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

B. Ginetti[a], S. Moricca[a]*, J. N. Squires[b], D. E. L. Cooke[b], A. Ragazzi[a] and T. Jung[cd]

[a]*Department of Agri-Food Production and Environmental Sciences, Plant Pathology and Entomology Division, University of Florence, Piazzale delle Cascine 28, 50144 Florence, Italy;* [b]*The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK;* [c]*Phytophthora Research and Consultancy, Thomastrasse 75, D-83098 Brannenburg, Germany; and* [d]*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*

A severe dieback of *Acer pseudoplatanus* trees was noticed in planted forest stands in northern Italy 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 analysis of ITS, *cox1* and β-tubulin gene regions demonstrated that *P. acerina* is unique and forms a separate cluster within the '*P. citricola* complex', closely related to *P. plurivora*. *Phytophthora acerina* is homothallic with smooth-walled oogonia, thick-walled, mostly aplerotic oospores with a high abortion rate, paragynous antheridia, and persistent, morphologically variable semipapillate sporangia. Four to 5-week-old cultures produced globose to subglobose, appressoria-like and coralloid hyphal swellings and characteristic stromata-like hyphal aggregations. Optimum and maximum temperatures for growth are 25°C and 32°C, respectively. Genetic uniformity of all 15 studied 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. Under-bark inoculation tests demonstrated high aggressiveness of *P. acerina* to *A. pseudoplatanus* indicating that this pathogen might be a serious risk to maple plantations and forests in Europe.

Keywords: biosecurity, Clade 2, invasive, nursery pathway, phylogeny, *Phytophthora citricola*

Introduction

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, 2013b; Vettraino *et al.*, 2002). 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 common European tree species including *Acer platanoides, Acer pseudoplatanus*, *F. sylvatica* and several *Quercus* spp. (Jung *et al.*, 1996, 2003; Vettraino *et al.*, 2002; Brasier & Jung, 2003; Jung & Burgess, 2009) strongly indicates 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 soil-borne *Phytophthora* species and the increasing frequency of climatic extremes are a major cause for the severe declines of oak and beech forests (Brasier & Scott, 1994; Jung *et al.*, 1996, 2013b; Jung, 2009).

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 (Jung & Burgess, 2009; Jung *et al.*, 2013b). 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.

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 (Cooke *et al.*, 2000; Kroon *et al.*, 2004; Blair *et al.*, 2008). Several well-known morphospecies such as *P. citricola*, *P. gonapodyides*, *P. megasperma*, *P. porri* or *P. syringae* have been shown to be complexes of morphologically and phys-

*E-mail: salvatore.moricca@unifi.it

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iologically almost indistinguishable species (Brasier *et al.*, 2003; Jung *et al.*, 2003; Jung & Burgess, 2009). The morphospecies *P. citricola sensu lato* was divided into *P. citricola sensu stricto* and seven new closely related taxa that together form the '*Phytophthora citricola* complex', i.e. *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 (Jung & Burgess, 2009; Scott *et al.*, 2009; Bezuidenhout *et al.*, 2010; Hong *et al.*, 2011; Kroon *et al.*, 2012).

In 2008, a severe decline and dieback of *A. pseudoplatanus* trees was observed in several planted forest stands in northern Italy. 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 2010 and 2011, *Phytophthora* isolates were consistently isolated from necrotic bark and stained xylem tissue and from rhizosphere soil of *A. pseudoplatanus* trees with symptoms in planted forest stands of the Boscoincittà Park in Milan. Being homothallic, with persistent semipapillate sporangia, the isolates resembled species from the '*P. citricola* complex'. Detailed morphological and physiological studies and a phylogenetic analysis of ITS, *cox1* and β-tubulin sequence data revealed that all isolates belong to an unknown unique taxon of the '*P. citricola* complex', which is described here as *Phytophthora acerina* sp. nov.

Materials and methods

Sampling and isolation procedures

Soils in the Boscoincittà Park, Milan, Italy (45°29′9″N, 9°5′19″E) have a sandy-clay texture (70% coarse and fine sand, 15% silt and 15% clay) and a slightly acidic to neutral reaction with pH values ranging from 5·5 to 7·3. Between 2007 and 2010 annual precipitation varied between 912 mm (2007) and 1395 mm (2008), with 1375 mm in 2010. Because of these favourable soil and climatic conditions, *Acer pseudoplatanus* stands were commonly planted in the park during previous decades.

Sampling was carried out from *c.* 30–35-year-old planted *A. pseudoplatanus* trees growing within a 2 km radius on non-flooded sites in four forest stands of the Boscoincittà Park during both summer (June 2010) and winter (December 2010 and February 2011). Affected trees showed symptoms characteristic of infection 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.

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.* 8 × 3 × 3 mm) were cut from different parts and depths of the phloem and xylem samples, blotted on filter paper, and plated onto selective PARPNH V8 agar (V8A; 16 g agar, 3 g $CaCO_3$, 100 mL Campbell's V8 juice, 900 mL distilled water) amended with 10 μg mL^{-1} pimaricin, 200 μg mL^{-1} ampicillin, 10 μg mL^{-1} rifampicin, 25 μg mL^{-1} PCNB, 50 μg mL^{-1} nystatin and 50 μg mL^{-1} hymexazol (Erwin & Ribeiro, 1996). 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 at a distance of 1–1·5 m from the stem base at four points around each tree. In addition, one sample of soil was taken from each of 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 bait (Erwin & Ribeiro, 1996; Jung *et al.*, 1996). After surface disinfection with 95% ethanol, four equidistant holes of *c.* 1 cm diameter and *c.* 2 cm depth were cut into each apple with a sterilized scalpel. Each hole was completely filled with subsamples of soil from one sample and subsequently wetted with sterile deionized water in order to induce the germination of potential *Phytophthora* resting structures. Each apple was finally wrapped in a transparent film of polymer material 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 course of a canal that runs through 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. After 7 days the apples were collected and transported to the laboratory where isolations were carried out as described before. A second survey was conducted in February 2011 in four small ponds located in the park following the same procedure.

Phytophthora isolates

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

DNA isolation, amplification and sequencing

The 24 *Phytophthora* isolates obtained were subsequently transferred to potato dextrose agar (39 g PDA, 5 g agar in 1 L deionized water) and grown at 22°C for 1 week. The mycelium was collected in sterile 1·5 mL microfuge tubes by gently scratching hyphae from the surface of the colony with the aid of a sterile scalpel and placed in the freezer (−20°C) for not less than 12 h. 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.

The ITS (internal transcribed spacer) region of the ribosomal DNA was amplified using the primers ITS-6 (5′-GAAGGTGAA GTCGTAACAAGG-3′) (Cooke *et al.*, 2000) and ITS-4 (5′-TCC TCCGCTTATTGATATGC-3′) (White *et al.*, 1990). The programme used for amplification of the ITS region was: 95°C for 3 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min. The final extension was carried out at 72°C for 5 min.

Primers OomCoxILevup (5′-TCAWCWMGATGGCTTTTTT CAAC-3′) and Fm85mod (5′-RRHWACKTGACTDATRATACC AAA-3′) modified by Robideau *et al.* (2011) were used to amplify a 727 bp fragment of the mitochondrial *cox1* gene from 15 isolates of *P. acerina*. To extend this, an overlapping 553 bp fragment was amplified from five isolates of *P. acerina* with

Figure 1 Symptoms caused by *Phytophthora acerina* on 30–35-year-old *Acer pseudoplatanus* trees in forest stands of the Boscoincittà Park, 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.

forward primer OomCoxI-Levlomod (5′-TTTGGTTTTTTGG TCATCCAGAGG-3′) and reverse primer Fm83mod (5′-CCAA TAAAAAATAACCAAAAATG-3′). The former primer anneals at the same location as OomCoxI-Levlo (Robideau *et al.*, 2011) but in the forward orientation and based on *P. infestans* accession U17009, and the latter is identical to Fm83 but shortened by two bases at the 5′ end to match the Tm of primer OomCoxI-Levlomod. The PCR programme used for amplification of both *cox1* fragments was: 95°C for 2 min, followed by 35 cycles of 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.

A fragment of the β-tubulin gene was amplified and sequenced from 13 isolates of *P. acerina* using primers Btub_F1A (5′-GCCAAGTTCTGGGARGTSAT-3′), Btub_R1A (5′-CCTGGTA CTGCTGGTAYTCMGA-3′), Btub_F2 (5′-CGGTAACAACTGG GCCAAGG-3′) and Btub_R2 (5′-GATCCACTCAACGAAGTA CG-3′) according to Blair *et al.* (2008).

All fragments were sequenced in both orientations in dye terminator cycle sequencing reactions using the primers used for PCR on an ABI 3730 Genetic Analyzer (Applied Biosystems) at The James Hutton Institute. ABI trace files were analysed using SEQUENCHER v. 4.6 (GeneCodes).

Table 1 Isolates of the '*Phytophthora citricola* complex' used in this study

Culture no.[a]	Identification	Host	Location, year	Reference	ITS	*cox1*	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[f]	*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[b]		–	Japan, (Argentina)	Uddin *et al.* (unpubl.)	AB367492	–	–
CBS 181.25, IMI 077970	*P. pini* (ex-type)	*Pinus resinosa*, roots	Minnesota, USA, 1925	Hong (unpubl.)	FJ392322	–	–
22F3, P33		–	Ohio, USA	Hong (unpubl.)	FJ392321	–	–
CIT-US1[cf]		*Fagus sylvatica*, canker	New York State, USA, 2003	Jung & Burgess (2009)	FJ665234	FJ665242	FJ665253
CIT-US10[cf]		*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[d]		–	Spain	Moralejo (unpubl.)	DQ648146	–	–
OH6/5		*Quercus rubra*, soil	Ohio State, USA, 2004	Balci *et al.* (2007)	EF032477	–	–
IMI 031372[d]	*P. citricola* E	*Rubus idaeus*	Ireland	Cooke *et al.* (2000)	AF266788	–	–
112		–	Switzerland	Bragante *et al.* (unpubl.)	EU263906	–	–
87-302		*R. idaeus*	Switzerland, Grandcours	Lefort *et al.* (unpubl.)	EU000100.1		
83-41[d]		–	Switzerland, Angers	Lefort *et al.* (unpubl.)	EU000081	–	–
WAC 13201, CBS 124094	*P. multivora* (ex-type)	*Eucalyptus marginata*	Yalgorup, WA, 2007	Scott *et al.* (2009)	FJ237521	FJ237508	FJ665260
WAC 13200[c]	*P. multivora*	*E. gomphocephala*	Yalgorup, WA, 2007	Scott *et al.* (2009)	FJ237522	FJ237509	FJ665261
WAC 13204[c]		*E. gomphocephala*	Yalgorup, WA, 2007	Scott *et al.* (2009)	FJ237518	FJ237507	FJ665259
WAC 13205, CBS124095[c]		*E. marginata*	Jarrahdale, WA, 1998	Scott *et al.* (2009)	FJ237517	FJ237506	–
VHS 16168[c]		*Banksia grandis*	Pemberton, WA	Scott *et al.* (2009)	FJ237513	–	FJ665257
IMI 329674[c]		Soil	Walpole, WA	Scott *et al.* (2009)	–	FJ237504	–
VHS 16439[c]		*B. littoralis*	Mandarah, WA	Scott *et al.* (2009)	FJ237516	FJ237505	FJ665258
P1817[d]		*Medicago sativa*	South Africa	Kroon *et al.* (2004)	AB367494	–	AY564055
P10458[d]		–	–	Blair *et al.* (2008)	–	–	EU079582
P7902[d]		*Pinus radiata*	USA, 1992	Blair *et al.* (2008)	–	–	EU080236
PLU-A5, CBS 124093[c]	*P. plurivora* (ex-type)	*F. sylvatica,* root lesion	Irschenberg, Germany, 2004	Jung & Burgess (2009)	FJ665225	FJ665236	FJ665247
PLU-A9[c]	*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[c]		*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[c]		*F. sylvatica*, canker	Munich, Germany, 1995	Jung & Burgess (2009)	FJ665228	–	–
PLU41, CBS 124091[c]		*A. saccharum,* root	Mount Royal, Canada, 1996	Jung & Burgess (2009)	FJ665229	FJ665238	FJ665249

(continued)

Table 1 (continued)

Culture no.[a]	Identification	Host	Location, year	Reference	ITS	*cox1*	β-tubulin
PLU77[c]		*Q. robur,* nursery soil	Nettetal, Germany, 1999	Jung & Burgess (2009)	FJ665230	FJ665239	FJ665250
PLU92[c]		*Quercus sp.,* soil	Turkey, 2000	Jung & Burgess (2009)	FJ665231	FJ665240	FJ665251
PLU255[c]		*F. sylvatica*, canker	Sumuva, Czech Republic, 2007	Jung & Burgess (2009)	FJ665232	–	–
PLU276, CBS 124092[c]		*Carpinus betulus,* soil	Snagov, Romania, 2008	Jung & Burgess (2009)	FJ665233	FJ665241	FJ665252
P10338[d]				Blair *et al.* (2008)	–	–	EU079526
MN21HH[d]		*Rhododendron* sp.	USA	Schwingle *et al.* (2007)	DQ486661	–	–
InfGaul[e]		*Gaultheria shalon*	Scotland	Schlenzig (2005)	AY879291	AY894685	–
IMI 342898[e]		*Syringa vulgaris*	UK	Cooke *et al.* (2000) - ITS, Kroon *et al.* (2004) - *cox1*	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	(ex-type)	*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, CBS 133931[cfg]	*P. acerina* (ex-type)	*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951285	KC156134	KC201283
B080[cf]		*A. pseudoplatanus,* soil	Milan, Italy, 2010	This study	JX951291	KC156140	KC201289
B035[cfg]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951282	KC156131	KC201281
B053[cf]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951283	KC156132	KC201282
B054[c,f]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951292	KC156141	KC201290
B055[cf]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951293	KC156142	KC201291
B056[c,f]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951284	KC156133	–
B058[cf]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951286	KC156135	KC201284
B060[cfg]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951287	KC156136	KC201285
B062[cf]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951294	KC156143	KC201292
B063[cf]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951295	KC156144	KC201293
B064[cfg]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951288	KC156137	KC201286
B071[cfg]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951289	KC156138	KC201287
B077[cfg]		*A. pseudoplatanus*, canker	Milan, Italy, 2010	This study	JX951290	KC156139	KC201288
B081[cf]		*A. pseudoplatanus,* soil	Milan, Italy, 2010	This study	JX951296	KC156145	KC201294
CH95PHE28[d]		*Eustoma grandiflorum*	Japan	Villa *et al.* (2006)	AB217676	–	–
CH95PHE[d]		*E. grandiflorum*	Japan	Villa *et al.* (2006)	AB217676·1		
TARI23044[d]		*Prunus persica*	Taiwan, 2009	Ann *et al.* (unpubl.)	GU111596	–	–
P10366[d]		–	–	Coffey *et al.* (unpubl.)	GU259257	–	–

[a]Abbreviations of isolates and culture collections: CBS, Centraalbureau voor Schimmelcultures Utrecht, Netherlands; IMI, CABI Bioscience, UK; WAC, Department of Agriculture and Food Western Australia Plant Pathogen Collection, Perth, Australia; VHS, Vegetation Health Service of the Department of Environment and Conservation, Perth, Australia; other isolate names and numbers are as given in GenBank.
[b]Same code as isolate of Oudemans *et al.* (1994) which was collected in Argentina.
[c]Isolates used in statistical analysis.
[d]Submitted to GenBank as *P. citricola.*
[e]Submitted to GenBank as *P. inflata.*
[f]Isolates used in the morphological studies.
[g]Isolates used in the growth temperature studies.

Phylogenetic analysis

Sequences of related taxa were downloaded from manually curated lists of GenBank BLAST hits and lists in relevant publications on ITS Clade 2 taxa. These were combined with sequences of outgroup taxa from GenBank. In the case of the ITS region, disruption of the Clade 2 taxa alignment was prevented by a prior alignment of only the Clade 2 taxa sequences and subsequent alignment against sequences of the outgroup taxa using the CLUSTALW 'Profile' option. Mitochondrial *cox1* and β-tubulin sequences were aligned using CLUSTALW under default settings. All alignments were inspected and adjusted manually if required. Bayesian phylogenetic analysis was conducted using a stand-alone version of MRBAYES v. 3.1.1 (Ronquist & Heulsenbeck, 2003) in combination with tools available in TOPALI v. 2.5 (Milne *et al.*, 2008). Two duplicate MRBAYES analyses were run simultaneously for 2 million generations with a sampling frequency of every tenth generation and the first 20% of samples discarded as burn-in. The general time reversible model with gamma-distributed rate variation and a proportion of invariable sites was selected on the basis of the model selection in TOPALI. The default settings of a flat Dirichlet prior using the six-rate parameter of the GTR substitution model with three heated chains (temperature = 0·2) and one cold chain were used. A 4 × 4 model of nucleotide evolution was used for the ITS analysis and in the case of the *cox1* gene data a codon position model was applied. At the end of each run the convergence diagnostic potential scale reduction factor (PSRF) values for the taxon bipartitions were examined to ensure they were at or approaching a value of 1.

Colony morphology, growth rates and cardinal temperatures

Morphology of hyphae and colony growth patterns were described from 7-day-old cultures grown at 20°C in the dark on V8A, malt extract agar (MEA) and half-strength PDA (19·5 g PDA, 7·5 g agar, 1 L distilled water; Difco). 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 days along two lines intersecting the centre of the inoculum at right angles.

Morphology of sporangia and gametangia

Sporangia and gametangia of 15 isolates of *P. acerina* were measured on V8A as described by Jung *et al.* (2003). Because morphometric data of the same isolates may differ considerably between different studies, the authentic type of *P. citricola s. str.* (CBS295.29), the ex-type of *P. plurivora* (CBS124093) and *P. pini* isolates CIT US1 and CIT US10 (all used by Jung & Burgess, 2009) were included to verify whether the measurements of the present study could be compared with those published by Jung & Burgess (2009). Sporangia were produced by immersing 15 × 15 mm square agar plugs taken from growing margins of 3–5-day-old colonies in 90 mm Petri dishes filled with deionized water, and young leaves of *Quercus ilex* floating at the surface. After 6 h the water was replaced by nonsterile pond water. The plates were incubated at 20–25°C. After 24–36 h, dimensions and characteristic features of 50 mature sporangia per isolate chosen at random were determined at ×400 magnification (Axiophot dissecting microscope; Carl Zeiss). 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 15 × 15 mm square agar plug cut from the centre of 14–21-day-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 *c.* 5–10 mm) were collected in the field from a single mature tree of *A. pseudoplatanus* and *Fagus sylvatica* in March and May, respectively, shortly after budburst; leaves were removed and the twigs were cut into sections of *c.* 12 cm length.

In the centre of each cutting a *c.* 0·5 cm diameter piece of bark was removed aseptically with a razor blade. An even-sized V8A disc cut from the margin of freshly growing cultures of *P. acerina* isolates was placed on the wound, covered with the removed bark piece and autoclaved wet cotton, and sealed with Parafilm and aluminium foil. Five isolates were tested. Controls received only sterile V8A discs. Ten twigs were inoculated per isolate or control and placed in autoclaved glass Petri dishes containing two layers of moist filter paper. The plates were sealed with Parafilm and incubated at 20°C in the dark. After 21 days, lesion length was measured after removal of the outer bark. Random reisolations were made using PARPNH to fulfill Koch's postulates.

Statistical analysis

Statistical analyses were carried out using STATA11 (Stata Statistical software) to determine if morphometric and growth rate differences between *P. acerina* and other taxa of the '*P. citricola* complex' were statistically significant. As the 50 measurements for every character in each isolate showed a normal distribution, parametric tests were used. 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 four 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 bark 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, i.e. *P. lacustris*, *P.* taxon 'PgChlamydo', *P. gonapodyides*, *P. inundata* and *P.* taxon 'Walnut'.

Phylogenetic analysis

The 760 bp ITS sequences of 15 isolates of *P. acerina* were identical to each other but were not an exact match to any sequence in GenBank. They were most closely

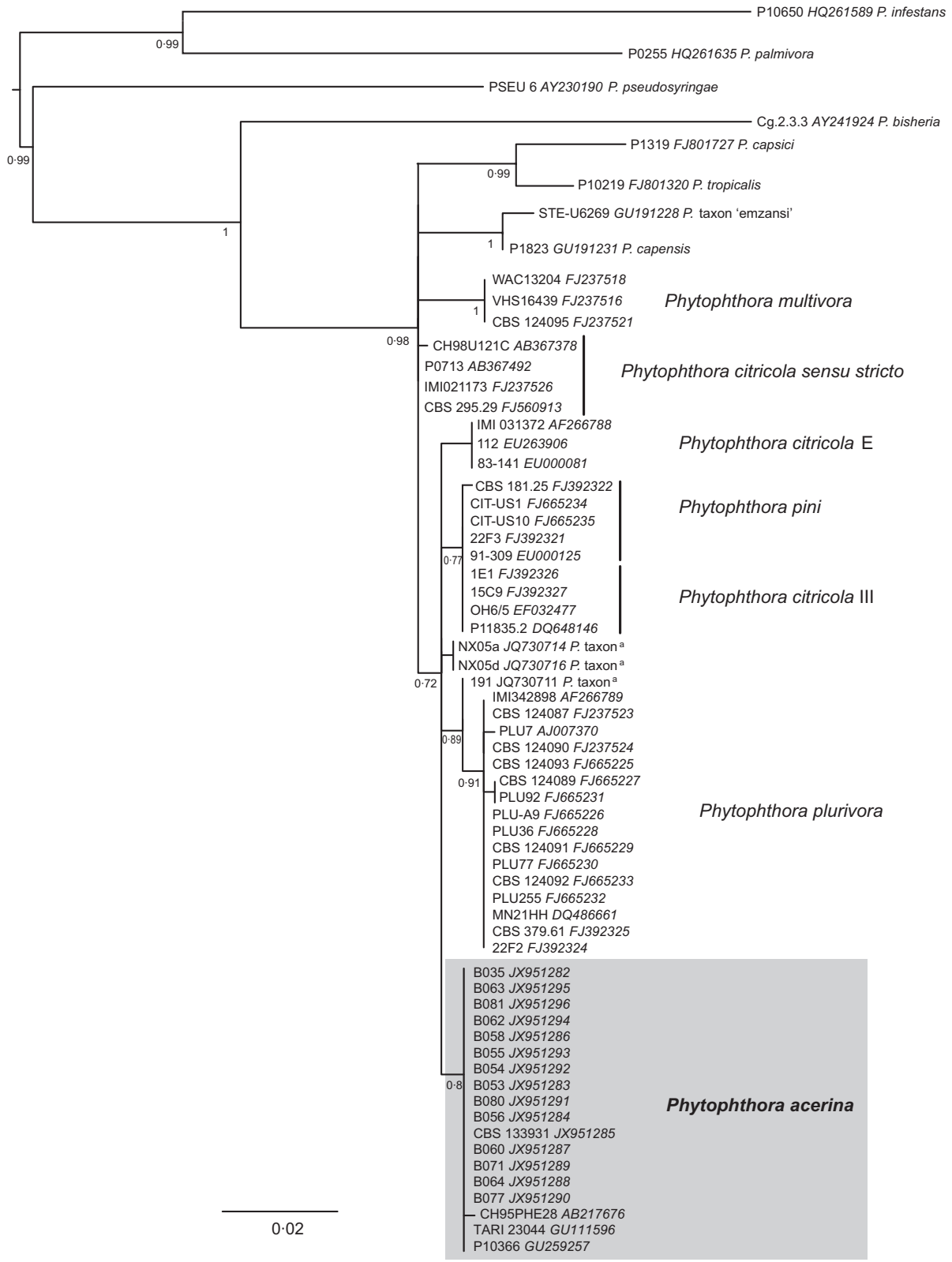

Figure 2 Bayesian inference tree based on the rDNA ITS sequences of isolates of *Phytophthora* in ITS Clade 2. Numbers below the branches indicate the posterior probability values. [a]Submitted to GenBank as *P. plurivora*.

Table 2 Polymorphic nucleotides (numbers to be read vertically) from aligned sequence data of ITS, *cox1* and β-tubulin gene regions showing the variation between isolates of *Phytophthora acerina*, *P. citricola s. str.*, *P. plurivora*, *P. pini*, *P. citricola* III and *P. citricola* E. Blue shading denotes polymorphisms found in *P. citricola s. str.*, green shows polymorphisms found in *P. plurivora*, orange shading is for polymorphisms found in *P. citricola* I, III and E and yellow shading highlights polymorphisms only found in *P. acerina*. Grey shading denotes no data available

ITS

Isolate no.	9	15	20	66	154	351	397	541	542	564	650	698	752
Phytophthora acerina[a]													
CBS 133931	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[b]													
TARI 23044	A	–	T	–	T	A	T	T	G	G	G	G	–
P10366	A	–	T	–	T	A	T	T	G	G	G	G	–
CH95PHE28	A	–	T	–	T	A	T	T	G	A	G	G	C
Phytophthora taxon[c]													
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.													
IMI 021173	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	–
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	–
PLU77	A	–	T	T	C	A	C	C	G	G	G	G	–
PLU92	A	–	T	T	C	T	C	C	G	G	G	G	–
InfGaul	A	–	T	T	C	A	C	C	G	G	G	G	–
PLU7	A	–	T	T	C	A	C	C	G	G	G	G	–
PLU-A9	A	–	T	T	C	A	C	C	G	G	G	G	–
PLU36	A	–	T	T	C	A	C	C	G	G	G	G	–
PLU255	A	–	T	T	C	A	C	C	G	G	G	G	–
IMI 342898	A	–	T	T	C	A	C	C	G	G	G	G	–
MN21HH	A	–	T	T	C	A	C	C	G	G	G	G	–
CBS 379.61	A	–	T	T	C	A	C	C	G	G	G	G	–
22F2	A	–	T	T	C	A	C	C	G	G	G	G	–
Phytophthora pini													
CIT-US1	A	A	T	–	T	A	T	C	G	G	G	A	–
CIT-US10	A	A	T	–	T	A	T	C	G	G	G	A	–
CBS 181.25	A	A	T	–	T	A	T	C	G	G	A	A	–
91-309	A	A	T	–	T	A	T	C	G	G	G	A	–
22F3	A	A	T	–	T	A	T	C	G	G	G	A	–

cox1

Isolate 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
Phytophthora acerina[a]																											
CBS 133931	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
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
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
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
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
Phytophthora acerina[b]																											
TARI 23044																											
P10366																											
CH95PHE28																											
Phytophthora taxon[c]																											
191																											
NX05a																											
NX05d																											
Phytophthora citricola s.str.																											
IMI 021173	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
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
PLU77									T	C	T	C	C	T	A	A	A	A	A	T	T	C	A	A	T	G	A
PLU92									T	C	T	C	C	T	A	A	A	A	A	T	T	C	A	A	T	G	A
InfGaul									T	C	T	C	C	T	A	A	A	A	A	T	T	C	A	A	T	G	A
PLU7																											
PLU-A9																											
PLU36																											
PLU255																											
IMI 342898																											
MN21HH																											
CBS 379.61																											
22F2																											
Phytophthora pini																											
CIT-US1									A	C	C	A	T	T	A	A	A	T	A	T	T	C	A	A	T	G	A
CIT-US10									A	C	C	A	T	T	A	A	A	T	A	T	T	C	A	A	T	G	A
CBS 181.25									A	C	C	A	T	T	A	T	A	T	A	T	T	C	A	A	T	A	A
91-309																											
22F3																											

β-tubulin

Isolate no.	308	382	414	829	1076
Phytophthora acerina[a]					
CBS 133931	G	C	A	T	T
B054	G	C	A	T	T
B055	G	C	A	T	T
B062	G	C	A	T	T
B063	G	C	A	T	T
B081	G	C	A	T	T
Phytophthora acerina[b]					
TARI 23044					
P10366					
CH95PHE28			T	T	
Phytophthora taxon[c]					
191					
NX05a					
NX05d					
Phytophthora citricola s.str.					
IMI 021173	G	C	A	C	T
CBS 295.29	G	C	A	C	T
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
PLU77	G	T	A	T	T
PLU92	G	C	A	T	T
InfGaul					
PLU7					
PLU-A9					
PLU36					
PLU255					
IMI 342898					
MN21HH					
CBS 379.61					
22F2					
Phytophthora pini					
CIT-US1	A	C	A	T	C
CIT-US10	A	C	A	T	C
CBS 181.25	A	C	A	T	C
91-309					
22F3					

Table 2 (Continued)

ITS

Isolate no.	9	15	20	66	154	351	397	541	542	564	650	698	752
Phytophthora citricola III													
1 E 1	A	–	T	–	T	A	T	C	G	G	G	A	–
15C9	A	–	T	–	T	A	T	C	G	G	G	A	–
P11835.2	A	–	T	–	T	A	T	C	G	G	G	A	–
OH6/5	A	–	T	–	T	A	T	C	G	G	G	A	–
Phytophthora citricola E													
IMI 031372	A	–	T	–	T	A	T	C	A	G	A	G	–
83-141	A	–	T	–	T	A	T	C	A	G	A	G	–
112	A	–	T	–	T	A	T	C	A	G	A	G	–

cox1

Isolate 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
Phytophthora citricola III																											
1 E 1									A	C	C	A	T	T	G	A	A	T	A	T	T	C	A	A	T	G	A
15C9									A	C	C	A	T	T	A	A	A	T	A	T	T	C	A	A	T	G	A
P11835.2																											
OH6/5																											
Phytophthora citricola E																											
IMI 031372																											
83-141																											
112																											

β-tubulin

Isolate no.	308	382	414	829	1076
Phytophthora citricola III					
1 E 1	A	C	A	T	C
15C9	A	C	A	T	C
P11835.2					
OH6/5					
Phytophthora citricola E					
IMI 031372					
83-141					
112					

[a]Isolates produced during this study.
[b]Submitted to GenBank as *P. citricola*.
[c]Submitted to GenBank as *P. plurivora*.

related to four GenBank accessions, three of which are shown in Figure 2. Each of the four accessions shared the base change at position 541 with all isolates of *P. acerina* and two accessions had a single discrete change at position 752 not shared with any isolate in the clade (Table 2). At position 564 all isolates of *P. acerina* had a clear heterozygous base (R = A/G) that was not reported in the GenBank accessions. However, two accessions had an A and two had a G at this location. All these isolates formed a distinct clade in the ITS tree within a closely related cluster of six taxa that also includes *P. plurivora*, *P. citricola* III, *P. pini* and *P. citricola* E, with *P. citricola s. str.* at a position slightly basal to the cluster (Fig. 2). Twelve ITS sequence differences have been shown to discriminate amongst these six taxa (Table 2).

Mitochondrial *cox1* sequence was generated for 14 isolates of *P. acerina,* with 1194 bp sequenced for five isolates and 694 bp for the remaining nine isolates. No sequence variation was observed amongst the *P. acerina* isolates. There were no exact matches on GenBank. Database coverage at the *cox1* locus is not as comprehensive as for the ITS region but sequences are available for isolates of the closely related *P. plurivora*, *P. citricola* III, *P. pini* and *P. citricola s. str.* Across these five taxa, 27 single base changes were observed over the 1194 bp of *cox1* sequence, eight of which were unique to isolates of *P. acerina* (Table 2). The phylogenetic analysis of *cox1* sequences indicated that isolates of *P. acerina* formed a distinct well-supported clade alongside the five other recognized taxa (Fig. 3).

Beta-tubulin gene sequences of 1100 bp in length were generated for 13 isolates of *P. acerina* and once again no intraspecific variation was observed. Five single base pair changes were observed amongst the closely related *P. acerina*, *P. plurivora*, *P. citricola* III, *P. pini* and *P. citricola s. str.* (Table 2) but there was insufficient phylogenetic signal (data not shown) in this gene to resolve these taxa. Two bases specific to *P. pini* and *P. citricola* III were noted but other differences were shared amongst *P. plurivora*, *P. citricola s. str.* and *P. acerina*.

Taxonomy

Phytophthora acerina B. Ginetti, T. Jung, D. E. L. Cooke, S. Moricca sp. nov. — MycoBank MB 804019; 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 homothallicum, solum partim functionale; oogonia in medio 50% abortiva, terminalia, lateralia

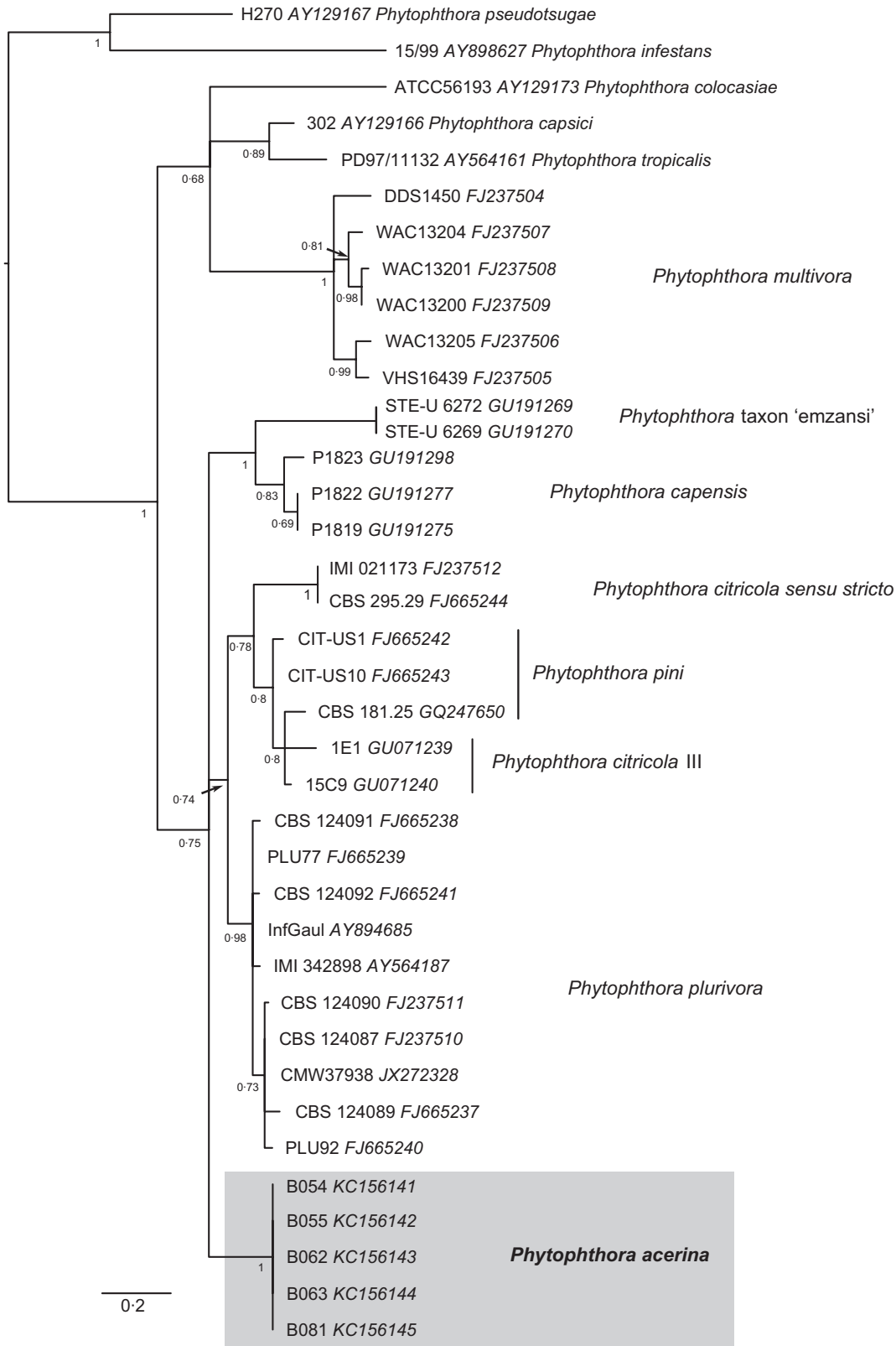

Figure 3 Bayesian inference tree based on sequences of the mitochondrial gene *cox1* of isolates of *Phytophthora* in ITS Clade 2. Numbers below the branches indicate the posterior probability values.

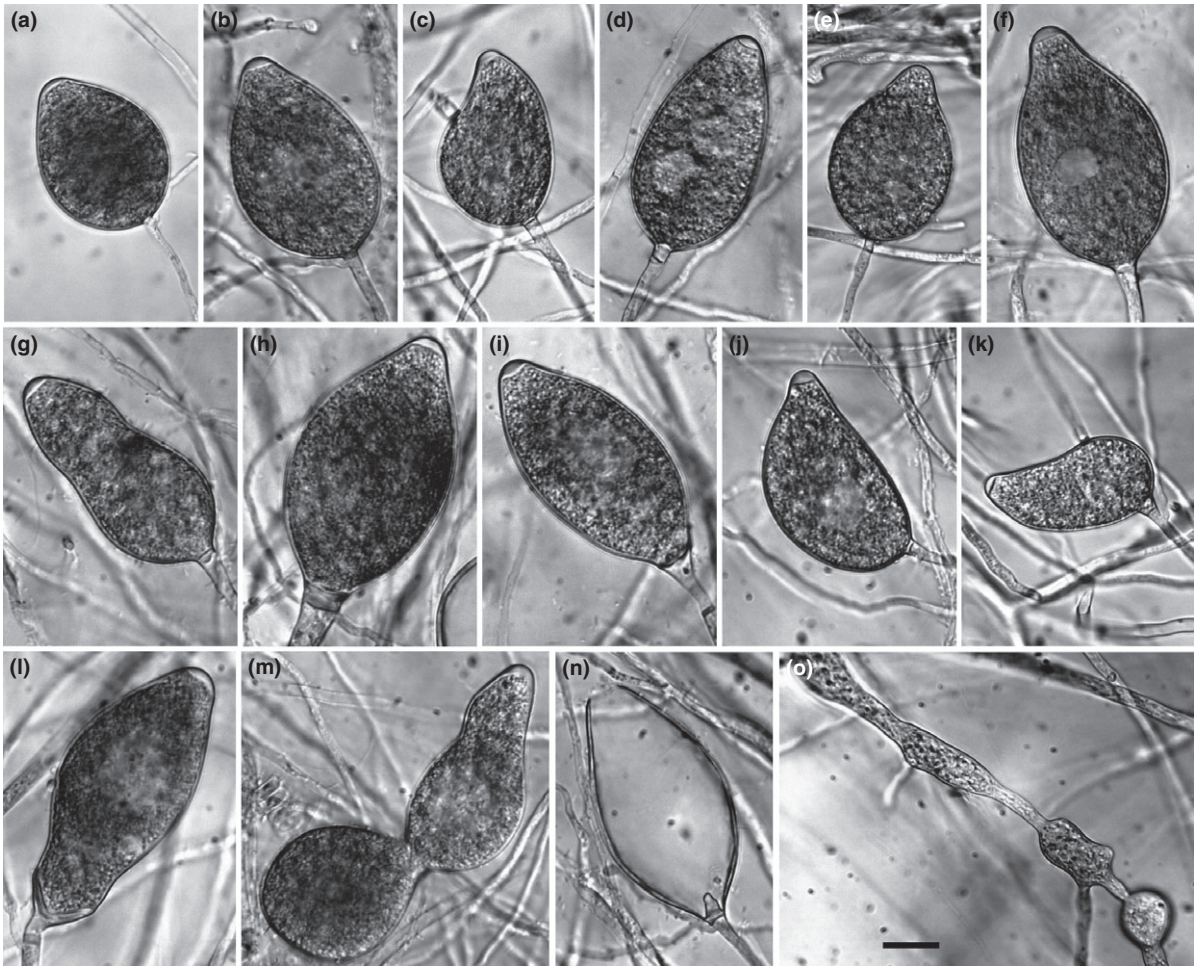

Figure 4 Semipapillate sporangia of *Phytophthora acerina* on V8 juice agar after 24–36 h flooding with nonsterile pond water. (a,b) ovoid; (c) mouse-shaped, intercalary inserted; (d) elongated-ovoid with vacuole; (e–g) obpyriform, (f,g) with vacuole; (h) limoniform, sporangiophore widening towards base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

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, in 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 crescentes in agaro 'V8A', optima *c.* 25°C et maxima c. 32°C. Coloniae in agaro 'V8A' chrysanthemum cum mycelio aerio restricto. Regiones 'rDNA ITS', '*cox1*' et 'β-tubulin' cum unica sequentia (GenBank JX951285, FJ665236, FJ665247).

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

Sporangia of *P. acerina* were rarely observed on solid agar but were produced abundantly in nonsterile pond water. Sporangia were typically borne terminally on unbranched sporangiophores. Small subglobose or irregular, sometimes catenulate hyphal swellings were infrequently formed on sporangiophores (Fig. 4o). Sporangia were persistent, semipapillate, less frequently bi- or tripapillate or bilobed (<1% over all isolates) and sometimes formed a conspicuous basal plug that protruded into the sporangium (Fig. 4n). In all isolates sporangial shapes showed a wide variation including ovoid (54·9% over all isolates; Fig. 4b), limoniform (14·1%; Fig. 4h), obpyriform (8·7%; Fig. 4e,f,g), ellipsoid (4·1%; Fig. 4i), elongated–ovoid (3·9%; Fig. 4d,n), obovoid (1·8%), broad–ovoid (1·5%; Fig. 4a), mouse-shaped (1·7%; Fig. 4c,j) and other distorted shapes (8·7%; Fig. 4l,m). Sporangia with special features such as curved apices (13·6% over all isolates; Fig. 4c,i,j,m), lateral attachment of the sporangiophore (10·3%; Fig. 4c), intercalary insertion (7·7%; Fig. 4k), hyphal swellings, sometimes catenulate, on the sporangiophore (2·4%; Fig. 4o), a short hyphal projection (1·2%; Fig. 4c), vacu-

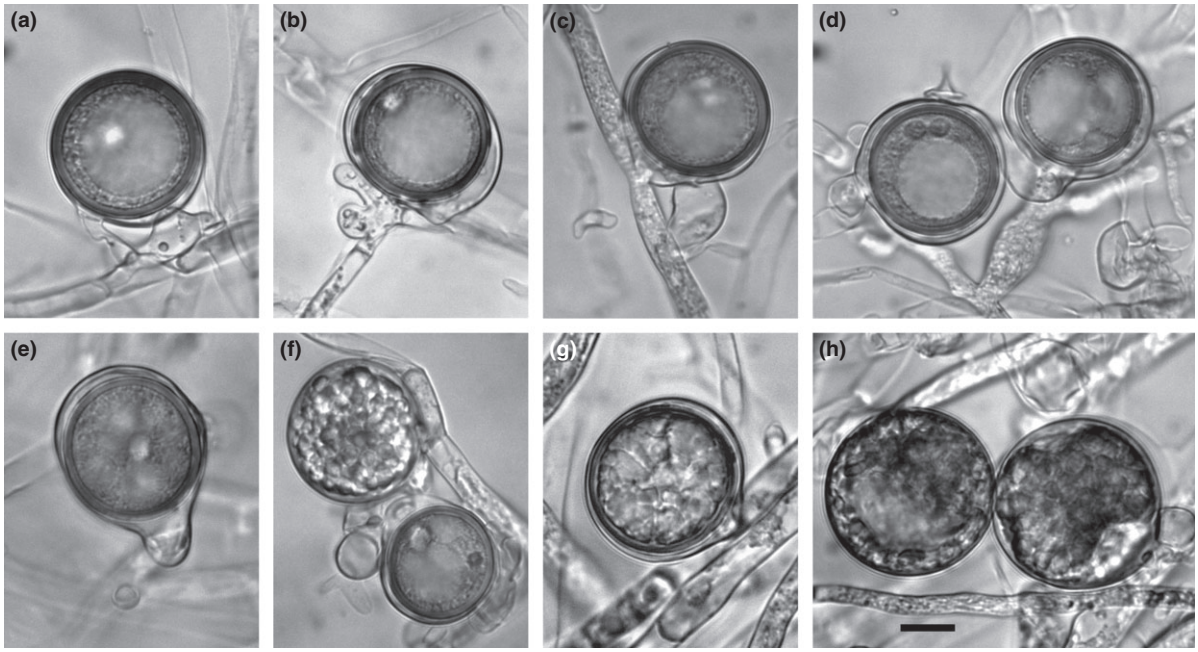

Figure 5 Oogonia of *Phytophthora acerina* formed in V8 juice agar. (a–e) Mature viable oogonia with oospores containing ooplasts, and paragynous antheridia; (a) plerotic oospore and paragynous antheridium with finger-like projection; (b) elongated oogonium with aplerotic oospore and paragynous antheridium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

oles (Fig. 4b,d,f,i,j,l,m) and a widening of the sporangiophore towards the base of the sporangium (Fig. 4h) 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. 4n). Sporangial dimensions of 15 isolates of *P. acerina* averaged 52·0 ± 13 × 32·8 ± 7·7 μm (overall range 20·3–105·7 × 11·1–51·3 μm) with a length/breadth (l/b) ratio of 1·6 ± 0·3 (overall range 1·2–2·6; range of isolate means 1·4–2·0). Mean sporangial dimensions of the authentic type of *P. citricola s. str.* (CBS295.29), the ex-type of *P. plurivora* (CBS124093) and *P. pini* isolates CIT US1 and CIT US10 deviated from the data in Jung & Burgess (2009) by less than 2 μm (data not shown).

Phytophthora acerina is homothallic with paragynous antheridia. Oogonia of *P. acerina* were borne terminally or were laterally sessile (Fig. 5c,g), had smooth walls and were usually globose to slightly subglobose (Fig. 5a, c,d,g,h). Elongated oogonia (8·6% over all isolates; Fig. 5e), slightly elongated oogonia (2·7%; Fig. 5f) and excentric oogonia (3·9%; Fig. 5b) were present in all isolates. In some isolates oogonial 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 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,d,e) 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. 5f,h) or immediately after the formation of the oospore wall (Fig. 5g). The mean abortion rate of 14 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 (overall range 0·8–3·4; range of isolate means 1·8–2·4) and a mean oospore wall 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–d,f), globose to obovoid, club-shaped or irregular, sometimes with a finger-like projection (2% over all isolates), and usually attached close to the oogonial stalk. They measured 12·8 ± 3·4 × 9·5 ± 1·6 μm. Mean oogonia and oospore diameters of the authentic type of *P. citricola s. str.* (CBS295.29), the ex-type of *P. plurivora* (CBS124093) and *P. pini* isolates CIT US1 and CIT US10 deviated from the data in Jung & Burgess (2009) by less than 2 μm and oospore wall diameters were almost identical to those reported by Jung & Burgess (2009) (data not shown).

In ageing cultures (3–6 weeks old) and in nonsterile pond water, *P. acerina* isolates produced globose to subglobose and coralloid hyphal swellings (Fig. 6a,b,c) and

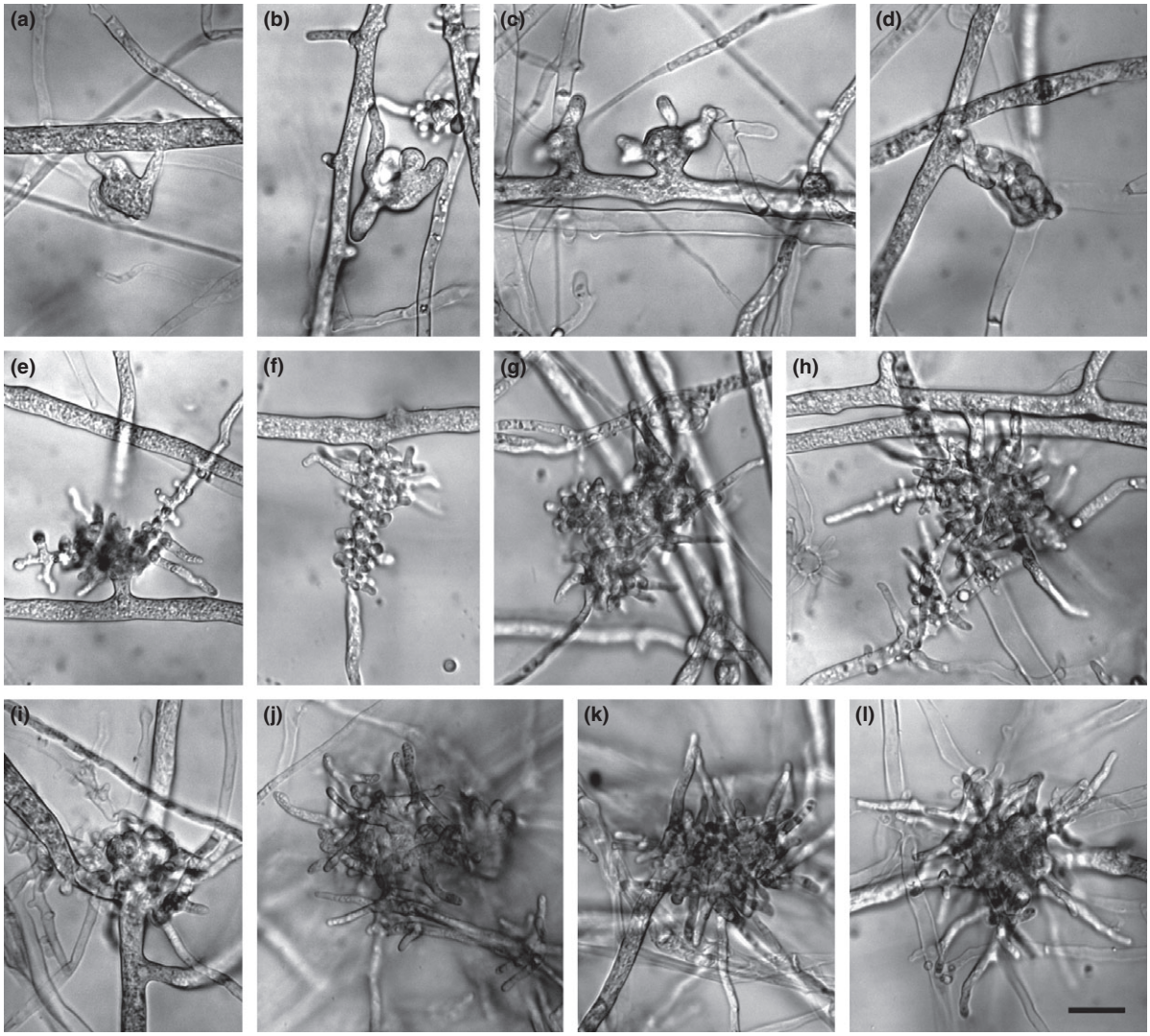

Figure 6 Vegetative structures formed by *Phytophthora acerina* in V8 juice agar. (a,b) Swollen lateral hyphal swellings; (c) lateral globose swelling with radiating short hyphae; (d) two lateral hyphae twisting around each other; (e) multiple branching at the end of a short lateral hypha; (f) multiple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

abundant hyphal aggregations (Fig. 6d–l). The hyphal aggregations started with multiple lateral branching of short sections along the hyphae (Fig. 6f,k,l) or by multiple branching at the end of main hyphae (Fig. 6i) 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. The authentic type of *P. citricola s. str.* (CBS295.29), the ex-type of *P. plurivora* (CBS124093) and *P. pini* isolates CIT US1 and CIT US10 did not form dense hyphal aggregations. Very rarely, in nonsterile pond water loose intermingling and twisting of individual hyphae could be observed in cultures of the authentic type of *P. citricola s. str.* and the ex-type of *P. plurivora*.

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 woolly aerial mycelium. Colony growth patterns of *P. citricola s. str.* (CBS295.29), the ex-type of *P. plurivora* (CBS124093) and *P. pini* isolates CIT US1 and CIT US10 on all three agar media corresponded well with those reported by Jung & Burgess (2009) (data not shown). Diameters of primary

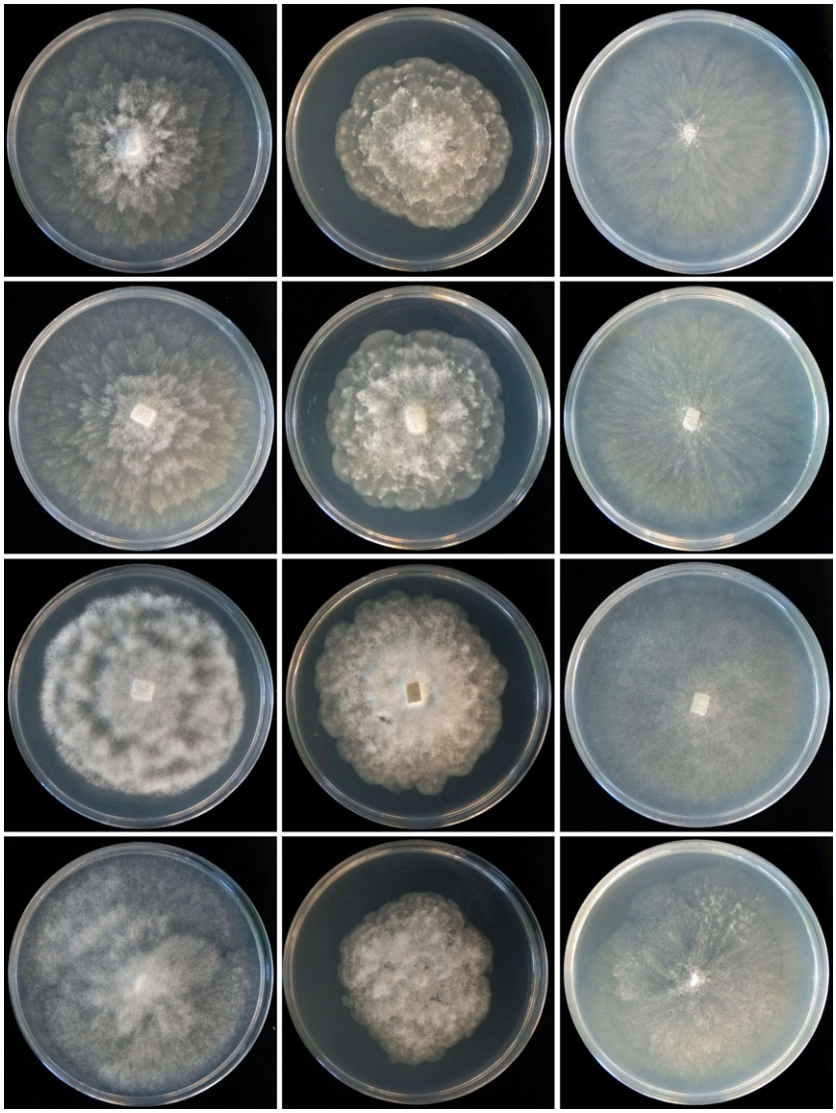

Figure 7 Colony morphology of *Phytophthora acerina* isolates B077, B063, B080 and CBS 133931 (from top to bottom) after 7 days' growth at 20°C on V8 juice agar, potato dextrose agar and malt extract agar (from left to right).

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 unable to grow at 35°C and did recommence growth when plates previously incubated for 5 days at 35°C were transferred to 18–20°C. Optimum temperature for growth was 25°C with radial growth rates ranging from 7·5 to 7·9 mm per day. At 20°C *P. acerina* showed growth rates of 6·5 ± 0·2 mm per day on V8A and 4·3 ± 0·2 mm per day on PDA.

Specimens examined

Typus: Italy: Boscoincittà Park, Milan, isolated from bleeding bark canker of *Acer pseudoplatanus*, 2010, B. Ginetti. Holotype B057 (dried culture on V8A, herbarium of the Museum of Natural History of the University of Florence, Italy, under accession no. FI AGR 057). Ex-type culture CBS 133931. For other specimens examined see Table 1.

Under-bark inoculation test

All five isolates of *P. acerina* were pathogenic to 1-year-old twigs from mature trees of both *A. pseudoplatanus* and *F. sylvatica* (Fig. 9).

After 22 days, mean lesion length on *A. pseudoplatanus* was 9·9 ± 0·3 cm (overall range 6·9–11·3 cm) while lesion 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

A detailed list of morphological and physiological characters and data of *P. acerina* and other known taxa of

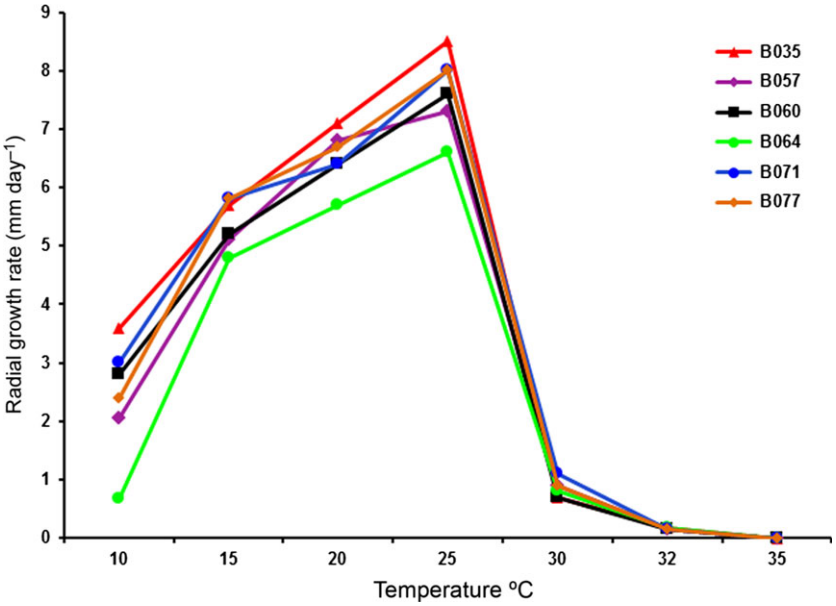

Figure 8 Radial growth rates of six isolates of *Phytophthora acerina* on V8 juice agar at different temperatures.

Figure 9 Lesions caused by *Phytophthora acerina* in the under-bark 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).

the '*P. citricola* complex' is given in Table 3. *Phytophthora acerina* differs from the other taxa by its abundant production of dense stromata-like hyphal aggregations in both solid agar and in nonsterile liquid culture. In addition, no other taxon except for *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.

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'. The oospore wall index of *P. acerina* was significantly lower than in *P. multivora* (0·37 vs 0·52; $P < 0{\cdot}001$) but significantly higher than in all other taxa ($P < 0{\cdot}001$; Table 3).

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 woolly mycelium (Fig. 7). On PDA the rosaceous to petaloid colonies clearly discriminated *P. acerina* from *P. plurivora* (chrysanthemum), *P. citricola s. str.* (striate) and *P. multivora* (uniform) (Jung & Burgess, 2009). Similar to *P. plurivora*, *P. citricola s. str.*, *P. citricola* E and *P. multivora*, *P. acerina* had a growth optimum at 25°C whereas the optimum temperature was 22·5°C for *P. capensis* and *P.* taxon 'emzansi' and 30°C for *P. pini* (Jung & Burgess, 2009; Bezuidenhout *et al.*, 2010). At 32·5°C the maximum temperature for growth in *P. acerina* was markedly higher than in *P. capensis* and *P.* taxon 'emzansi' (27·5°C; Bezuidenhout *et al.*, 2010).

Discussion

In the first comprehensive phylogenetic analysis of the genus *Phytophthora* the major ITS Clade 2 contained seven species of which only *P. citricola* and *P. inflata*

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*[a]	*P. pini*	*P. plurivora*	*P.* taxon 'emzansi'
No. of isolates investigated	15[b]	3[c]	2[d]	6[e]	2[d]	4[f]	7[d]	2[c]
Sporangia								
l × b mean	52 ± 13 × 32·8 ± 7·7	39·1 ± 6 × 24 ± 3·3	52 ± 7·9 × 29·9 ± 5·1	51 ± 10·4 × 30 ± 5·1	53·7 ± 6·5 × 33·8 ± 3·9	47·4 × 31·5	47·4 ± 7·7 × 33·5 ± 5·9	46·9 ± 8·6 × 27·4 ± 5·7
Range of isolate means	42·5–61·6 × 26·8–38·3		50·9–52 × 29·9	44·2–62·1 × 26·2–34·2	51·2–56·2 × 33·5–34·1		39·6–52·3 × 28·9–38·8	
Total range	20·3–105·7 × 11·1–51·3	27·5–50 × 17·5–32·5	36–75 × 21–40	36–58 × 13–33	39–70 × 20–42·1	31·5–75·3 × 22·9–38·9	27·5–80·5 × 16·7–69·6	35–67·5 × 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 diameter	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
Range of isolate means	28·1–36·3		29·7–30·3	25·5–27·8	30·9–31·4		27·5–29·9	
Total 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
Oospores								
Aplerotic oospores	69·6% (40–96%)		44% (32–56%)	45% (36–52%)	43% (38–48%)		44·3% (22–62%)	47%
Mean diameter	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
Total 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 diameter	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[g]	0·33 ± 0·05	0·52 ± 0·07	0·34 ± 0·05		0·3 ± 0·06	0·45[h]
Antheridia								
l × b mean	12·8 ± 3·4 × 9·48 ± 1·6	9 ± 1·8 × 9·1 ± 1·7	12·8 ± 2·7 × 8·2 ± 1·7	12·9 ± 1·9 × 8·7 ± 1·3	12·2 ± 2·1 × 9·0 ± 1·6	12·6 × 11·1	11·1 ± 4·4 × 8·4 ± 3·1	14·5 ± 2·1 × 14 ± 1·4
l × b range	5·4–28·5 × 5·1–15·2	5–12·5	7·5–18·5 × 5·4–14·4	8–20 × 5–14	7·7–16·9 × 6·1–12·6	10·2–15 × 10·2–12·9	7–21 × 5·3–16	10–20 × 12·5–17·5
Abortion rate	38·5% (10–99%)							42–46%[i]
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 per day)	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 per day)								
V8A	6·52 ± 0·20	6·6[g]	6·2 ± 0·04	4·8 ± 0·6	6·3 ± 0·23		6·3 ± 0·1	5[g]
PDA	4·28 ± 0·16		2·0 ± 0·2	3·3 ± 0	6·5 ± 0·42		3·2 ± 0·2	

[a]Designated as *P. citricola* I in Jung & Burgess (2009).
[b]Six of the 15 isolates were included in the growth tests.
[c]Data from: Bezuidenhout *et al.* (2010).
[d]Data from: Jung & Burgess (2009).
[e]Data from: Scott *et al.* (2009).
[f]Data from: Hong *et al.* (2011).
[g]Growth rate on CA at 20°C.
[h]Values calculated from data in Bezuidenhout *et al.* (2010).
[i]Aborted plus immature oospores.

were homothallic with paragynous antheridia (Cooke *et al.*, 2000). This clade has since expanded considerably including, in 2012, 25 species and informally designated taxa (Jung & Burgess, 2009; Bezuidenhout *et al.*, 2010; Hong *et al.*, 2011; Kroon *et al.*, 2012). A molecular re-evaluation demonstrated that the original *P. inflata* 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* 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 subclade 2a and the two more distantly related new species *P. mengei* and *P. elongata* belonging to subclade 2c (Jung & Burgess, 2009; Bezuidenhout *et al.*, 2010; Hong *et al.*, 2011; Kroon *et al.*, 2012).

Phylogenetic analysis of the ITS, *cox1* 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 analysed isolates of *P. acerina* shared identical ITS, *cox1* and β-tubulin sequences. In GenBank, ITS sequences from four isolates designated as *P. citricola* can be found that are similar (but not completely homologous) to *P. acerina*. The ITS sequence of 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, are identical to each other (GU111596.1 and GU259257.1, respectively). *Phytophthora acerina* differs from these two isolates by 1 bp. 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* by 2 bp. The presence of A and G in these isolates at position 564 where *P. acerina* has an R suggests these isolates might be ancestral to *P. acerina* but mtDNA data is required to verify this. In the ITS region, *P. acerina* differs from *P. citricola s. str.* 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 *cox1* sequence data clearly discriminated *P. acerina* as an evolutionarily distinct lineage amongst the '*P. citricola* complex'. Differences between *P. acerina* and other taxa ranged from 15–16 bp (*P. plurivora*) to 23 bp (*P. citricola s. str.*). As shown in other studies, the levels of variation are much lower within the β-tubulin gene (Jung & Burgess, 2009; Hong *et al.*, 2011).

Besides many smaller differences to individual taxa which are listed in the Notes, the high oospore abortion rate of 40–96% and the abundant production of dense stromata-like hyphal aggregations clearly discriminate *P. acerina* from all other members of the '*P. citricola* complex'. Such hyphal aggregations are not produced by any other taxon of the '*P. citricola* complex' but are known from *P. cinnamomi*, *P. lateralis* and *P. gregata*, a species with a comparably high oospore abortion rate, (Brasier *et al.*, 2010; Jung *et al.*, 2011, 2013a). The hyphal aggregations of *P. acerina* resembled the stromata formed by *P. ramorum* beneath the cuticle of infected leaves (Moralejo *et al.*, 2006), and it is likely that they play a role as survival structures in the life cycle of *P. acerina*.

The genetic uniformity of all 15 sequenced isolates, pathogenicity 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 nursery stands, horticultural plantations and planted and seminatural forest stands in Europe is identical to *P. acerina,* strongly supports the hypothesis that there has been a relatively recent clonal introduction of *P. acerina* to northern Italy. The origin of *P. citricola s. str.* in Japan and Taiwan and recent findings of *P. acerina*-like isolates in Japan (CH95PHE28 and CH95PHE31) and Taiwan (TARI 23044), and of *P. plurivora* or *P. plurivora*-like isolates in remote, undisturbed healthy forests in Nepal (Vettraino *et al.*, 2011), Taiwan (T. Jung and M. Horta Jung, University of Algarve, Faro, Portugal, unpublished) and Yunnan (GenBank nos. JQ730711.1, JQ730714.1, JQ730715.1 and JQ730716.1; Huai *et al.*, 2013) provide indirect evidence that the centre of origin of *P. acerina* might also lie in Eastern Asia.

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 shoot dieback on multiple hosts from different genera and families. In particular, *P. plurivora* has a wide host range with 45 species in 16 dicotyledonous and four coniferous families, respectively, listed in the original description including *A. pseudoplatanus* and several other *Acer* spp. (Jung & Burgess, 2009). In the pathogenicity test of the present study, *P. acerina* was aggressive to bark of its host species *A. pseudoplatanus,* indicating that it might pose a serious threat to maple stands in Italy and the rest of Europe if it cannot be contained in the forest stands of the Boscoincittà Park near Milan and if it is not immediately eradicated wherever it may turn up in nurseries and plantations in the future. More pathogenicity tests are required to determine the potential host ranges of *P. acerina*, *P. pini*, *P. multivora*, *P. citricola* III and the not yet 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 was introduced a long time ago and has become well established in seminatural ecosystems, plantings and nurseries across Europe, and the 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.

Their most likely pathway into Europe and between and within countries of Europe is the import of plants-for-planting and the intense European nursery trade (Brasier, 2008; Jung & Burgess, 2009; Jung *et al.*, 2013b). Consequently, a pathway regulation approach based on pathway risk analyses and recent scientific knowledge about pathogen biology, intense inspection regimes and the regular use of modern molecular detection tools and protocols at the ports of entry are urgently required to minimize the risks of further introductions of both known and unknown potential pathogens to Europe.

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References

Balci Y, Balci S, Eggers J *et al.*, 2007. *Phytophthora* spp. associated with forest soils in eastern and north-central U.S. oak ecosystems. *Plant Disease* **91**, 705–10.

Bezuidenhout CM, Denman S, Kirk SA, Botha WJ, Mostert L, McLeod A, 2010. *Phytophthora* taxa associated with cultivated *Agathosma*, with emphasis on the *P. citricola* complex and *P. capensis* sp. nov. *Persoonia* **25**, 32–49.

Blair JE, Coffey MD, Park SY, Geiser DM, Kang S, 2008. A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology* **45**, 266–77.

Brasier CM, 2008. The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* **57**, 792–808.

Brasier CM, Jung T, 2003. Progress in understanding *Phytophthora* diseases of trees in Europe. In: McComb JA, Hardy GEStJ, eds. Phytophthora *in Forests and Natural Ecosystems*. Proceedings of the 2nd Int. IUFRO Working Party 7.02.09 Meeting, Albany, Western Australia. Perth, Australia: Murdoch University Print, 4–18.

Brasier CM, Scott J, 1994. European oak declines and global warming: a theoretical assessment with special reference to the activity of *Phytophthora cinnamomi*. *EPPO Bulletin* **24**, 221–32.

Brasier CM, Cooke DEL, Duncan JM, Hansen EM, 2003. Multiple new phenotypic taxa from trees and riparian ecosystems in *Phytophthora gonapodyides–P. megasperma* ITS Clade 6, which tend to be high-temperature tolerant and either inbreeding or sterile. *Mycological Research* **107**, 277–90.

Brasier CM, Vettraino AM, Chang TT, Vannini A, 2010. *Phytophthora lateralis* discovered in an old growth *Chamaecyparis* forest in Taiwan. *Plant Pathology* **59**, 595–603.

Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM, 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology* **30**, 17–32.

Dick MW, 1990. *Keys to* Pythium. Reading, UK: University of Reading Press.

Erwin DC, Ribeiro OK, 1996. *Phytophthora Diseases Worldwide*. St Paul, MN, USA: APS Press.

Hong C, Gallegly ME, Richardson PA, Kong P, 2011. *Phytophthora pini* Leonian resurrected to distinct species status. *Mycologia* **103**, 351–60.

Huai W-X, Tian E, Hansen EM *et al.*, 2013. Identification of *Phytophthora* species baited and isolated from forest soil and streams in northwestern Yunnan province, China. *Forest Pathology* **43**, 87–103.

Jung T, 2009. Beech decline in central Europe driven by the interaction between *Phytophthora* infections and climatic extremes. *Forest Pathology* **39**, 73–94.

Jung T, Burgess TI, 2009. Re-evaluation of *Phytophthora citricola* isolates from multiple woody hosts in Europe and North America reveals a new species, *Phytophthora plurivora* sp. nov. *Persoonia* **22**, 95–110.

Jung T, Blaschke H, Neumann P, 1996. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. *European Journal of Forest Pathology* **26**, 253–72.

Jung T, Blaschke H, Osswald W, 2000. Involvement of soilborne *Phytophthora* species in central European oak decline and the effect of site factors on the disease. *Plant Pathology* **49**, 706–18.

Jung T, Nechwatal J, Cooke DEL *et al.*, 2003. *Phytophthora pseudosyringae* sp. nov., a new species causing root and collar rot of deciduous tree species in Europe. *Mycological Research* **107**, 772–89.

Jung T, Stukely MJC, Hardy GEStJ *et al.*, 2011. Multiple new *Phytophthora* species from ITS Clade 6 associated with natural ecosystems in Australia: evolutionary and ecological implications. *Persoonia* **26**, 13–39.

Jung T, Colquhoun IJ, Hardy GEStJ, 2013a. New insights into the survival strategy of the invasive soilborne pathogen *Phytophthora cinnamomi* in different natural ecosystems in Western Australia. *Forest Pathology* **43**, 266–88.

Jung T, Vettraino AM, Cech TL, Vannini A, 2013b. The impact of invasive *Phytophthora* species on European forests. In: Lamour K, ed. *Phytophthora: A Global Perspective*. Wallingford, UK: CABI, 146–58.

Kroon LPNM, Bakker FT, van den Bosch GBM, Bonants PJM, Flier WG, 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology* **41**, 766–82.

Kroon LP, Brouwer H, de Cock AW, Govers F, 2012. The genus *Phytophthora* anno 2012. *Phytopathology* **102**, 348–64.

Milne I, Lindner D, Bayer M *et al.*, 2008. TOPALi v. 2: a rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops. *Bioinformatics* **25**, 126–27.

Moralejo E, Puig M, García JA, Descals E, 2006. Stromata, sporangiomata and chlamydosori of *Phytophthora ramorum* on inoculated Mediterranean woody plants. *Mycological Research* **110**, 1323–32.

Oudemans P, Förster H, Coffey MD, 1994. Evidence for distinct isozyme subgroups within *Phytophthora citricola* and close relationships with *P. capsici* and *P. citrophthora*. *Mycological Research* **98**, 189–99.

Robideau GP, de Cock AWAM, Coffey MD *et al.*, 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I (COI) and internal transcribed spacer (ITS). *Molecular Ecology Resources* **11**, 1002–11.

Ronquist F, Heulsenbeck JP, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–4.

Schlenzig A, 2005. First report of *Phytophthora inflata* on nursery plants of *Rhododendron* spp., *Gaultheria shalon*, and *Vaccinium vitis-idaea* in Scotland. *Plant Pathology* **54**, 582.

Schubert R, Bahnweg G, Nechwatal J *et al.*, 1999. Detection and quantification of *Phytophthora* species which are associated with root-rot diseases in European deciduous forests by species-specific polymerase chain reaction. *European Journal of Forest Pathology* **29**, 169–88.

Schwingle BW, Smith JA, Blanchette RA, 2007. *Phytophthora* species associated with diseased woody ornamentals in Minnesota nurseries. *Plant Disease* **91**, 97–102.

Scott PM, Burgess TI, Barber PA *et al.*, 2009. *Phytophthora multivora* sp. nov., a new species recovered from declining *Eucalyptus*, *Banksia*, *Agonis* and other plant species in Western Australia. *Persoonia* **22**, 1–13.

Vettraino AM, Barzanti GP, Bianco MC *et al.*, 2002. Occurrence of *Phytophthora* species in oak stands in Italy and their association with declining oak trees. *Forest Pathology* **32**, 19–28.

Vettraino AM, Brasier CM, Brown AV, Vannini A, 2011. *Phytophthora himalsilva* sp. nov. an unusually phenotypically variable species from a remote forest in Nepal. *Fungal Biology* **115**, 275–87.

Villa NO, Kageyama K, Asano T, Suga H, 2006. Phylogenetic relationships of *Pythium* and *Phytophthora* species based on ITS rDNA, cytochrome oxidase II and beta-tubulin gene sequences. *Mycologia* **98**, 410–22.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: a Guide to Methods and Applications*. San Diego, CA, USA: Academic Press, 315–22.