RESEARCH ARTICLE

Re-evaluation of *Phytophthora citricola* isolates from multiple woody hosts in Europe and North America reveals a new species, *Phytophthora plurivora* sp. nov.

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Abstract   During large-scale surveys for soilborne *Phytophthora* species in forests and semi-natural stands and nurseries in Europe during the last decade, homothallic *Phytophthora* isolates with paragynous antheridia, semi-papillate persistent sporangia and a growth optimum around 25 °C which did not form catenulate hyphal swellings, were recovered from 39 host species in 16 families. Based on their morphological and physiological characters and the similarity of their ITS DNA sequences with *P. citricola* as designated on GenBank, these isolates were routinely identified as *P. citricola*. In this study DNA sequence data from the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S gene of the rRNA operon, the mitochondrial *cox*1 and β-tubulin genes were used in combination with morphological and physiological characteristics to characterise these isolates and compare them to the ex-type and the authentic type isolates of *P. citricola*, and two other taxa of the *P. citricola* complex, *P. citricola* I and the recently described *P. multivora*. Due to their unique combination of morphological, physiological and molecular characters these semipapillate homothallic isolates are described here as a new species, *P. plurivora* sp. nov.

INTRODUCTION

Phytophthora is a major genus of plant pathogens within the *Oomycota*, kingdom *Straminipila*. Until the mid 1990s, only 54 *Phytophthora* species had been described worldwide (Erwin & Ribeiro 1996). Since then, knowledge on *Phytophthora* species and the diseases they are causing in trees has been growing rapidly. In Europe alone, a remarkable array of 23 new taxa have been recovered from forests and semi-natural ecosystems, of which 13 have been officially described. The main reasons for this discovery boom were large-scale surveys for soilborne *Phytophthora* species in more than a thousand forest and semi-natural stands and in nurseries stimulated by several devastating declines and diebacks of major forest tree species, in particular oak decline, beech decline and alder dieback (Jung et al. 2000, Vettraino et al. 2002, Balci & Halmschlager 2003a, b, Jung & Blaschke 2004, Jung 2009). Additionally, the development and increased availability of molecular tools has helped to uncover new *Phytophthora* species which had previously been misidentified as known species because they are morphologically and physiologically indistinguishable or almost so (Man in't Veld et al. 2002, Brasier et al. 2003, Jung et al. 2003, de Cock & Lévesques 2004).

During these extensive surveys in Europe, homothallic *Phytophthora* isolates with paragynous antheridia, semipapillate persistent sporangia and a growth optimum around 25 °C which did not form catenulate hyphal swellings were recovered from many host species showing high transparency and dieback of crowns, small-sized and often yellowish foliage, extensive fine root losses, root lesions, collar rots, aerial cankers and shoot dieback, respectively (Fig. 1). Based on their morphological and physiological characters and similarity of their ITS sequence data to other GenBank sequences designated as *P. citricola*, these isolates were identified as *P. citricola*. This species was first described by Sawada (1927) from brown rot of citrus in Taiwan. Unfortunately, the lack of a formal diagnosis caused considerable confusion, and in early identification keys *P. citricola* was considered being conspecific with *P. cactorum* (Tucker 1931, Leonian 1934). In 1932 homothallic isolates with a flat, wide papilla were described as *P. cactorum* var. *applanata* (Chester 1932) which was accepted by Leonian (1934). Eventually, Waterhouse (1957) investigated both original *P. citricola* isolates from Sawada and isolates designated as *P. cactorum* var. *applanata* and concluded that they belong to the same species, *P. citricola* having priority. However, morphological variation within *P. citricola* isolates has repeatedly been reported (Zentmyer et al. 1974, Oudemans et al. 1994, Balci & Halmschlager 2003a, b, Jung et al. 2005). Morphological and molecular studies using a broad range of *P. citricola* isolates have demonstrated that *P. citricola* is very diverse (Oudemans et al. 1994, Bhat & Browne 2007, Moralejo et al. 2008). In the isozyme study of Oudemans et al. (1994) a global collection of 125 isolates of *P. citricola* clustered into five distinct subgroups (CIT1-5). Using an SSCP fingerprinting technique *P. citricola* was divided into four different subgroups, *P. citricola* I to IV (Kong et al. 2003, Gallegly & Hong 2008). These observations, in addition to the wide host and geographic range of *P. citricola* (Erwin & Ribeiro 1996, Fontaneto et al. 2008), strongly suggested a species complex comprising of several morphologically similar, but genetically distinct species. With this in mind, a large group of isolates from Western Australia obtained from dead or dying plants in natural ecosystems by the Vegetation Health Service (VHS) and misidentified as *P. citricola* for over 30 years, have recently been described as *P. multivora* (Scott et al. 2009). Several isolates on GenBank identified as *P. citricola* had identical sequences to *P. multivora*.

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Fig. 1   a. Upper crown of a mature declining beech (*Fagus sylvatica*) with high transparency, brush- and claw-like structures and severe dieback of branches due to extensive fine root losses; b. crown of a mature declining oak (*Quercus robur*) with high transparency, formation of leaf clusters and dieback of branches due to extensive fine root losses; c. small woody root (diam 2–3 mm) of a declining mature beech with severe losses of lateral roots and fine roots caused by *P. plurivora*; d. small woody roots (diam 2–3 mm) of a declining mature oak with severe losses of lateral roots and fine roots caused by *P. plurivora*; e. collar rot of mature beech caused by *P. plurivora* with tarry spots on the outer bark; f. stem of declining mature beech in a mountain forest in Bavaria with a series of isolated aerial cankers caused by *P. plurivora*; g. leaf necrosis and shoot dieback of *Rhododendron* sp. caused by *P. plurivora*.

In this study DNA sequence data from the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S gene of the rRNA operon and part of the mitochondrial *cox*1 and β-tubulin genes were used in combination with morphological and physiological characteristics to characterise these European *P. 'citricola'* isolates, and compare them to the ex-type and the authentic type isolates of *P. citricola*, *P. multivora* and *P. citricola* I. Due to their unique combination of morphological and physiological characters and sequence data, the semipapillate homothallic isolates from a multitude of hosts in Europe and North America are described here as a new species, *P. plurivora* sp. nov.

MATERIAL AND METHODS

Sampling and Phytophthora isolation

Sampling of rhizosphere soil and necrotic bark were according to Jung (2009). Soil samples were taken from mature and young declining trees of 39 species from 12 dicotyledonous and four coniferous families (see detailed species list in Table 1) in forests and parks, and from a multitude of nurseries across Europe. Necrotic bark was sampled from mature trees of European beech, Black and Grey alder (*Alnus glutinosa*, *Al. incana*), Norway maple (*Acer platanoides*), Common horse chestnut (*Aesculus hippocastanum*) and Canadian hemlock (*Tsuga canadensis*) in Germany, Austria, Italy and Switzerland. Necrotic fine roots were sampled from mature declining *Quercus robur* and *Q. petraea* trees across Germany and from mature declining Sugar maple trees (*Acer saccharum*) at Mount Royal in Montreal, Canada.

Isolations from soil samples were carried out at 18−20 °C using 2−7 d old leaflets of *Q. robur* and *F. sylvatica* seedlings as baits (Jung et al. 1996, Jung 2009). Isolations were also made from water of four streams and Lake Constance in Germany by floating apple fruits and rhododendron leaves as baits for 7 d on the surface of the water bodies. Infected baits were blotted dry on filter paper, cut into small pieces 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) and incubated at 20 °C. Necrotic bark and root samples were flooded for 2−3 d to remove excess polyphenols, and then cut into small pieces and plated directly onto PARPNH-agar (Jung 2009). Colonies growing from plated bait and bark sections were transferred to V8 agar for initial confirmation as *Phytophthora* species.

Table 1 Host range and distribution of *Phytophthora plurivora*.

Host	Sample type	Country[1] (year of first isolation)	References
Abies alba	Nursery soil	D (1998)	This study
Ab. fraseri	n.k.	USA (n.k.)	GenBank
Acer campestre	Rhizosphere soil	D (2007), CH (2000)	Jung et al. 2009, this study
Ac. platanoides	Aerial canker, collar rot, rhizosphere soil	D (1995), CH (2000)	Jung & Blaschke 1996, Jung et al. 2009, this study
Ac. pseudoplatanus	Aerial canker, collar rot rhizosphere soil	D (2007), A (2007)	Jung et al. 2009, this study
Ac. saccharum	Fine roots	CDN (1996)	This study
Aesculus hippocastanum	Aerial canker, collar rot, nursery & rhizosphere soil	D (1995), CH (2000), NL (2005)	Jung & Blaschke 1996, this study
Alnus glutinosa	Aerial canker, collar rot, nursery & rhizosphere soil	D (1998), A (2005), RO (2008)	Jung & Blaschke 2004, this study
Al. incana	Aerial canker, collar rot, nursery & rhizosphere soil	D (1998), A (2005)	Jung & Blaschke 2004, this study
Al. viridis	Nursery soil	D (2000)	Jung & Blaschke 2004
Betula pendula	Rhizosphere soil	D (2007)	Jung et al. 2009, this study
Buxus sempervirens	n.k.	CH (n.k.)	GenBank
Calluna vulgaris	Fine roots, nursery soil	D (2005)	This study
Carpinus betulus	Rhizosphere soil	D (1998), CH (2000), RO 2008)	This study
Carya sp.	Rhizosphere soil	D (2008)	This study
Castanea sativa	Rhizosphere soil	I (1998)	Vettraino et al. 2001
Chamaecyparis lawsoniana	Collar rot, rhizosphere soil	D (2006), I (2007)	This study
Cornus mas	n.k.	BG (n.k.)	GenBank
Corylus colurna	Nursery soil, rhizosphere soil	D (2007)	This study
Fagus sylvatica	Collar rot, aerial canker, root rot, fine roots, nursery soil, rhizosphere soil	D (1995), CH (2000), A (2007), CZ (2007), SLO (2007)	Jung & Blaschke 1996, Jung 2009, Munda et al. 2007, this study
Fragaria × ananassa	n.k.	USA (n.k.)	GenBank
Hedera helix	Rhizosphere soil	I (2007)	This study
Ilex aquifolium	n.k.	CH (n.k.)	GenBank
Malus domestica	Rhizosphere soil	A (2007)	This study
Juglans regia	Rhizosphere soil	I (2001), D (2005)	Vettraino et al. 2003, this study
Panax quinquefolium	n.k.	USA (n.k.)	GenBank
Picea abies	Nursery soil, Rhizosphere soil	D (1998)	Jung & Blaschke 2004, this study
Pinus silvestris	Rhizosphere soil	D (2007)	This study
Pseudotsuga menziesii	Rhizosphere soil	D (2007)	This study
Quercus cerris	Rhizosphere soil	D (1995), I (1998), TR (1999)	Jung et al. 1996, Vettraino et al. 2002, Balci & Halmschlager 2003b
Q. petraea	Fine roots, rhizosphere soil	SLO (1995), D (1996), F (1996), I (1998), SRB (2002)	Jung et al. 1996, 2000, Vettraino et al. 2002, this study
Q. pubescens	Rhizosphere soil	I (1997)	Vettraino et al. 2002
Q. robur	Fine roots, rhizosphere soil	D (1994), CH (1995), I (1995), HU (1995), F (1998), L (1998), UK (1999), A (2000) SRB (2003)	Jung & Blaschke 1996, Jung et al. 1996, 2000, Balci & Halmschlager 2003a, Vettraino et al. 2001, this study
Q. rubra	Rhizosphere soil	D (1995),	Jung & Blaschke 1996
Rhododendron sp.	Shoot dieback, leaf necrosis, nursery & rhizosphere soil	D (1999), I (2006), USA (n.k.)	This study, GenBank
Robinia pseudacacia	Rhizosphere soil	I (1995)	Jung & Blaschke 1996
Salix alba	Nursery soil	D (1999)	This study
Sambucus nigra	Rhizosphere soil	D (2007)	This study
Sequoiadendron giganteum	Rhizosphere soil	D (2006)	This study
Taxus baccata	Rhizosphere soil	D (2006)	This study
Thuja plicata	Rhizosphere soil	D (2008)	This study
Tilia cordata	Nursery & rhizosphere soil	D (2000)	Jung et al. 2009, this study
T. × europaea	Nursery & rhizosphere soil	D (2006)	Jung et al. 2009, this study
T. platyphyllos	Rhizosphere soil	D (2007)	Jung et al. 2009, this study
Tsuga canadensis	Collar rot	D (2006)	This study

[1] A = Austria, BG = Bulgaria, CDN = Canada, CH = Switzerland, CZ = Czech Republic, F = France, D = Germany, GR = Greece, HU = Hungary, I = Italy, L = Luxembourg, NL = Netherlands, RO = Romania, SLO = Slovenia, SRB = Serbia, TR = Turkey, UK = United Kingdom, n.k. = not known.

Table 2 Isolates of *Phytophthora citricola*, *P. citricola* I–IV and E, *P. multivora* and *P. plurivora* considered in the phylogenetic study.

Identification	Culture no.[1]	Host	Location, year	Reference	ITS	cox1	β-tubulin
P. citricola (type)	IMI 021173, CBS 221.88[2]	*Citrus sinensis*, fruit	Taiwan, 1927	Scott et al. (2009)	FJ237526	FJ237512	FJ665255
P. citricola (authentic type)	CBS 295.29[2]	*Citrus* sp., leaf	Japan, 1929	This study	FJ560913	FJ665244	FJ665256
P. citricola	CH98U121C	–	Japan,	Uddin et al. (unpubl.)	AB367378	–	–
	Citri-P0713[3]	–	Japan, (Argentina)	Uddin et al. (unpubl.)	AB367492	–	–
P. citricola I	CBS 181.25, IMI 077970	*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	This study	FJ665234	FJ665242	FJ665253
	CIT-US10[2]	*F. sylvatica*, canker	New York State, USA, 2003	This study	FJ665235	FJ665243	FJ665254
	91-309	*Thuja* sp., canker	Maumens, Switzerland	Lefort et al. (unpubl.)	EU000125	–	–
P. citricola II	CBS 379.61	*Rhododendron* sp.	Germany, 1958	Hong (unpubl.)	FJ392325	–	–
	22F2, P52	*Acer saccharum*	New York State, USA, 1987	Hong (unpubl.)	FJ392324	–	–
P. citricola III	15C9	Irrigation water	Wisconsin, USA, 1985	Hong (unpubl.)	FJ392327	–	–
	1E1	–	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	–	–
P. citricola IV	15C8	Field soil	South Carolina, USA, 1997	Hong (unpubl.)	FJ392329	–	–
	15C7	*Hedera helix*	South Carolina, USA, 1997	Hong (unpubl.)	FJ392328	–	–
P. citricola E	IMI 031372[4]	*Rubus idaeus*	Ireland	Cooke et al. (2000)	AF266788	–	–
	112[4]	–	Switzerland	Bragante et al. (unpubl.)	EU263906	–	–
	83-141[4]	–	Switzerland, Angers	Lefort et al. (unpubl.)	EU000081	–	–
P. multivora (type)	WAC 13201, CBS 124094[2]	*Eucalyptus marginata*	Yalgorup, WA, 2007	Scott et al. (2009)	FJ237521	FJ237508	FJ665260
P. multivora	WAC 13200	*E. gomphocephala*	Yalgorup, WA, 2007	Scott et al. (2009)	FJ237522	FJ237509	FJ665261
	WAC 13204	*E. gomphocephala*	Yalgorup, WA, 2007	Scott et al. (2009)	FJ237518	FJ237507	FJ665259
	WAC 13205, CBS 124095[2]	*E. marginata*	Jarrahdale, WA, 1988	Scott et al. (2009)	FJ237517	FJ237506	–
	VHS 16168	*Banksia grandis*	Pemberton, WA	Scott et al. (2009)	FJ237513	FJ237502	FJ665257
	IMI 329674	Soil	Walpole, WA	Scott et al. (2009)	FJ237515	FJ237504	–
	VHS 16439	*B. littoralis*	Mandarah, WA	Scott et al. (2009)	FJ237516	FJ237505	FJ665258
	P1817[4]	*Medicago sativa*	South Africa	Kroon et al. (2004)	–	–	AY564055
	P10458[4]	–	–	Blair et al. (2007)	–	–	EU079582
	P7902[4]	*Pinus radiata*	USA, 1992	Blair et al. (2007)	–	–	EU080236
P. plurivora (type)	PLU-A5, CBS 124093[2]	*F. sylvatica*, root lesion	Irschenberg, Germany, 2004	This study	FJ665225	FJ665236	FJ665247
P. plurivora	PLU-A9	*F. sylvatica*, canker	Irschenberg, Germany, 2004	This study	FJ665226	–	–
	PLU7	*Q. robur*, soil	Pulling, Germany, 1994	Schubert et al. (1999)	AJ007370	–	–
	PLU9, CBS 124087[2]	*Q. robur*, soil	Pulling, Germany, 1994	Scott et al. (2009)	FJ237523	FJ237510	FJ665245
	PLU30, CBS 124089[2]	*Q. robur*, soil	Cornuda, Italy, 1995	Scott et al. (2009)	FJ665227	FJ665237	FJ665248
	PLU35, CBS 124090[2]	*Q. petraea*, soil	Ljubljana, Slovenia, 1995	Scott et al. (2009)	FJ237524	FJ237511	FJ665246
	PLU36	*F. sylvatica*, canker	Munich, Germany, 1995	This study	FJ665228	–	–
	PLU41, CBS 124091[2]	*Ac. saccharum*, root	Mount Royal, Canada, 1996	This study	FJ665229	FJ665238	FJ665249
	PLU77[2]	*Q. robur*, nursery soil	Nettetal, Germany, 1999	This study	FJ665230	FJ665239	FJ665250
	PLU92	*Quercus* sp., soil	Turkey, 2000	This study	FJ665231	FJ665240	FJ665251
	PLU255	*F. sylvatica*, canker	Sumava, Czech Republic, 2007	This study	FJ665232	–	–
	PLU276, CBS 124092[2]	*Carpinus betulus*, soil	Snagov, Romania, 2008	This study	FJ665233	FJ665241	FJ665252
	P10338[4]	–	–	Blair et al. (2007)	–	–	EU079526
	MN21HH[4]	*Rhododendron* sp.	USA	Schwingle et al. (2007)	DQ486661	–	–
	InfGaul[5]	*Vaccinium vitis-idaea*	Scotland	Schlenzig (2005)	AY879292	AY894684	–
	IMI 342898[5]	*Syringa vulgaris*	UK	Cooke et al. (2000) - ITS, Kroon et al. (2004) - cox1	AF266789	AY564187	–
Phytophthora sp.	P4242	*Ceanothus* sp.	Spain	Moralejo et al. (unpubl)	AY946259	–	EF050526

[1] 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 on GenBank.
[2] Isolates used in the morphological and growth-temperature studies.
[3] Same code as isolate of Oudemans et al. (1994) which was collected in Argentina.
[4] Submitted to GenBank as *P. citricola*.
[5] Submitted to GenBank as *P. inflata*.

Phytophthora isolates

The isolates used in the phylogenetic, morphological and physiological studies are given in Table 2. To avoid confusion with the different *P. citricola* subgroups, the ex-type and the authentic type isolates are referred to as *P. citricola* s.str. *Phytophthora citricola* I and III as designated by Kong et al. (2003) are retained and the group of isolates with identical ITS sequence to IMI 031372, the isolate used to represent *P. citricola* by Cooke et al. (2000) will be referred to as *P. citricola* E.

DNA isolation, amplification and sequencing

The *Phytophthora* isolates were grown on half strength potato-dextrose agar (PDA; 19.5 g Difco PDA from Becton, Dickinson & Company, Sparks, USA, 7.5 g of agar and 1 L of distilled water, Burgess et al. 2009) at 20 °C for 2 wk and the mycelium was harvested by scraping from the agar surface with a sterile blade and placed in a 1.5 ml sterile Eppendorf® tube. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder and genomic DNA was extracted according to Andjic et al. (2007). The region spanning the internal transcribed spacer (ITS)1-5.8S-ITS2 region 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 PCR reaction mixture, PCR conditions, the clean-up of products and sequencing were as described by Andjic et al. (2007).

For selected isolates two additional gene regions were sequenced. The mitochondrial gene *cox*1 was amplified with primers Fm84 (5' TTT AAT TTT TAG TGC TTT TGC) and Fm83 (5' CTC CAA TAA AAA ATA ACC AAA AAT G) (Martin & Tooley 2003). The PCR reaction mixture was the same as for the ITS region, but the PCR conditions were as described previously (Martin & Tooley 2003). Templates were sequenced in both directions with primers used in amplification, as well as primers FM 85 (5' AAC TTG ACT AAT AAT ACC AAA) and FM 50 (5' GTT TAC TGT TGG TTT AGA TG) (Martin & Tooley 2003). The β-tubulin region was amplified using the primers Btub F1 (5' GCC AAG TTC TGG GAG GTC ATC) (Blair et al. 2008) and Btub R1 (5' CCT GGT ACT GCT GGT ACT CAG) (Kroon et al. 2004). The PCR reaction mixture was the same as for the ITS region, but the PCR conditions were as described previously (Blair et al. 2008). Templates were sequenced in both directions with primers used in amplification. The clean-up of products and sequencing were the same as for the ITS region. All sequences derived in this study were deposited in GenBank and accession numbers are shown in Table 2.

Phylogenetic analysis

The *Phytophthora* isolates used in this study were compared with other closely related species (ITS clade 2, Cooke et al. 2000) and other *Phytophthora* species representative of other ITS clades. The ITS dataset contains additional representative sequences obtained from GenBank, including isolates designated as *P. citricola* I–IV (Kong et al. 2003, Gallegly & Hong 2008) (Table 2). Sequence data for the ITS region were initially assembled using Sequence Navigator v1.01 (Perkin Elmer) and aligned in Clustal X (Thompson et al. 1997). Manual adjustments were made visually by inserting gaps where necessary in Bioedit v5.0.6 (Hall 2001). Few sequences are available for other gene regions and thus the datasets for *cox*1 or β-tubulin are smaller. The first 540 bp of the *cox*1 dataset and 215 bp of the β-tubulin dataset were excluded to allow alignment with other sequence available on GenBank. There were no gaps in the *cox*1 or β-tubulin alignments. There were no isolates on GenBank for which all three gene regions used in this study were available, thus a combined analysis was not possible.

Parsimony analysis was performed in PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford 2003). The most parsimonious trees were obtained using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis 1992). Branch and branch node support was determined using 1 000 bootstrap replicates (Felsenstein 1985).

Bayesian analysis was conducted on the same individual dataset as that used in the parsimony analysis. First, MrModeltest v2.5 (Nylander 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v3.1 (Ronquist 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Two independent runs of Markov Chain Monte Carlo (MCMC) using 4 chains were run over 1 000 000 generations. Trees were saved each 1 000 generations, resulting in 10 001 trees. Burn-in was set at 51 000 generations (i.e. 51 trees), well after the likelihood values converged to stationary, leaving 9 950 trees from which the consensus trees and posterior probabilities were calculated.

All datasets and trees arriving from parsimony and Baysian analyses are available from TreeBASE (SN SN4309; http://www.treebase.org/treebase/index.html).

Colony morphology, growth rates and cardinal temperatures

Hyphal morphology 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 (all from Becton, Dickinson & Company, Sparks, USA). Colony morphologies were described according to Zentmyer et al. (1974), Erwin & Ribeiro (1996) and Jung et al. (2003).

For temperature-growth relationships, V8A plates of five isolates of *P. plurivora* and two isolates each of *P. multivora*, *P. citricola* I and *P. citricola* s.str. were incubated for 24 h at 20 °C to stimulate onset of growth. Then three replicate plates per isolate were transferred to 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 flooding 15 × 15 mm square agar discs taken from growing margins of 3–5 d old colonies, just over its surface, with non-sterile soil extract (200 g soil from a *Eucalyptus marginata* stand suspended in 500 mL demineralised water for 24 h at 18 °C and then the supernatant taken with a syringe and diluted to 10 % with deionised water) in 90 mm Petri dishes and incubating them in the dark at 18–22 °C at natural daylight. The soil extract was decanted and replaced again after 6 and 12 h, and after 24 to 36 h dimensions and characteristic features of 50 mature sporangia per isolate chosen at random were determined at ×400 magnification (BX51, Olympus). 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).
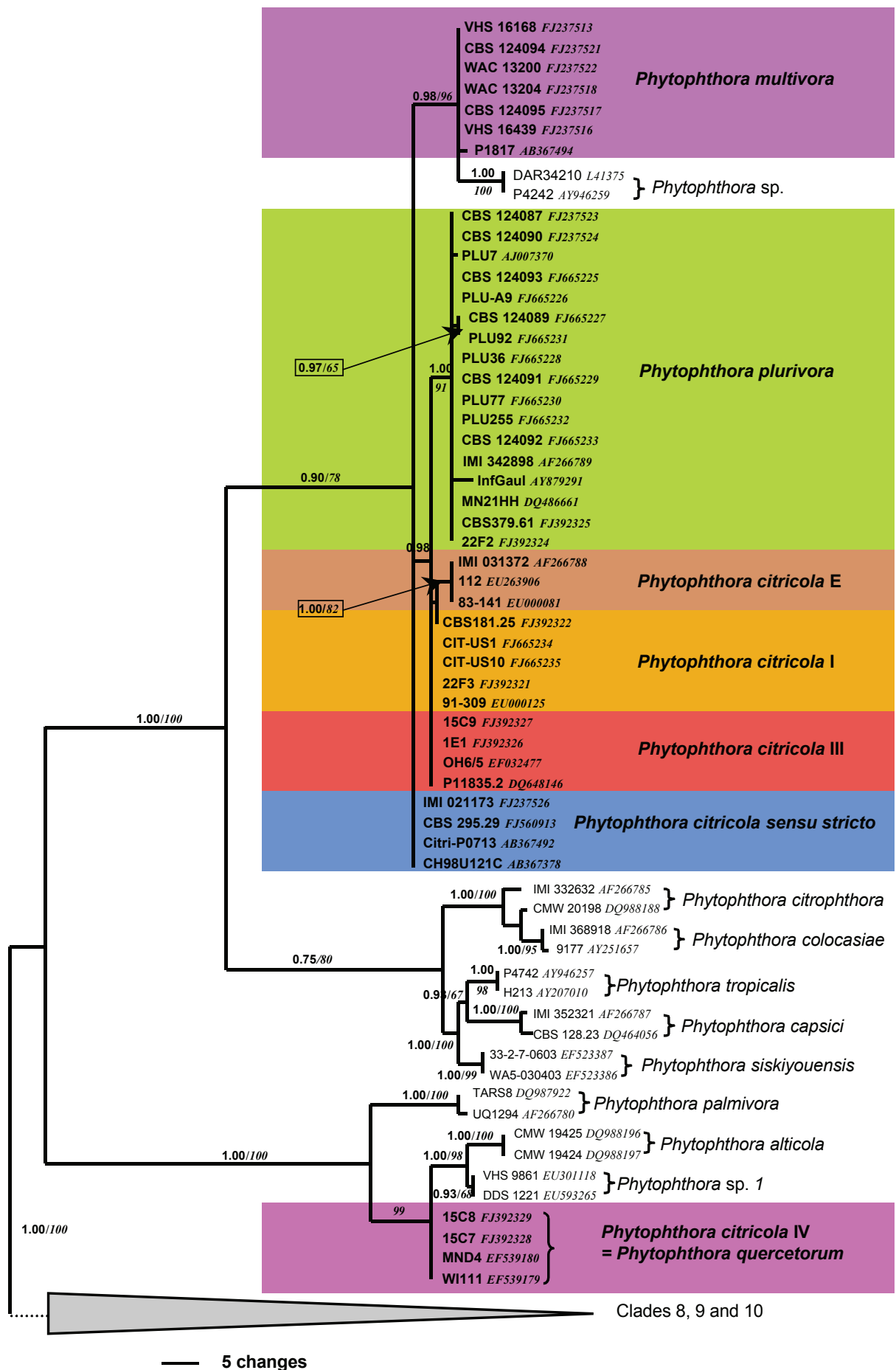
Fig. 2 Bayesian inference tree using rDNA ITS sequences showing phylogenetic relationships within the *P. citricola* complex. Numbers above branches in **bold** represent posterior probability based on Bayesian analysis of the dataset, numbers in *italics* represent bootstrap support for the nodes. Different colour boxes are used to differentiate the species recognised in the *P. citricola* complex.
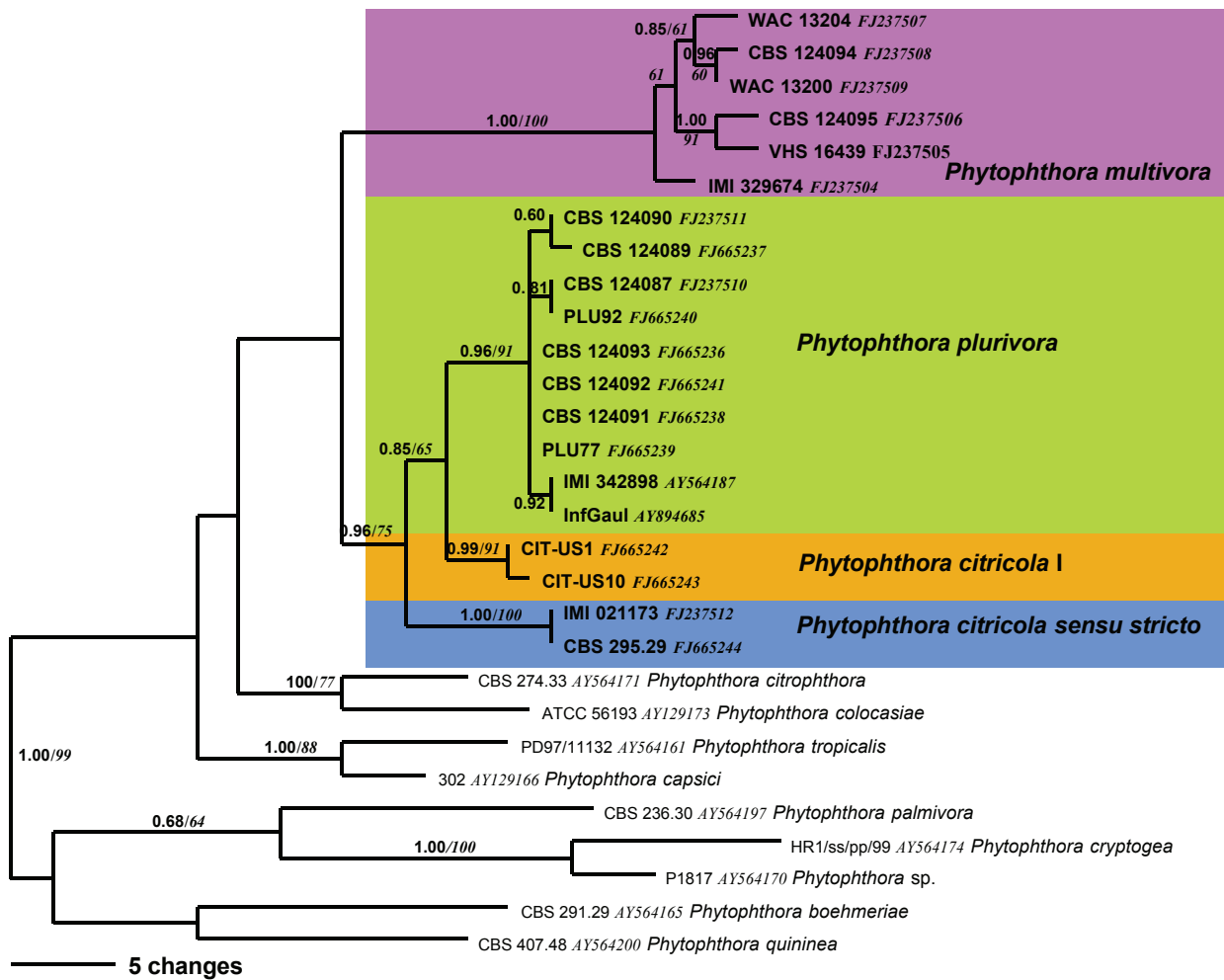
Fig. 3 One of 16 most parsimonious trees of 200 steps based on analysis of mitochondrial gene *cox*1 sequence, showing phylogenetic relationships within the *P. citricola* complex. Numbers above branches in **bold** represent posterior probability based on Bayesian analysis of the dataset, numbers in *italics* represent bootstrap support for the nodes. Different colour boxes are used to differentiate the species recognised in the *P. citricola* complex.

Statistical analysis

Analyses of Variances were carried out using Prism3 (Graph-Pad, San Diego, USA) to determine whether morphological and physiological measurements were significantly different between the different taxa.

RESULTS

Phylogenetic analysis

The ITS dataset consisted of 895 characters of which 258 were parsimony informative. The dataset contained significant phylogenetic signal compared to 1 000 random trees (p < 0.01, g1 = -1.12). Heuristic searches resulted in 6 most parsimonious trees of 453 steps (CI = 0.80, RI = 0.95). The topology of the Bayesian tree was very similar (TreeBASE SN4309) and is presented here (Fig. 2). The difference between the Bayesian and parsimony analysis is that in the former analysis isolates of *P. citricola* s.str. reside in a separate clade, whilst in the later analysis there is no bootstrap support to separate them from isolates of *P. citricola* I and III. In both analyses, isolates of *P. plurivora* reside in a strongly supported terminal clade along with several other sequences obtained from GenBank, including isolates designated as *P. inflata*. The additional isolates selected from GenBank for the analysis represent just a few over 100 sequences available which match *P. plurivora*. Most of this sequence data is for isolates from Europe. A few isolates

identical to that of IMI031372 (AF266788), the isolate used in the study of Cooke et al. (2000), form their own small moderately supported clade (*P. citricola* E). There are two sequences identical to *P. citricola* s.str., AB367492 and AB367378. Compared to *P. plurivora* there are far fewer sequences available which correspond to *P. citricola* I or III. Of those available more are from North America than from Europe and those from Europe have been found in nursery studies. Isolates designated as *P. citricola* IV (Kong et al. 2003) are identical to *P. quercetorum* (Balci et al. 2008) and are found in ITS clade 4, not ITS clade 2 with the isolates from the *P. citricola* complex.

The *cox*1 dataset consisted of 742 characters of which 93 were parsimony informative. The dataset contained significant phylogenetic signal compared to 1 000 random trees (p < 0.01, g1 = -1.00). Heuristic searches resulted in 16 most parsimonious trees of 200 steps (CI = 0.60, RI = 0.78). The topology of the Bayesian tree was very similar (TreeBASE SN4309) (Fig. 3). Species from ITS clade 2 group together with strong support. Isolates of *P. plurivora* reside in a strongly supported terminal clade separate from *P. citricola* s.str., *P. citricola* I and *P. multivora.*

There were few informative sites in the β-tubulin dataset among isolates from the *P. citricola* complex and the resultant trees are not presented (but available on TreeBASE, SN4309). Interestingly, β-tubulin sequences of *P. citricola* submitted to GenBank in previous studies match *P. multivora* rather than *P. plurivora* or *P. citricola* (Table 2).

Table 3 Polymorphic nucleotides from aligned sequence data of ITS, *cox*1 and β-tubulin gene regions showing the variation between isolates of *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* and orange shading is for those polymorphisms only found in *P. citricola* s.l. Grey shading denotes no data available.

Culture no.	ITS									*cox*1																β-tubulin			
	15	20	66	154	351	397	542	650	697	6	18	21	60	78	159	207	231	321	420	426	549	576	585	645	705	308	382	829	1076
Phytophthora citricola s.str.																													
CBS 221.88	–	C	T	T	A	T	G	G	G	A	T	C	A	T	G	A	T	T	A	A	G	T	T	C	T	G	C	C	T
CBS 295.29	–	C	T	T	A	T	G	G	G	A	T	C	A	T	G	A	T	T	A	A	G	T	T	C	T	G	C	C	T
Citri-P0713	–	C	T	T	A	T	G	G	G																				
CH98U121C	–	C	T	T	A	T	G	G	G																				
Phytophthora plurivora																													
CBS 124093	–	T	T	C	A	C	G	G	G	T	C	T	C	C	A	A	A	A	A	T	A	A	T	T	A	G	T	T	T
CBS 124087	–	T	T	C	A	C	G	G	G	T	C	T	C	C	A	A	A	A	C	T	A	A	T	T	A	G	T	T	T
CBS 124089	–	T	T	C	T	C	G	G	G	T	C	T	C	C	A	A	A	A	T	T	A	A	T	T	A	G	T	T	T
CBS 124090	–	T	T	C	A	C	G	G	G	T	C	T	C	C	A	A	A	A	T	T	A	A	T	T	A	G	T	T	T
CBS 124091	–	T	T	C	A	C	G	G	G	T	C	T	C	C	A	A	A	A	A	T	A	A	T	T	A	G	T	T	T
PLU77	–	T	T	C	A	C	G	G	G	T	C	T	C	C	A	A	A	A	A	T	A	A	T	T	A	G	T	T	T
PLU92	–	T	T	C	T	C	G	G	G	T	C	T	C	C	A	A	A	A	C	T	A	A	T	T	A	G	T	T	T
CBS 124092	–	T	T	C	A	C	G	G	G	T	C	T	C	C	A	A	A	A	A	T	A	A	T	T	A	G	T	T	T
PLU7	–	T	T	C	A	C	G	G	G																				
PLU-A9	–	T	T	C	A	C	G	G	G																				
PLU36	–	T	T	C	A	C	G	G	G																				
PLU255	–	T	T	C	A	C	G	G	G																				
IMI 342898	–	T	T	C	A	C	G	G	G																				
InfGaul	–	T	T	C	A	C	G	G	G																				
MN21HH	–	T	T	C	A	C	G	G	G																				
CBS 379.61	–	T	T	C	A	C	G	G	G																				
22F2	–	T	T	C	A	C	G	G	G																				
Phytophthora citricola I																													
CIT-US1	A	T	–	T	A	T	G	G	A	A	C	C	A	T	A	T	T	A	A	T	A	A	C	T	A	A	C	T	C
CIT-US10	A	T	–	T	A	T	G	G	A	A	C	C	A	T	A	T	T	A	A	T	A	A	C	T	A	A	C	T	C
91-309	A	T	–	T	A	T	G	G	A																				
CBS 181.25	A	T	–	T	A	T	G	A	A																				
22F3	A	T	–	T	A	T	G	G	A																				
Phytophthora citricola III																													
1E1	–	T	–	T	A	T	G	G	A																				
15C9	–	T	–	T	A	T	G	G	A																				
OH6/5	–	T	–	T	A	T	G	G	A																				
P11835.2	–	T	–	T	A	T	G	G	A																				
Phytophthora citricola E																													
IMI 031372	–	T	–	T	A	T	A	A	G																				
112	–	T	–	T	A	T	A	A	G																				
83-141	–	T	–	T	A	T	A	A	G																				

Across the three gene regions sequenced there are 18 fixed polymorphisms separating *P. plurivora* from *P. citricola* s.str.; three in ITS region, 13 in *cox*1 and 2 in β-tubulin (Table 3). For *P. citricola* III there are 2 fixed polymorphisms in the ITS region separating these isolates from *P. citricola* s.str. and 5 fixed polymorphisms in the ITS region separating them from *P. plurivora*. *Phytophthora citricola* E is separated from *P. citricola* s.str. by 3 fixed polymorphisms and 6 fixed polymorphisms separate *P. citricola* E from *P. plurivora* (Table 3). Two isolates sequenced in this study (CIT-US1 and CIT-US10) had ITS sequences identical to *P. citricola* I. Across the three gene regions these isolates as representatives of *P. citricola* I differed from *P. citricola* s.str. by 17 fixed polymorphisms and from *P. plurivora* by 15 fixed polymorphisms (Table 3).

Taxonomy

Phytophthora plurivora T. Jung & T.I. Burgess, *sp. nov.* — MycoBank MB512914; Fig. 4, 5

Sporangia abundantia in cultura liquida, persistentia, terminalia, interdum lateralia aut intercalaria, semi-papillata, ovoidea, obpyriformia aut limoniformia, rare distorta vel bipapillata, saepe cum obturamento conspicuo basale, apex interdum arcuatus, 49.9 ± 6.9 × 31.3 ± 4.8 µm, ratio longitudo ad altitudinem 1.6 ± 0.2 µm. Sporangiophora simplicia aut ramosa sympodiis laxis irregularibus; interdum inserta lateraliter ad sporangia, inflationes ad nodos rarae. Systema sexus homothallica; oogonia globosa vel rare subglobosa ad elongata, 28.4 ± 1.9 µm. Oosporae pleroticae aut apleroticae, 25.6 ± 1.6 µm, paries 1.6 ± 0.2 µm. Antheridia paragynosa, 10.6 ± 2.7 × 7.4 ± 1.5 µm. Chlamydosporae et inflationes hypharum non observatae. Temperaturae crescentiae in agaro 'V8A', optima c. 25 °C et maxima c. 32 °C. Coloniae in agaro 'V8A' radiatae cum mycelio aerio restricto. Regiones 'rDNA ITS', '*cox*1' et 'β-tubulin' cum unica sequentia (GenBank FJ665225, FJ665236, FJ665247).

Etymology: Name refers to the wide host range (pluri Lat = many, -vora Lat = feeding).

Sporangia (Fig. 4): Sporangia of all four taxa were rarely observed on solid agar but were produced abundantly in non-sterile soil extract. Interestingly, on the underside of 6 wk old cultures many viable sporangia were formed by both isolates of *P. citricola* s.str. but not by any isolates of *P. plurivora*, *P. multivora* and *P. citricola* I. Sporangia of *P. plurivora* were typically borne terminally on unbranched sporangiophores or more often in irregular lax or regular dense sympodia (Fig. 4b), and some were laterally attached or intercalary (Fig. 4g). Small subglobose hyphal swellings were sometimes formed at the nodes. Sporangia were non-caducous, semipapillate, less frequently bi- or tripapillate or bilobed (over all isolates < 5 %; Fig. 4h–j), and usually formed a conspicuous basal plug that protruded into the empty sporangium (Fig. 4b–d, k). Within all *P. plurivora* isolates sporangial shapes showed a wide variation ranging from ovoid (over all isolates 68 %; Fig. 4a, b, g, k) or limoniform (11.2 %; Fig. 4c–e) to obpyriform (7.2 %; Fig. 4f), ellipsoid (5.6 %), or distorted shapes (6.8 %; Fig. 4h–j, k). Sporangia with unusual features such as lateral attachment of the sporangiophore (over all isolates 17.6 %; Fig. 4b), markedly curved apices (16.4 %; Fig. 4b, c), a widening of the sporangiophore towards the base of the sporangium (1.6 %; Fig. 4d) or a short hyphal extension (1.2 %) were common in all
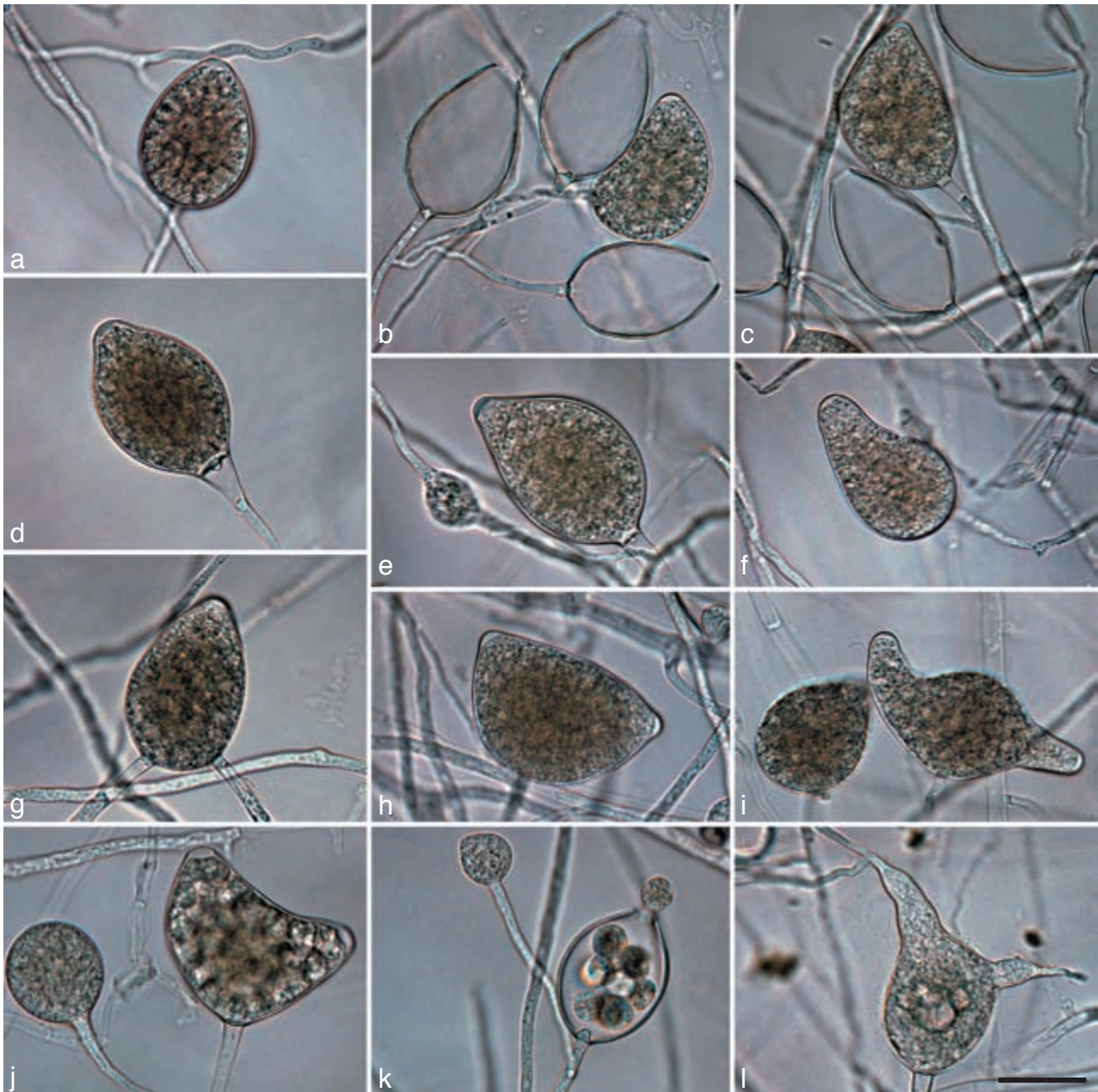
Fig. 4 Semipapillate sporangia of *Phytophthora plurivora* on V8 agar flooded with soil extract: a–i after 24–36 h flooding. a. Ovoid, the cytoplasm differenti-
ating into zoospores; b. laterally inserted mature sporangium with markedly curved apex, and dense sympodium of empty ovoid sporangia with conspicuous
basal plugs; c. mature sporangium with markedly curved apex, and empty limoniform sporangium; d. limoniform with tapering base and conspicuous basal
plug; e. limoniform, in the background small hyphal swelling on a sporangiophore; f. obpyriform; g. ovoid, intercalary inserted; h. bipapillate; i. bilobed; limoni-
form; j. young growing sporangium and bipapillate mature sporangium shortly before release of the zoospores; k. ovoid sporangium with conspicuous basal
plug releasing zoospores and proliferating externally; l. bipapillate sporangium germinating directly through both papillae after 48 h flooding with soil extract.
— Scale bar = 50 µm, applies to a–l.

isolates. In *P. plurivora* the proportion of sporangia with either
lateral attachment of the sporangiophore or curved apices was
higher than in *P. citricola* s.str. (6 % and 12 %, respectively),
P. citricola I (6 % and 11 %, respectively) and *P. multivora*
(9.3 % and 1.7 %, respectively). Zoospores of *P. plurivora* were
discharged through an exit pore 5–10 µm wide (av. 7.5 ± 1.0
µm) (Fig. 4k). They were limoniform to reniform whilst motile,
becoming spherical (av. diam = 10.1 ± 1.4 µm) on encystment.
Direct germination of sporangia was common in older water
cultures (Fig. 4l). Sporangial dimensions wood of seven isolates of
P. plurivora averaged 47.4 ± 7.7 × 33.5 ± 5.1 µm (overall range
27.5–80.5 × 16.7–69.6 µm) with a range of isolate means of
39.6–52.3 × 28.9–38.8 µm. The mean sporangial dimensions
of the ex-type and the authentic type of *P. citricola* s.str. (52 ±
7.9 × 29.9 ± 5.1 µm), two isolates of *P. citricola* I (53.7 ± 6.5 ×
33.8 ± 3.9) and six isolates of *P. multivora* (51.0 ± 10.4 ×

30.0 ± 5.1 µm) were on average significantly larger (p < 0.05)
than those of *P. plurivora*, but the ranges overlapped widely
(Table 4). With a length/breadth ratio of 1.43 ± 0.19 (range of
isolate means 1.25–1.61) the sporangia of *P. plurivora* were
on average significantly (p < 0.05) more squat than those of
P. citricola s.str. (1.73 ± 0.28, both isolates av. 1.73), *P. citricola* I
(1.6 ± 0.16) and *P. multivora* (1.7 ± 0.22, range of isolate
means 1.54–1.81). *Oogonia, oospores and antheridia* (Fig.
5a–g): Gametangia were readily produced in single culture
by all isolates of *P. plurivora*, *P. citricola* I, *P. multivora* and
P. citricola s.str. on V8A within 4 d. Oogonia of all four taxa were
borne terminally, had smooth walls and were usually globose to
slightly subglobose (Fig. 5a–d, f, g). In *P. plurivora*, *P. citricola*
s.str. and *P. multivora* elongated oogonia with a long tapering
base occurred only rarely (Fig. 5e) while in *P. citricola* I 16 % of
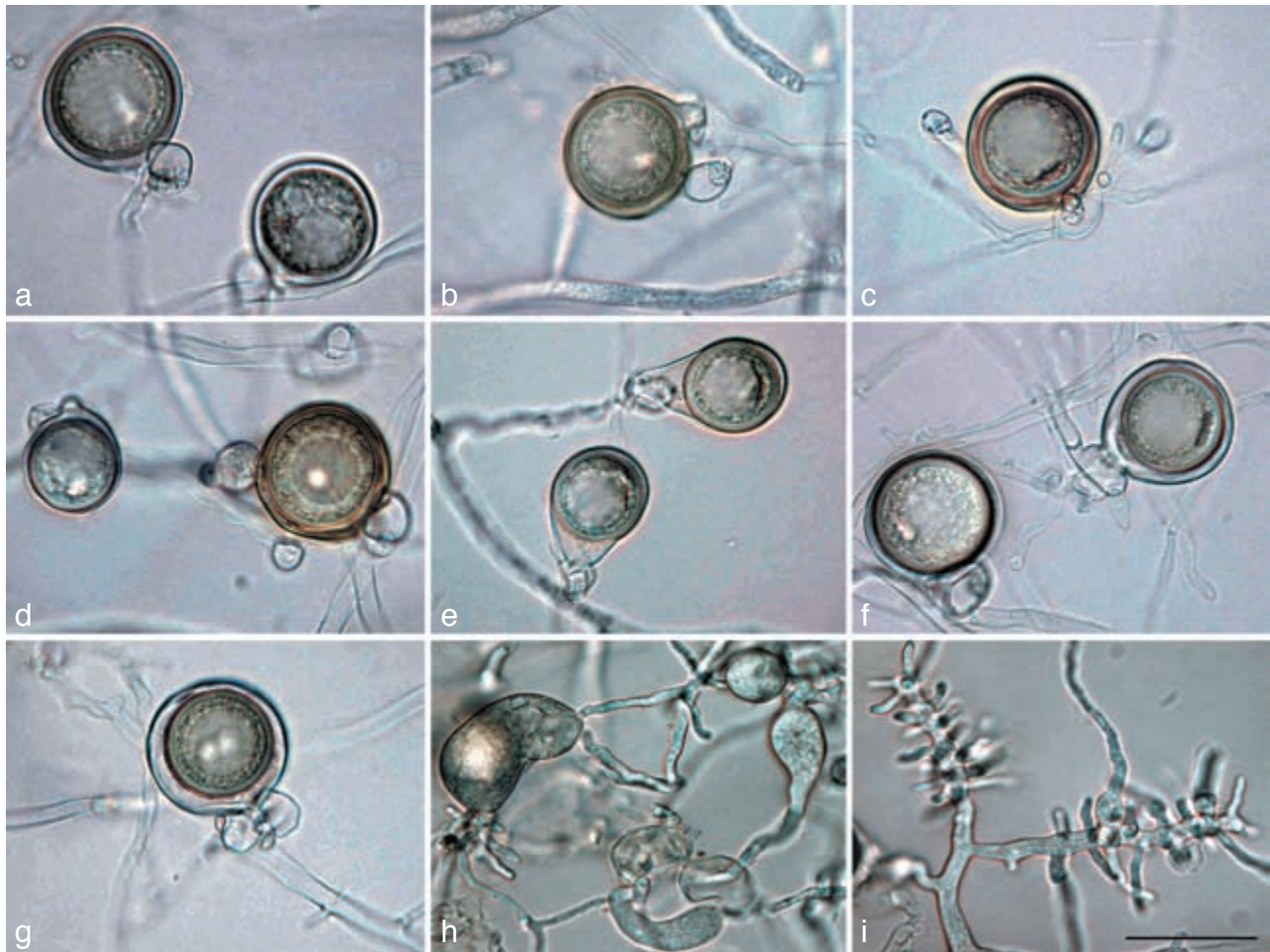the oogonia were elongated and another 18 % slightly excen-

Fig. 5 Morphological structures of *Phytophthora plurivora* formed on solid V8 agar. a–g. Mature oogonia with oospores containing ooplasts: a. oogonium with slightly aplerotic oospore and paragynous antheridium; b. oogonium with plerotic golden-brown oospore and paragynous antheridium; c. oogonium with slightly aplerotic golden-brown oospore and paragynous antheridium with finger-like hyphal projections; d. oogonium with plerotic golden-brown oospore and multiple paragynous antheridia; e. elongated oogonia with long tapering bases and plerotic oospores; f. oogonium with markedly aplerotic oospore and paragynous antheridium with finger-like hyphal projections; g. oogonium with markedly aplerotic oospore and amphigynous antheridium; h. hyphal swellings on the underside of a six weeks old culture; i. brush-like dense clusters of lateral hyphae on the underside of a 6 wk old culture. — Scale bar = 25 µm, applies to all.

tric resembling oogonia of *P. quercina* (Jung et al. 1999). In all isolates of *P. plurivora* and the other three taxa older oogonial walls usually turned golden-yellow to golden-brown (Fig. 5a–d). With a mean diam of 28.5 ± 3.3 µm (overall range 15–37.5 µm and range of isolate means 27.5–29.9 µm) the oogonia of the seven *P. plurivora* isolates were on average slightly smaller than those of *P. citricola* s.str. and *P. citricola* I, and slightly larger than those of *P. multivora* (Table 4). The means of all four species were significantly different (p < 0.05) and the ranges of isolate means had almost no overlap. However, the overall ranges were broadly overlapping (Table 4). As in the other three species oospores of *P. plurivora* were usually globose (Fig. 5b–d) but could be subglobose in elongated oogonia (Fig. 5e). The mean proportion of aplerotic oospores in *P. plurivora* (mean 44.3 %, range of isolate means 22–62 %) was similar to *P. multivora* (mean 45 %; range of isolate means 36–52 %) and *P. citricola* I (mean 43 %; range of isolate means 38–48 %) but significantly lower than in *P. citricola* s.str. (53 %, range of isolate means 50–56 %). Averaging 1.45 ± 0.35 µm in diam (range 0.4–2.5 µm), the oospore walls of *P. plurivora* were on average slightly thinner than in *P. citricola* s.str. and *P. citricola* I, while *P. multivora* isolates produced significantly (p < 0.05) thicker oospore walls (2.6 ± 0.5 µm, overall range 1.4–4.6 µm) than any of the other species. With 0.30 ± 0.06 the oospore wall index of *P. plurivora* was slightly lower than in *P. citricola* s.str. (0.33 ± 0.05) and *P. citricola* I (0.34 ± 0.05) and significantly lower than that of *P. multivora* (0.52 ± 0.07). In cultures of all six

P. multivora isolates growing for 6 wk at 20 °C, the majority of the oospores had germinated directly by multiple germinating hyphae while direct germination of oospores was only rarely observed in older cultures of *P. plurivora*, *P. citricola* s.str. and *P. citricola* I. The antheridia of all four species were obovoid, club-shaped or irregular, sometimes with one or more finger-like projections (Fig. 5c, f), almost exclusively paragynous, and usually attached close to the oogonial stalk. In some oogonia of *P. plurivora* and *P. citricola* I two or more antheridia were attached (Fig. 5d). Intercalary and amphigynous antheridia (Fig. 5g) were only rarely observed.

Colony morphology, growth rates and cardinal temperatures — Colony growth patterns of two isolates of *P. plurivora* (CBS 124091 and CBS 124093), one isolate of *P. citricola* I (CIT-US10), the ex-type isolate and the authentic type isolate of *P. citricola* s.str. (CBS 221.88 and CBS 295.29) and the ex-type isolate of *P. multivora* (CBS 124094) are shown in Fig. 6. All *P. plurivora* isolates formed similar colony growth patterns on the three different types of media. On V8A and MEA *P. plurivora* isolates produced colonies with limited aerial mycelium in the centre of the colonies and radiate to slightly chrysanthemum growth patterns. On PDA all *P. plurivora* isolates formed chrysanthemum colonies with markedly more aerial mycelium than on the other media. The growth patterns of both isolates of *P. citricola* s.str. were relatively similar to those of *P. plurivora* on V8A and MEA. However, on PDA colonies of *P. citricola* s.str. were clearly different from those of *P. plurivora*, forming
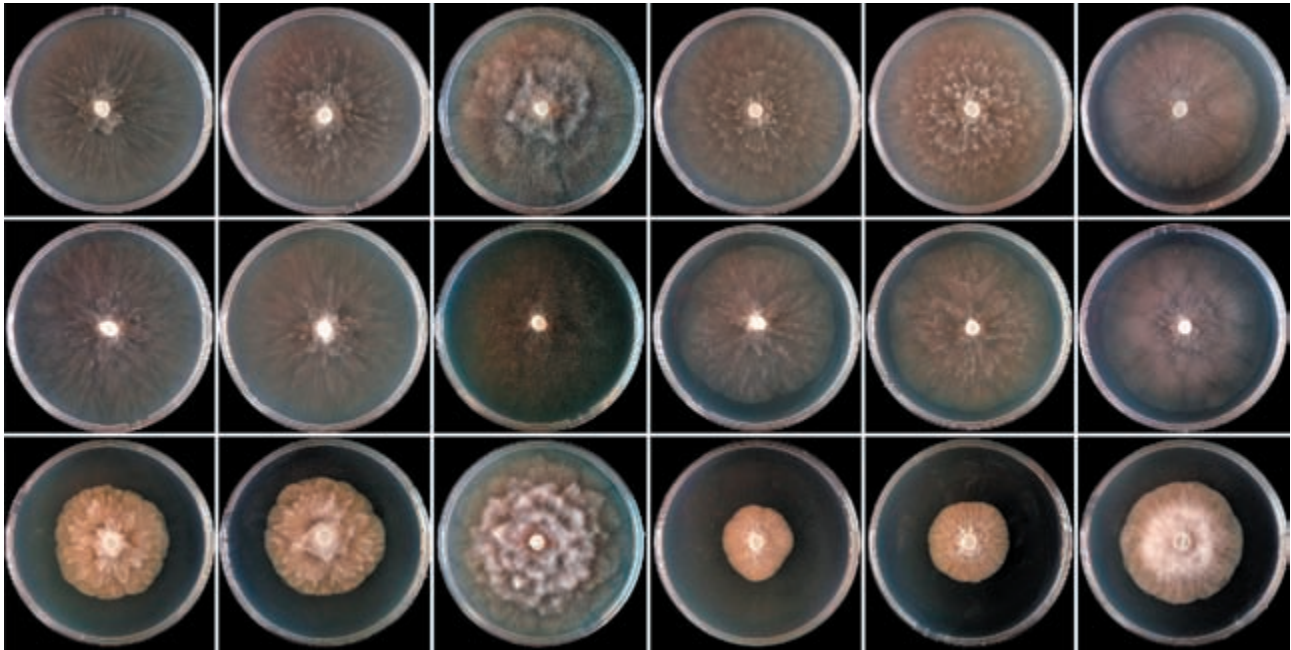
Fig. 6   Colony morphology of isolates CBS 124093 (ex-type) and CBS 124091 of *Phytophthora plurivora*, CIT-US10 of *P. citricola* I, the ex-type (CBS 221.88) and the authentic type isolate (CBS 295.29) of *P. citricola* s.str. and the ex-type isolate of *P. multivora* (CBS 124094) after 7 d growth at 20 °C on V8 agar, malt extract agar and potato-dextrose agar (from top to bottom).

Table 4   Morphological characters and dimensions (μm) and temperature-growth relations of *Phytophthora plurivora*, *P. citricola* s.str., *P. multivora*, *P. citricola* I and *P. inflata*.

	P. plurivora	*P. citricola* s.str.	*P. multivora*	*P. citricola* I	*P. inflata*[3]
No. of isolates investigated	7[1]	2	6[2]	2	n.k.
Sporangia					
l × b mean	47.4 ± 7.7 × 33.5 ± 5.9	52 ± 7.9 × 29.9 ± 5.1	51.0 ± 10.4 × 30.0 ± 5.1	53.7 ± 6.5 × 33.8 ± 3.9	38 × 23
Range of isolate means	39.6−52.3 × 28.9−38.8	50.9−52 × 29.9	44.2−62.1 × 26.2−34.2	51.2−56.2 × 33.5−34.1	
Total range	27.5−80.5 × 16.7−69.6	36−75 × 21−40	36−58 × 13−33	39−70 × 20−42.1	20−67 × 15−32
l/b ratio	1.43 ± 0.19	1.73 ± 0.28	1.7 ± 0.22	1.6 ± 0.16	1.65
Oogonia					
Mean diam	28.5 ± 3.3	30.0 ± 3.0	26.5 ± 1.9	31.2 ± 2.6	34
diam range	15−37.5	16.7−35.9	19−37	21.3−36	30−42.7
Range of isolate means	27.5−29.9	29.7−30.3	25.5−27.8	30.9−31.4	
Oospores					
aplerotic oospores	44.3 % (22−62 %)	44 % (32−56 %)	45 % (36−52 %)	43 % (38−48 %)	
mean diam	25.9 ± 3.1	27.1 ± 2.8	23.6 ± 1.8	27.7 ± 2.3	31.3
diam range	14−35.8	15.3−30.9	17.3−33.1	18.4−33.2	26−39.3
wall diam	1.45 ± 0.35	1.68 ± 0.35	2.6 ± 0.5	1.8 ± 0.36	3−4
oospore wall index	0.3 ± 0.06	0.33 ± 0.05	0.52 ± 0.07	0.34 ± 0.05	
Antheridia					
lxb mean	11.1 ± 4.4 × 8.4 ± 3.1	12.8 ± 2.7 × 8.2 ± 1.7	12.9 ± 1.9 × 8.7 ± 1.3	12.2 ± 2.1 × 9.0 ± 1.6	n.k.[4]
lxb range	7−21 × 5.3−16	7.5−18.5 × 5.4−14.4	8−20 × 5−14	7.7−16.9 × 6.1−12.6	
Maximum temperature (°C)	32	32	32	32	< 35
Optimum temperature (°C)	25	25	25	30	25−30
Growth rate on V8A at optimum (mm/d)	8.1 ± 0.18	6.9 ± 0.1	6.5 ± 0.02	9.2 ± 0.74	
Growth rate at 20 °C (mm/d)					
V8A	6.3 ± 0.1	6.2 ± 0.04	4.8 ± 0.6	6.3 ± 0.23	
MEA	6.2 ± 0.2	4.8 ± 0.3	4.8 ± 0.1	6.2 ± 0.14	
PDA	3.2 ± 0.2	2.0 ± 0.2	3.3 ± 0	6.5 ± 0.42	

[1]   Five of the seven isolates of P. plurivora were included in the growth tests.
[2]   Two of the six isolates of P. multivora were included in the growth tests; the morphometric data of six isolates were taken from Scott et al. 2009.
[3]   Data from Caroselli & Tucker (1949).
[4]   Size of antheridia not known. According to Caroselli & Tucker (1949) antheridia are very characteristic: "inflated, usually variously contorted, often twining or twisted about oogonial stalk, often irregularly lobed or branched".

striate growth patterns with only sparse aerial mycelium. Colony growth patterns of *P. citricola* I were also clearly different from *P. plurivora*, slightly petaloid with limited aerial mycelium on V8A and petaloid with moderate aerial mycelium on PDA. The colony morphology of *P. multivora* isolates was different from the colony morphologies of the other three taxa on all three media. Diameters of primary hyphae of *P. plurivora* varied from 2.6−7.5 μm. In ageing cultures, in particular on their underside, six of the seven *P. plurivora* isolates, including the ex-type, produced

globose to subglobose or appressoria-like hyphal swellings (Fig. 5h) and dense, brush-like to coralloid clusters of lateral hyphae (Fig. 5i) which were not observed in the other three taxa.

Temperature growth relations on V8A of five isolates of *P. plurivora*, the ex-type and the authentic type of *P. citricola* s.str., and each two isolates of *P. multivora* and *P. citricola* I are shown in Fig. 7. All five isolates of *P. plurivora* had identical cardinal temperatures and similar growth rates at all temperatures. The maximum growth temperature for *P. plurivora* and the other
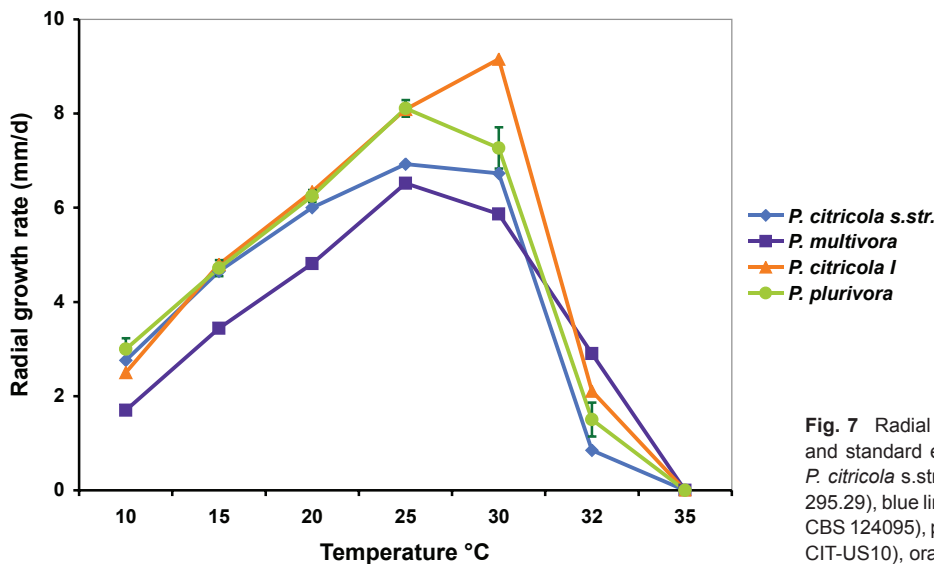
Fig. 7 Radial growth rates of *Phytophthora plurivora* (means and standard errors calculated from five isolates), green line, *P. citricola* s.str. (ex-type CBS 221.88 and authentic type CBS 295.29), blue line, *P. multivora* (ex-type CBS 124094 and isolate CBS 124095), purple line, and *P. citricola* I (isolates CIT-US1 and CIT-US10), orange line, on V8 agar at different temperatures.

three taxa was 32 °C. All four taxa were unable to grow at 35 °C, and isolates did not revive when plates that were incubated for 7 d at 35 °C were transferred to 20 °C. *Phytophthora plurivora*, *P. citricola* s.str. and *P. multivora* had a growth optimum at 25 °C. With radial growth rates at 25 °C ranging from 8.0–8.4 mm/d *P. plurivora* isolates were markedly faster than isolates of *P. citricola* (6.9–7.0 mm/d) and *P. multivora* (both isolates 6.5 mm/d) (Table 4). Having a growth optimum at 30 °C *P. citricola* I was markedly different from *P. plurivora*, *P. citricola* s.str. and *P. multivora*. At 20 °C *P. plurivora* showed significantly higher growth rates on MEA and PDA than *P. citricola* s.str. and was faster growing than *P. multivora* on all three media (Table 4). Compared to *P. citricola* I *P. plurivora* had similar growth rates at 20 °C on V8A and MEA, but was only half as fast on PDA (Table 4).

A summary of decisive morphological and physiological characters discriminating *P. plurivora* from *P. citricola* s.str., *P. citricola* I, *P. multivora* and the original *P. inflata* of Caroselli & Tucker (1949) is given in Table 5.

Specimens examined. CANADA, Montreal, from necrotic fine root of declining *Acer saccharum*, Dec. 1996, *T. Jung*, CBS 124091. – GERMANY, Irschenberg, from root lesion of declining mature *Fagus sylvatica*, Feb. 2004, *T. Jung*, holotype MURU 433 (dried culture on V8A, Herbarium of Murdoch University, Western Australia), culture ex-type CBS 124093; Pulling, from rhizosphere soil of declining mature *Quercus robur*, July 1994, *T. Jung*, CBS 124087; Munich, from collar rot of declining mature *Fagus sylvatica*, May 1995, *T. Jung*, PLU36; Nettetal, from rhizosphere soil of *Q. robur* in a nursery, Feb. 1999, *T. Jung*, PLU77. – ITALY, Cornuda, from rhizosphere soil of declining mature *Q. robur*, June 1995, *T. Jung*, CBS 124089. – ROMANIA, Snagov, from rhizosphere soil of declining *Carpinus betulus*, Jan. 2008, *T. Jung*, CBS 124092. – SLOVENIA, Ljubljana, from rhizosphere soil of declining mature *Q. petraea*, Aug. 1995, *T. Jung*, CBS 124090.

Table 5 Morphological and physiological characters discriminating *Phytophthora plurivora* from *P. citricola* s.str., *P. multivora*, *P. citricola* I and *P. inflata*.

	Characters discriminating from *P. plurivora*[1]			
	P. citricola s.str.	*P. multivora*	*P. citricola* I	*P. inflata*[2]
Sporangia	On av. slightly larger, lower proportion of lateral attachment and curved apices	On av. slightly larger, less variable, lower proportion of lateral attachment, curved apices and distorted shapes	On av. significantly larger	On av. markedly smaller
l/b ratio	Significantly higher	Significantly higher	Significantly higher	Higher
Oogonia and oospores	On av. slightly larger	On av. slightly smaller with significantly thicker oospore walls, germination of most oospores after 4 weeks at 20 °C	On av. slightly larger	On av. markedly larger with much thicker oospore walls
Antheridia				Often inflated, contorted, irregularly lobed or branched, twisted around oogonial stalk
Structures formed on the underside of older colonies	Production of viable sporangia, absence of hyphal swellings, appressoria and hyphal clusters	Absence of hyphal swellings, appressoria and hyphal clusters	Absence of hyphal swellings, appressoria and hyphal clusters	n.a.
Colony growth patterns different from *P. plurivora* on	PDA	V8A, MEA, PDA	V8A, PDA	n.a.
Optimum temp.			Higher	
Maximum temp.				
Growth rate on V8A at optimum (mm/d)	Significantly slower	Significantly slower	Significantly higher	n.a.
Growth rate at 20 °C (mm/d)				
V8A		Significantly slower		n.a.
MEA	Significantly slower	Significantly slower		n.a.
PDA	Significantly slower	Significantly slower	Significantly higher	n.a.

[1] For morphometric and growth-temperature data see Table 4.
[2] Data from Caroselli & Tucker (1949); n.a. = not available.

Notes — In previous studies *P. plurivora* is referred to as *P. citricola* (Jung et al. 1996, 1999, 2000, 2003, 2005, Jung & Blaschke 1996, 2004, Heiser et al. 1999, Schubert et al. 1999, Nechwatal et al. 2001, Vettraino et al. 2001, 2002, 2003, Fleischmann et al. 2002, 2004, Nechwatal & Oßwald 2001, Munda et al. 2007, Jung & Nechwatal 2008, Jung 2009, Scott et al. 2009), *P. inflata* (Cooke et al. 2000, Schlenzig 2005) and *P. citricola* II (Kong et al. 2003, Gallegly & Hong 2008). Many isolates from a wide range of host species in Europe and North America that had been identified as *P. citricola* or *P. inflata* in the past must be reassigned to *P. plurivora*. In this study and previous studies *P. plurivora* has been isolated from fine roots, collar rots, aerial bark cankers, dying shoots and necrotic leaves (Fig. 1), respectively, of 12 woody host species and from rhizosphere soil of another 27 woody species (Table 1). ITS sequences from GenBank add another six species to the host list so that *P. plurivora* has so far been recovered from 45 species in 16 dicotyledonous and 4 coniferous families. Under the original morphological identification as *P. citricola*, pathogenicity of *P. plurivora* to a series of host species was demonstrated in many studies. In underbark inoculation tests *P. plurivora* caused extensive bark necroses on stems of both young and mature trees of *Q. robur* and *F. sylvatica* (Jung & Blaschke 1996), and shoots of mature trees of *F. sylvatica*, *Syringa vulgaris* and *Alnus glutinosa* (Jung et al. 2005, Jung & Nechwatal 2008). In soil infestation tests isolates of *P. plurivora* caused extensive fine root losses, dieback and necrotic lesions of suberised long roots on seedlings and young trees of *Q. robur* (Jung et al. 1996, 1999, 2003), *F. sylvatica* (Nechwatal & Oßwald 2001, Fleischmann et al. 2002, 2004, Jung et al. 2003), *Acer platanoides* and *T. cordata* (unpubl. results), and *Picea abies* (Nechwatal & Oßwald 2001). *Phytophthora plurivora* occurs on a wide range of sites with gritty-loamy, sandy-loamy to loamy, silty or clayey soils, usually rich in base cations with a pH between 3.5–7.2 (in $CaCl_2$), which are mainly derived from limestone, base-rich moraine sediments and gravels from the last ice age, alluvial deposits, flysch and loess or less frequently from sandstones and claystones (Jung et al. 2000, Jung 2009) or volcanic gabbro (Mount Royal, Canada). The vertical limit of its distribution in the Bavarian Alps was 870 m a.s.l.

DISCUSSION

Phytophthora plurivora was previously identified as *P. citricola* or less frequently as *P. inflata* in Europe and North America based solely on morphological and physiological characters. Likewise in Western Australia, *Phytophthora* isolates with the same combination of morphological and physiological characters were routinely identified as *P. citricola* for over 30 years until a recent re-evaluation using ITS and *cox*1 DNA sequence analyses demonstrated that they comprised a new species, *P. multivora* sp. nov. (Scott et al. 2009). In Western Australia, another taxon currently designated as *P.* sp. 2 was also misidentified as *P. citricola*, but was found to be phylogenetically distant, being most closely related to *P. bisheria* (Burgess et al. 2009). The present study is pursuing earlier approaches to unravel the *P. citricola* complex (Oudemans et al. 1994, Bhat & Browne 2007, Gallegly & Hong 2008, Scott et al. 2009). Phylogenetic analyses of the ITS, *cox*1 and β-tubulin gene regions as well as detailed morphological and physiological comparisons with *P. citricola* s.str. and several isolates of *P. multivora* and *P. citricola* I show that *P. plurivora* is unique and comprises a discrete cluster within the major ITS clade 2 of Cooke et al. (2000) with its present closest relative being *P. citricola* s.str.

In our study we have sequenced three gene regions for four clades within the *P. citricola* complex, which (based on ITS sequence data alone) correspond to *P. citricola* s.str., *P. multi-*

vora, *P. plurivora* (= *P. citricola* II) and *P. citricola* I. In both the parsimony and Bayesian analyses of the ITS sequence data, isolates of *P. plurivora* were found identical to *P. citricola* II of Kong et al. (2003) and reside in a strongly supported terminal clade. We consider the type isolates of *P. citricola* collected by Sawada in Taiwan and Japan to be *P. citricola* s.str. In both the parsimony and Baysian analyses of the ITS sequence data, there was no branch support to separate *P. citricola* I and III. Isolates designated as *P. citricola* IV by Kong et al. (2003) were identical to *P. quercetorum* (Balci et al. 2008) and fall into ITS clade 4, not ITS clade 2 with the isolates from the *P. citricola* complex.

Analysis of *cox*1 sequence data, clearly separated *P. plurivora*, *P. multivora*, *P. citricola* s.str. and *P. citricola* I. There are more informative sites in the *cox*1 dataset compared to the ITS dataset as evidenced by intraspecific variation in sequence resulting in substructuring within species clades. The mitochondrial genome is evolving more rapidly than genomic DNA, and intraspecific variation may prove to be linked to host species or geographic location (Kroon et al. 2004).

Oudemans et al. (1994) divided a world-wide collection of *P. citricola* isolates into five groups (CIT1-5) based on a profile generated from 14 isozyme loci. These groups do not correspond directly to *P. citricola* I–IV of Kong et al. (2003). Unfortunately, very few of the isolates used by Oudemans et al. (1994) have been subsequently sequenced; *P. citricola* s.str. (designated P3911 and P0716) was resolved as CIT1 (Oudemans et al. 1994). CIT1 included isolates from a wide host range and many geographic locations including Europe. The sequence of the ITS1 region of isolate P1805 (AF242792) from California (Förster et al. 2000), belonging to CIT1 is identical to that of *P. plurivora*.

CIT2 included an isolate (P1321) from California for which the ITS1 region (AF242786) was sequenced by Förster et al. (2000). The ITS1 region of P1321 is identical to that of isolate IMI031372 (AF266788), designated in this study as *P. citricola* E. Interestingly, an isolate called Citri-P1321 found only on GenBank as AB367493 (submitted 2007, country of origin Japan) is also identical to IMI031372. We believe that these isolates are the same and the country information supplied for AB367493 is incorrect. Two additional isolates Citri-P0713 (AB367492) and Citri-P1817 (AB367494) also have Japan designated as country of origin, but these codes also match codes used by Oudemans et al. (1994); P0713 from Argentina belonging to CIT1 and P1817 from South Africa belonging to CIT3. Citri-P0713 (AB367492) has identical sequence to *P. citricola* s.str. Citri-P1817 (AB367494) has identical sequence to *P. multivora*. If we accept that Citri-P1817 is the same as the isolate used by Oudemans et al. (1994) then CIT3 corresponds to *P. multivora*.

The ITS1 region of isolate P3049, belonging to CIT5, was sequenced by Förster et al. (2000) (AF242796) and is identical to *Phytophthora* sp. CH-2008C (EU748547) (Gallegly & Hong 2008); the closest described species is *P. capsici*. CIT4 was represented by three isolates (P1822, P1819 and P1823) from South Africa, no sequence is available for these isolates. In the study of Oudemans et al. (1994), CIT1-3 clustered separately from CIT4 and CIT5. From our investigations it appears that the isozyme profiles which placed isolates in CIT1 could not resolve the *P. citricola* complex which includes *P. citricola* s.str., *P. plurivora*, *P. citricola* I and *P. citricola* III. CIT2 corresponds to a group of isolates represented by IMI031372 (*P. citricola* E) and CIT 3 corresponds to *P. multivora*. CIT4 and CIT5 may represent new species. The nomenclature of studies focussed on the *P. citricola* complex is compared in Table 6.

Table 6 Comparison of nomenclature between studies focussed on the *P. citricola* complex. Names in brackets represent groups for which there is no molecular data to make the connection between the different studies.

Present study	Oudemans et al. 1994	Kong et al. 2003, Gallegly & Hong 2008
P. citricola s.str.	CIT1; P0713	n.a.
P. citricola I	(CIT1)	*P. citricola* I
P. plurivora	CIT1; P1805	*P. citricola* II
P. citricola III	(CIT1)	*P. citricola* III
P. citricola E	CIT2; P1321	n.a.
P. multivora	CIT3; P1817	n.a.
n.a.	CIT4	n.a.
n.a.	CIT5; P3049	*Phytophthora* sp. CH-2008C

n.a. = not available

The original *P. inflata* ex-type from pit cankers of elm trees in the United States (Caroselli & Tucker 1949) has been lost and it is likely that designated isolates of *P. inflata* from other hosts in Europe (Hall et al. 1992) are conspecific with *P. citricola* (Cooke et al. 2000). ITS sequences of isolates listed on GenBank as *P. inflata* are dispersed among the *P. citricola* complex (Scott et al. 2009). Due to the taxonomic uncertainty of this species, few isolates designated as *P. inflata* were considered in the current study, but we included InfGaul and IMI342898 as sequence for both ITS and *cox*1 regions were available. These isolates were found to be identical to *P. plurivora* in both gene regions. However, the original *P. inflata* is morphologically clearly different from *P. plurivora*, *P. citricola* s.str., *P. citricola* I and *P. multivora* by having much smaller sporangia, larger oogonia and oospores with markedly thicker walls, and large inflated and contorted antheridia which are often irregularly lobed and twining or twisted around the oogonial stalk (Caroselli & Tucker 1949, Table 4). It is unlikely that any of the sequence on GenBank designated as *P. inflata* represent the original *P. inflata* and these isolates must be re-assigned to other species of the *P. citricola* complex including *P. plurivora*.

Despite the morphological and physiological similarities between *P. plurivora* and *P. citricola* s.str., there are clear differences. The most decisive characters for discriminating between *P. plurivora* and *P. citricola* s.str. are the significantly lower length/breadth ratio of *P. plurivora* sporangia, differences of morphological structures formed on the underside of older colonies, colony growth patterns on PDA, and higher radial growth rates of *P. plurivora* on MEA and PDA at 20 °C and on V8A between 20 and 30 °C. *Phytophthora plurivora* is also clearly different from *P. citricola* I and *P. multivora* by morphological and physiological characters.

Interestingly, *P. plurivora*, *P. multivora* and *P. citricola* I seem to occupy similar niches causing fine root destructions, collar rots and aerial bark cankers (Jung et al. 2000, 2005, Jung & Blaschke 2004, Jung 2009, Scott et al. 2009). The close relationship and the morphological and ecological similarities between the '*P. citricola*-like' lineages, suggests recent divergence from a common ancestor. The question how or why the multiple lineages have emerged requires some speculation. Driving forces for speciation are geographical divergence leading to genetically isolated populations and selection caused by different environmental conditions; climatic conditions and available host plants being the most important for *Phytophthora* species. In fact, most '*P. citricola*-like' lineages appear to have emerged in different geographical areas. The basal species of the *P. citricola* complex, *P. multivora*, is widespread in natural ecosystems across Western Australia (Scott et al. 2009) which is characterised by a dry climate and a diverse flora. *Phytophthora multivora* has developed the thickest oospore walls of all '*P. citricola*-like' taxa, most likely as adaptation to the dry

climate. All the other lineages are distributed in geographical areas with higher humidity which is reflected by thinner oospore walls. Outside Australia, *P. multivora* has only been recovered from nurseries in Europe, with the notable exception of one oak stand in Hungary (Ilona Szàbo pers. comm.), and from ornamentals and agricultural crops in nurseries and plantations in California and South Africa (subgroup CIT3 of Oudemans et al. 1994) indicating recent introductions.

Isolates with ITS sequences identical to *P. citricola* s.str. are so far only known from *Citrus* species in Taiwan, Japan and Argentina and have never been recovered from native forest trees in the southern Hemisphere, Europe or North America. *Phytophthora plurivora* is widespread in forests, semi-natural ecosystems and nurseries across Europe and has been recovered here from diseased tissues of 11 woody host species and from rhizosphere soil of another 31 species. Reports of *P. plurivora* from North America all come from highly managed plantations or nurseries indicating a recent introduction. The only record from a natural ecosystem was from a highly frequented sugar maple stand at Mount Royal in Montreal. In contrast, *P. citricola* I is found widespread in the eastern USA causing damage and mortality to the introduced species *F. sylvatica* (Jung et al. 2005) but not to native species which might indicate a co-evolution between *P. citricola* I and the plant species in the eastern USA. Likewise, *P. citricola* III has yet only been recovered from natural stands in the USA. In Europe, *P. citricola* I and III have only been found in nurseries suggesting recent introduction. The inability to separate between *P. citricola* I and III in both the parsimony and the Bayesian analyses of their ITS sequences may indicate incomplete speciation in North America from a common ancestor. The true *P. inflata* of Caroselli & Tucker (1949) has also only been recorded from the USA but it is uncertain whether it belongs to ITS clade 2 or like *P. citricola* IV (= *P. quercetorum*) to another ITS clade.

Under the original morphological identification as *P. citricola*, pathogenicity of *P. plurivora* to fine root systems of young trees of *Q. robur*, *F. sylvatica*, *Ac. platanoides* and *T. cordata* and to bark of both young and mature trees of *Q. robur*, *F. sylvatica*, *Syringa vulgaris* and *Al. glutinosa* was demonstrated in many studies (see Notes). In soil infestation tests, *F. sylvatica* (80–90 % root rot and 67–90 % mortality; Fleischmann et al. 2002, 2004, Jung et al. 2003) and *Ac. platanoides* (88 % root rot and 20 % mortality; unpubl. results) were the most susceptible species to *P. plurivora*.

Also under the original identification as *P. citricola*, *P. plurivora* was shown to be strongly involved in several devastating declines of forests and semi-natural ecosystems across Europe. *Phytophthora plurivora* was regularly associated with a widespread chronic decline and dieback of oak species across Europe causing a progressive destruction of fine root systems and predisposing the trees to droughts and attacks by secondary pests and pathogens (Hansen & Delatour 1999, Jung et al. 2000, Vettraino et al. 2002, Balci & Halmschlager 2003a, b). Since the late 1990s, and in particular after the wet year 2002 and the dry year 2003, an increasing number of trees and stands of European beech are showing high transparency and dieback of crowns, small-sized and often yellowish foliage and high mortality. *Phytophthora plurivora*, *P. cambivora* and various other *Phytophthora* species were consistently associated with the disease causing root and collar rot, aerial bleeding cankers and extensive fine root losses (Jung et al. 2005, Brown & Brasier 2007, Munda et al. 2007, Jung 2009). During this study, *P. plurivora* was found associated with declining maple, linden, horse chestnut and birch trees in 29 out of 40 stands (71 %) investigated in central Europe. *Phytophthora plurivora* was also often isolated alongside *P. alni* from collar rot lesions of alder trees in riparian stands in Germany and

Austria (Jung & Blaschke 2004) which is probably due to the regular occurrence of the pathogen in streams and lakes as shown exemplarily in this study.

The demonstrated high aggressiveness of *P. plurivora* to major native tree species, i.e. *F. sylvatica*, *Q. robur*, *Al. glutinosa* and *Ac. platanoides*, and its regular involvement in devastating declines of forests and semi-natural ecosystems in Europe indicate a lack of co-evolution between these hosts and this pathogen. *Phytophthora plurivora* or an ancestor was most likely introduced from overseas on living plant stock (Brasier 2008), and became widespread and well established due to its almost ubiquitous presence in the European nursery sector. Under its original identification as *P. citricola*, *P. plurivora* was recovered from alder, beech, oak, maple, linden, willow and horse chestnut fields of 21 out of 34 nurseries tested across Germany (Jung & Blaschke 2004, Jung 2009).

Considering the wide host range of *P. multivora* in Western Australia (Scott et al. 2009) and the regular association of *P. citricola* I with collar rots and aerial bark cankers of European beech in the eastern US (Jung et al. 2005) the recent appearance of these species in European nurseries is posing a serious threat to the nursery, forestry and horticultural industries in Europe. Their distribution via infested nursery stock must be stopped before they might become invasive like *P. plurivora*.

In conclusion, due to its wide host range, its high aggressiveness to major native tree species and the involvement in several widespread, devastating tree declines, *P. plurivora* (possibly together with *P. cambivora*), is currently the most threatening *Phytophthora* species, and generally one of the most important pathogens of forests and semi-natural ecosystems in Europe. New silvicultural concepts in Europe, and in particular in Germany, are aiming to replace non-natural pure conifer stands by mixed forests of beech and oaks in order to stabilise these ecosystems against predicted risks of climate change (Ammer et al. 2005) and millions of seedlings are currently planted every year. Due to the widespread infestations of nursery stock by *P. plurivora* and other aggressive *Phytophthora* species these silvicultural concepts are likely to fail. Therefore, management concepts for the production of non-infested nursery stock and quarantine regulations and procedures to prevent the introduction of potentially invasive *Phytophthora* species are urgently required at a European scale (Brasier 2008).

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