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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Key words beech citricola decline dieback forest multivora nursery oak phylogeny

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, semipapillate 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 cox1 and β-tubulin genes were used in combination with morphological and physiological characters 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.

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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 & Halschlager 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 roots, root lesions, collar roots, 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 & Halschlager 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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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 cox1 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.

Table 1 Host range and distribution of Phytophthora plurivora.

| Host          | Sample type                  | Country1 (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 et al. 2009, this study    |
| Ac. pseudoalpinus| Aerial canker, collar rot rhizosphere soil | D (2007), A (2007) | Jung et al. 2009, this study    |
| Ac. saccharum | Fine roots                   | CDN (1996)                        | This study                      |
| A. esculentus 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)                          | Jung et al. 2001                |
| Chamaecyparis lawsoniana| Collar rot, rhizosphere soil | D (2006), I (2007)              | This study                      |
| Cornus mas    | n.k.                         | BG (n.k.)                         | GenBank                         |
| Corylus columa| 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 |
| Fragraia × ananassa| n.k.                        | USA (n.k.)                        | GenBank                         |
| Hedera helix  | Rhizosphere soil             | I (2007)                          | This study                      |
| Illex aquifolium| n.k.                        | CH (n.k.)                         | GenBank                         |
| Malus domestica| Rhizosphere soil             | A (2007)                          | This study                      |
| Juglans regia | Rhizosphere soil             | I (2001), D (2005)               | Veittraino et al. 2003, this study |
| Panax quinquifolium| n.k.                       | USA (n.k.)                        | GenBank                         |
| Picea abies   | Nursery soil, Rhizosphere soil| D (1998)                          | Jung & Blaschke 2004            |
| 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, Veittraino et al. 2002, Balci & Halmischlager 2003b |
| Q. petraea    | Fine roots, rhizosphere soil | SLO (1995), D (1996), F (1996), I (1996), SRB (2002) | Jung et al. 1996, 2000, Veittraino et al. 2002, Jung & Blaschke 1996, Jung et al. 1996, 2000, Balci & Halmischlager 2003a, Veittraino et al. 2001, this study |
| Q. pubescens  | Rhizosphere soil             | I (1997)                          | Jung et al. 1996, Veittraino et al. 2002, this study |
| 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 et al. 1996, Veittraino et al. 2002, Jung & Blaschke 1996, Jung et al. 1996, 2000, Balci & Halmischlager 2003a, Veittraino 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                      |
| Thujia 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. platyphylos| 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.

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 bark and bark sections were transferred to V8 agar for initial confirmation as Phytophthora species.
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. | Host                  | Location, year | Reference                                      | ITS            | cox1        | β-tubulin   |
|----------------|-------------|-----------------------|----------------|------------------------------------------------|----------------|-------------|-------------|
| P. citricola (type) | IM0121173, CBS 221.88 | Citrus sinensis, fruit | Taiwan, 1927  | Scott et al. (2009)                             | FJ237526       | FJ237512    | FJ665255    |
| P. citricola (authentic type) | CBS 295.20 | Citrus sp., leaf       | Japan, 1929    | This study                                     | FJ660913       | FJ665244    | FJ665256    |
| P. citricola I | CH98U121C   | –                     | Japan, (Argentina) | Uddin et al. (unpubl.) | –               | –           | –           |
| P. citricola II | CBS 181.25, IMI 077970 | Pinus resinosa, roots | Minnesota, USA, 1925 | Hong (unpubl.) | –               | –           | –           |
|                | 2F33, P33   | –                     | Ohio, USA      | Hong (unpubl.)                                | FJ692321       | –           | –           |
| P. citricola I | CIT-US1214f | Fagus sylvatica, canker | New York State, USA, 2003 | This study | FJ665234       | FJ665242    | FJ665253    |
| P. citricola II | CBS 379.61  | Rhododendron sp.       | Germany, 1958  | Hong (unpubl.)                                | FJ392325       | –           | –           |
| P. citricola II | 22F2, P52   | –                     | New York State, USA, 1987 | Hong (unpubl.) | FJ692324       | –           | –           |
| P. citricola III | 15C9        | Acer saccharum         | Wisconsin, USA, 1985 | Hong (unpubl.) | FJ392327       | –           | –           |
| P. citricola III | 15C8        | Field soil             | South Carolina, USA, 1997 | Hong (unpubl.) | FJ692329       | –           | –           |
| P. citricola I | IM0131372f  | Rubus idaeus           | Ireland        | Cooke et al. (2000)                           | AF266788       | –           | –           |
|                | 112*        | –                     | Switzerland    | Bragante et al. (unpubl.)                     | E083906        | –           | –           |
| P. multivora (type) | WAC 13201, CBS 124094 | Eucalyptus marginata | Yalgorup, WA, 2007 | Scott et al. (2009) | FJ237521       | FJ237508    | FJ665260    |
| P. multivora | WAC 13230 | E. gomphocephala       | Yalgorup, WA, 2007 | Scott et al. (2009) | FJ237522       | FJ237509    | FJ665261    |
| P. plurivora | WAC 13205 | E. gomphocephala       | Yalgorup, WA, 2007 | Scott et al. (2009) | FJ237518       | FJ237507    | FJ665259    |
|                | CBS 13205, CBS 124095 | E. marginata         | Jarrahdale, WA, 1998 | Scott et al. (2009) | FJ237517       | –           | –           |
| P. plurivora | VHS 16168   | Banksia grandis        | Pemberton, WA   | Scott et al. (2009)                           | FJ237513       | FJ237502    | FJ665257    |
| P. plurivora | IMI 329674 | Soil                   | Walpole, WA     | Scott et al. (2009)                           | FJ237515       | FJ237504    | FJ665255    |
|                | VHS 164343  | B. littoralis           | Mandareah, WA   | Scott et al. (2009)                           | FJ237516       | FJ237505    | FJ665258    |
| P. plurivora | P18177†     | Medicago sativa        | South Africa    | Koon et al. (2004)                           | –              | –           | AY640585    |
| P. plurivora | P10458†     | –                      | –              | Blair et al. (2007)                           | –              | –           | EU079592    |
|                | P7902*      | Pinus radiata          | USA, 1992       | Blair et al. (2007)                           | –              | –           | EU080236    |
| P. plurivora (type) | PLU-AS, CBS 124093 | F. sylvatica, root lesion | Ilschenberg, Germany, 2004 | This study | FJ665225       | FJ665236    | FJ665247    |
| P. plurivora | PLU-9b | F. sylvatica, carker   | Ilschenberg, Germany, 2004 | This study | FJ665226       | –           | –           |
| P. plurivora | PLU7        | Q. rubor, soil         | Pulling, Germany, 1994 | Schubert et al. (1999) | A007370       | –           | –           |
| P. plurivora | PLU9, CBS 12408f | Q. rubor, soil         | Pulling, Germany, 1994 | Scott et al. (2009) | FJ237523       | FJ237510    | FJ665245    |
| P. plurivora | PLU30, CBS 12408g | Q. rubor, soil         | Comorda, Italy, 1995 | FJ665227       | FJ665237    | FJ665248    |
| P. plurivora | PLU35, CBS 12409c | Q. petreae, soil      | Ljubljana, Slovenia, 1995 | Scott et al. (2009) | FJ237524       | FJ237511    | FJ665246    |
| P. plurivora | PLU6        | F. sylvatica, carker   | Munich, Germany, 1995 | This study | FJ665228       | –           | –           |
| P. plurivora | PLU41, CBS 12409c | Ac. saccharum, root   | Mount Royal, Canada, 1996 | Scott et al. (2009) | FJ665229       | FJ665238    | FJ665249    |
| P. plurivora | PLU77†     | Q. rubor, nursery soil | Nettetal, Germany, 1999 | This study | FJ665230       | FJ665239    | FJ665250    |
| P. plurivora | PLU92       | Quercus sp., soil      | Turkey, 2000    | This study | FJ665231       | FJ665240    | FJ665251    |
| P. plurivora | PLU265      | F. sylvatica, carker   | Sumava, Czech Republic, 2007 | This study | FJ665232       | –           | –           |
| P. plurivora | PLU276      | Carpinus betulus, soil | Snagov, Romania, 2008 | This study | FJ665233       | FJ665241    | FJ665252    |
|                | P10339     | –                      | –              | Blair et al. (2007)                           | –              | –           | EU079592    |
|                | MN21H4     | Rhododendron sp.       | USA, 2007       | Schwingel et al. (2007)                      | DQ486661       | –           | –           |
|                | InfGaul8†  | Vaccinium vitis-idaea   | Scotland        | Schlenzig (2005)                            | AY792929       | AY694848    | –           |
|                | IM134289b | Syringa vulgaris        | UK              | Cooke et al. (2000) – ITS, Kroon et al. (2004) – cox1 | AF266788       | AY64187     | –           |

1 Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelculturen 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 (ITS1-5.8S-ITS2) region of the ribosomal DNA was amplified using the primers ITS6 (5' GAA GGT GAA GTC GTA ACA AGG 3') (Cooke et al. 2000) and ITS4 (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 *cox1* was amplified with primers Fm684 (5' TTT AAT TTT TAG TGC TTT TGC) and Fm683 (5' GCC AAG TTC TGG GAG GTC ATC) (Blair et al. 2008) and *cox*1 was amplified with *Btub* F1 (5' CCT GGT ACT GCT GGT ACT CAG) (Kroon et al. 2003). Templates were sequenced in both directions with primers used in amplification, as well as primers FM 85 (5' AAC TTG ACT AAT ACC AAA AAT G) 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 TGC GAG GTC ATC) (Blair et al. 2008) and Btub R1 (5' CCT GGT ACT GCT GCT 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 (Martin & Tooley 2003). Templates were sequenced in both directions with primers used in amplification. The clean-up of products and sequencing were as the same 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 *cox1* or *β*-tubulin are smaller. The first 540 bp of the *cox1* 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 *cox1* 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 Bayesian 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 CaCO₃, 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, 35 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.

5 changes

Clades 8, 9 and 10
### 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 = 0.60, RI = 0.78). The topology of the Bayesian tree was very similar (TreeBASE SN4309) and is 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 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. mulivora* rather than *P. plurivora* or *P. citricola* (Table 2).

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**Statistical analysis**

Analyses of Variances were carried out using Prism3 (GraphPad, San Diego, USA) to determine whether morphological and physiological measurements were significantly different between the different taxa.
Table 3  Polymorphic nucleotides from aligned sequence data of ITS, cox1 and β-tubulin gene regions showing the variation between isolates of *P. citricola* s.str., *P. plurivora* and *P. citrica* s.l. (including *P. citrica* I, III and E). Blue shading denotes polymorphisms found in *P. citrica* s.str., green shading is for those found in *P. plurivora* and orange shading is for those polymorphisms only found in *P. citrica* s.l. Grey shading denotes no data available.

|     | ITS         | cox1      | β-tubulin |
|-----|-------------|-----------|-----------|
|     | Culture no. |           |           |
|     | 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 |
| CBS 221.88 | T T A T G G G | A T C A T G | G C C T T |
| CBS 295.29 | T T A T G G G | A T C A T G | G C C T T |
| CBS 124093 | T T A C G G G | T C T C C A | G T T T T |
| CBS 124087 | T T A C G G G | T C T C C A | G T T T T |
| CBS 124099 | T T A C G G G | T C T C C A | G T T T T |
| PLU77     | T T A C G G G | T C T C C A | G T T T T |
| PLU92     | T T A C G G G | T C T C C A | G T T T T |
| CBS 124092 | T T A C G G G | T C T C C A | G T T T T |
| PLU4     | T T A C G G G | T C T C C A | G T T T T |
| PLU36     | T T A C G G G | T C T C C A | G T T T T |
| PLU255   | T T A C G G G | T C T C C A | G T T T T |
| IMI 34298 | T C A T G G G | T C T C C A | G T T T T |
| InfGaul   | T T A C G G G | T C T C C A | G T T T T |
| MN211HH   | T T A C G G G | T C T C C A | G T T T T |
| CBS 379.61 | T T A C G G G | T C T C C A | G T T T T |
| 22F2     | T T A C G G G | T C T C C A | G T T T T |

Across the three gene regions sequenced there are 18 fixed polymorphisms separating *P. plurivora* from *P. citrica* s.str.; three in ITS region, 13 in cox1 and 2 in β-tubulin (Table 3). For *P. citrica* III there are 2 fixed polymorphisms in the ITS region separating these isolates from *P. citrica* s.str. and 5 fixed polymorphisms in the ITS region separating them from *P. plurivora*. *Phytophthora citrica* E is separated from *P. citrica* s.str. by 3 fixed polymorphisms and 6 fixed polymorphisms separate *P. citrica* E from *P. plurivora* (Table 3). Two isolates sequenced in this study (CIT-US1 and CIT-US10) had ITS sequences identical to *P. citrica* I. Across the three gene regions these isolates as representatives of *P. citrica* I differed from *P. citrica* 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, persistencia, terminalia, interdum lateralia aut intercalaria, semi-papillata, ovoides, obpyriformia aut limoniformia, rare distorta vel bipapillata, saepe cum obturamento conspicuo basale, apicem interdum arcuatum, 49.9 ± 1.9 µm, Antheridia paragyna, 10.6 ± 2.7 × 7.4 ± 1.5 µm. Chlamydosporeae et infusoriae hypharum non observatae. Temperatura variantes ranging from ovoid (over all isolates < 5 %; Fig. 4h–j), and usually formed a conspicuous basal plug that protruded into the empty sporangium (Fig. 4b–d, k). Smaller 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 sporangium (over all isolates 17.6 %; Fig. 4g, j) or a short hyphal extension (1.2 %) were common in all

**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. citrica* s.str. but not by any isolates of *P. plurivora*, *P. multivora* and *P. citrica* 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). 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).
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 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, oosporas 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 4](image-url)
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 36–52 %) and *P. citricola* I (mean 43 %; range of isolate means 38–48 %) but significantly lower than in *P. multivora* (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 all *P. plurivora* isolates formed a chrysanthemum growth pattern on the underside of a soil 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.

![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.](image-url)
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<sup>3</sup> |
|---------------------|--------------|---------------------|--------------|----------------|------------------------|
| No. of isolates investigated | 7<sup>1</sup> | 2 | 6<sup>2</sup> | 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 | 36 × 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 | 36–56 × 13–33 | 39–70 × 20–42.1 | 20–67 × 15–32 |
| l/b ratio           | 1.43 ± 0.19 | 1.73 ± 0.2 | 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 |
| Range               | 15–37.5 | 16.7–35.9 | 19–37 | 21.3–36 | 30–42.7 |
| **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 |
| 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 |
| Wall oospore wall index | 0.3 ± 0.06 | 0.33 ± 0.05 | 0.52 ± 0.07 | 0.34 ± 0.05 |                        |
| **Antheridia**      |              |                     |              |                |                        |
| lx b 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.<sup>4</sup> |
| lx b 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 |

<sup>1</sup> Five of the seven isolates of P. plurivora were included in the growth tests.
<sup>2</sup> 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.
<sup>3</sup> Data from Caroselli & Tucker (1949).
<sup>4</sup> 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
Antheridia

Often inflated, contorted, after 4 weeks at 20 °C germination of most oospores oospore walls significantly thicker, with much thicker appressoria and hyphal clusters l/b ratio Significantly higher Significantly higher Significantly higher Higher

Oogonia and oospores On av. slightly larger On av. slightly smaller with significantly larger On av. markedly larger

Underside of older colonies absence of hyphal swellings, appressoria and hyphal apices and distorted shapes

MEA Significantly slower Significantly slower n.a.

V8A Significantly slower n.a.

Growth rate on V8A at optimum (mm/d) Significantly slower Significantly slower Significantly higher n.a.

V8A MEA PDA

Characteristics discriminating from Phytophthora plurivora

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

| Character                                      | P. citricola s.str. | P. multivora | P. citricola I | P. inflata |
|------------------------------------------------|--------------------|--------------|----------------|------------|
| 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                                     |                      |              |                |            |
| 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.                                  |                     |              |                |            |
| 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)                    | Significantly slower | Significantly slower | Significantly slower | n.a. |
| V8A MEA PDA                                    | Significantly slower | Significantly slower | Significantly slower | Significantly higher | n.a. |

A summary of decisive morphological and physiological characters discriminating Phytophthora 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, Irshenberg, 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 12407; Munich, from collar rot of declining mature Fagus sylvatica, May 1995, T. Jung, PLUS6; Nettetal, from rhizosphere soil of Q. robur in a nursery, Feb. 1999, T. Jung, PLUT7. – Italy, Comuda, 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.

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, Mundal 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, Gallegh & 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 CaCl₂), which are mainly derived from limestone, base-rich soils, usually rich in base cations with a pH between 3.5 – 7.2, and 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 cox1 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, Galleghy & Hong 2008, Scott et al. 2009). Phylogenetic analyses of the ITS, cox1 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 Ishow 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. multivora, 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 Bayesian 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 cox1 sequence data, clearly separated P. plurivora, P. multivora, P. citricola s.str. and P. citricola I. There are more informative sites in the cox1 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 IM031372 (AF266788), designated in this study as P. citricola. Interestingly, an isolate called Citri-P1321 found only on GenBank as AB367493 (submitted 2007, country of origin Japan) is also identical to IM031372. 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) (Galleghy & Hong 2008); the closest described species is P. capsici. CIT4 was represented by three isolates (P1822, P1819 and P1823) from 14 isozyme loci. These groups do not correspond to CIT4 and CIT5. From our investigations it appears that CIT4 and CIT5 may be P. multivora. CIT4 and CIT5 may represent new species. The nomenclature of studies focussed on the P. citricola complex is compared in Table 6.
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 cox1 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 (Ibóna Szabó 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* I (= *P. querceotorum*) 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 *A. 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 & Halmischlager 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

### 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 | |
| *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
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-native pure conifer stands with 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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