC. Within 7 days spherical oogonia with a smooth surface and predominantly paragynous antheridia formed; sporadic amphyginous antheridia could be observed. Colony squares (1 cm2) were then placed in filtered and sterilized pond water. After 48 hours ovoid, obpyriform or clavate, nonpapillate, persistent sporangia with internal nested and extended proliferation were formed. Fifty oogonia and 30 sporangia were measured. The diameter of the 50 spherical oogonia varied from 33.6 to 44.9 Am (avg. 39 Am); dimensions of the 30 sporangia were 42.6-59.8 x 28.9-47.8 Am (avg. 52.95 x 38.98 Am; 1:b ratio 1.37). The isolate was identified as *Phytophthora humicola* W. H. Ko & Ann on the basis of colony type, size and morphology of oogonia and sporangia, average length/width ratio of sporangia, the homothallic formation of oogonia (4), and ITS rDNA sequence information (GenBank accession no. JQ757060). A BLAST search of the ITS sequence of *P. humicola* isolate B33 revealed a 99% identity with the *Phytophthora* ITS Clade 6 species *P. humicola* and *P. inundata* (2). This latter species could be ruled out, however, since it is self-sterile, whereas our isolate B33 was self-fertile (3). A strain of *P. humicola* was deposited in the CBS-KNAW Fungal Biodiversity Centre, strain no. CBS129249. Pathogenicity tests were conducted on 10 one-year-old twigs of *Pinus pinea*. A bark portion was removed aseptically and a V8-Agar disc (0.5 cm diam.) of *P. humicola* mycelium was placed on the wound. Control twigs (3) received sterile V8A discs. Inoculated and control twigs were incubated at 20°C in the dark. Clearly noticeable necrotic lesions (avg. length 2.2 x 0.68 cm) were observed after 15 days on inoculated twigs. Control twigs showed no symptoms. Reisolations on selective V8-PARPNHagar confirmed *P. humicola* as the causal agent. *P. humicola* is mainly associated with woody horticultural crops (1, 3), while the other taxa grouped with this species in Clade 6 are mainly found in forest and riparian ecosystems (1). These aquatic *Phytophthora* species normally have a saprophytic lifestyle, but under favourable environmental circumstances can act as opportunistic pathogens, attacking susceptible trees and causing scattered mortality in forest stands and natural ecosystems (3). To our knowledge, this is the first report of *P. humicola* from a pine stand. It is supposed that the pathogen reached the stand through infected plant material or infested soil introduced into the stand.



A, Young *Pinus pinea* individual infected with *Phytophthora humicola* showing evident stuntedgrowth, with needle desiccation and crown thinning. **B,** Lesion produced on a *P. pinea* twig following artificial inoculation with *P. humicola* isolate B33 after 2 weeks incubation at 20°C in the dark. **C,** 7- day-old colony of *P. humicola*, with a cottony mycelium texture, growing at 24°C on V8-agar. **D,** Typical ovoid, nonpapillate sporangium of *P. humicola* produced after 48 hours of flooding in filtered and sterilized pond water (scale bar = 10 Am).

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