**Phytophthora multivora** sp. nov., a new species recovered from declining Eucalyptus, Banksia, Agonis and other plant species in Western Australia

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Key words: decline, dieback, forest, jarrah, phylogeny, Phytophthora citricola, tuart

Abstract: A new Phytophthora species, isolated from rhizosphere soil of declining or dead trees of Eucalyptus gomphocephala, E. marginata, Agonis flexuosa, and another 13 plant species, and from fine roots of E. marginata and collar lesions of Banksia attenuata in Western Australia, is described as Phytophthora multivora sp. nov. It is homothallic and produces semipapillate sporangia, smooth-walled oogonia containing thick-walled oospores, and paragynous antheridia. Although morphologically similar to *P. citricola*, phylogenetic analyses of the ITS and cox1 gene regions demonstrate that *P. multivora* is unique. *Phytophthora multivora* is pathogenic to bark and cambium of *E. gomphocephala* and *E. marginata* and is believed to be involved in the decline syndrome of both eucalypt species within the tuart woodland in south-west Western Australia.

Article info: Received: 21 October 2008; Accepted: 3 December 2008; Published: 2 February 2009.

INTRODUCTION

The oomycete genus *Phytophthora* includes many well-known species that contribute to and often drive tree declines worldwide. Knowledge about the diversity and significance of *Phytophthora* species in forest ecosystems has significantly increased in recent years as research has focussed on new and devastating tree declines in natural ecosystems in Europe and the Americas (Jung et al. 1999, 2000, 2002, Vettriano et al. 2001, 2002, Rizzo et al. 2002, Jung & Blaschke 2004, Brasier et al. 2005, Balci et al. 2007, Greslebin et al. 2007, Jung 2008) and advances in molecular techniques have improved our phylogenetic understanding of the genus (Cooke et al. 2000, Kroon et al. 2004). Since the discovery of *P. cinnamomi* in the south-west of Western Australia (WA) (Podger et al. 1965), this introduced pathogen has become renowned for its unparalleled impact on flora biodiversity with 40 % of the 5710 species in the south-west Botanical Province found to be susceptible and 14 % highly susceptible (Shearer et al. 2004).

As a result of the wide scale forest quarantine and management of *P. cinnamomi* in WA, extensive and regular testing of soil and plant tissue samples for *P. cinnamomi* at the Vegetation Health Service (VHS) laboratory of the Department of Environment and Conservation has led to the isolation of a large range of *Phytophthora* spp. and undescribed *Phytophthora* taxa (Stukely et al. 1997, 2007a, b, Burgess et al. 2009). The recovery of *Phytophthora* taxa other than *P. cinnamomi* from some sites with declining vegetation in WA has recently focussed attention on their role in the decline of these woodland and forest ecosystems (Shearer & Smith 2000).

Across the south-west of WA there are a number of recently observed and significant forest declines occurring. In particular, the declines of *Corymbia calophylla* (Paap et al. 2008), *Eucalyptus wandoo* (Hooper & Sivasithamparam 2005), *E. gomphocephala* (tuart, Fig. 1a, b), *E. marginata* (jarrah, Fig. 1c, d), *E. rudis*, *Agonis flexuosa* and Banksia spp. (Fig. 1e, f) are causing concern to land managers and the general community. Within the tuart woodland of Yalgorup National Park on the Swan Coastal Plain south of Perth, a significant decline and substantial numbers of deaths of *E. gomphocephala* have been observed together with localised declines and mortality of *E. marginata* since the 1990s and *A. flexuosa* since 2006. A range of biotic and abiotic factors has been shown to contribute to tuart decline (Edwards 2004, Archibald 2006), although as yet, no satisfactory aetiology has been established. The progressive canopy thinning and dieback, and the heterogeneous distribution of the decline, are similar to Jarrah dieback (Shearer & Tippett 1989) and suggest the potential involvement of a *Phytophthora* species.

In May and June 2007 *Phytophthora* isolates were recovered from the rhizosphere of declining *E. gomphocephala*, *E. marginata* and *A. flexuosa* in Yalgorup National Park. These isolates morphologically resembled *P. citricola*, which has been recovered over the past three decades throughout the south-west of WA by the VHS (Stukely et al. 1997). However, recent re-evaluation of the VHS culture collection using molecular techniques has identified most of these isolates as a new taxon (*Phytophthora* sp. 4) in the *P. citricola* complex (Burgess et al. 2009). DNA sequence data from the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S gene of the rRNA operon and the mitochondrial cox1 gene were used in combination with morphological and physiological characteristics to characterise these isolates and compare them to the ex-type isolate of *P. citricola* as described by Sawada (1927). Due to their unique combination of morphological and physiological characters, and ITS and cox1 sequences, these semipapillate homothallic isolates from the south-west of WA are described here as a new species, *P. multivora* sp. nov.

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MATERIAL AND METHODS

Sampling and Phytophthora isolation

Phytophthora isolates sampled from the tuart forest were obtained using soil sampling, baiting and isolation techniques modified from Jung et al. (1996, 2000). Soils were sampled beneath trees of *E. gomphocephala*, *E. marginata*, or *A. flexuosa* from 32 sites (four trees per site). Sites sampled included 24 sites with all stages of crown dieback, and 8 sites without visible signs of canopy decline. From each tree a total of 4 L of soil was collected from 4 points, at a distance of 50–150 cm from the stem base. Soils were sampled below the upper 5 cm organic layer to a depth of 30 cm, with attention paid to sampling along main lateral roots. The four subsamples from each tree were bulked and baited in 35 × 35 cm plastic trays. Samples were pre-moistened for 12 h before flooding with distilled water to 3–4 cm in depth above the soil line. Floating organic material was moved to the side of the baiting tray with flyscreen meshing and any remaining organic material floating on the surface of the baiting water was removed with paper towelling. Juvenile leaves of *Quercus ilex*, *Q. suber* and *Pittosporum undulatum* were floated on the water as baits. Leaves with brownish le-

![Fig. 1 a. Severe dieback and mortality of a forest stand of *Eucalyptus gomphocephala*; b. crown symptoms of a declining *E. gomphocephala* including thinning, clustering of leaves, and dieback of branches and parts of the crown; c. dieback and mortality of a forest stand of *E. marginata*; d. crown symptoms of a declining *E. marginata* including thinning, clustering of leaves and dieback of branches and parts of the crown; e, f. collar rot of *Banksia attenuata* caused by *Phytophthora multivora*; e. sudden wilting and death due to the girdling of the collar; f. tongue-shaped, orange-brown necrosis of the inner bark.](image-url)
sions appearing after 48–96 h were examined for the presence of Phytophthora sporangia using a light microscope. Leaflets with sporangia were blotted dry, and the lesions cut into 1–2 mm sections and plated onto Phytophthora selective PARPNH medium (Jung et al. 2000). Colonies growing from the plated lesion sections were transferred to V8 agar for confirmation as Phytophthora isolates.

**Phytophthora isolates**

In addition to the five semi-papillate *Phytophthora* isolates (WAC13200–WAC13204) collected in the present study, another two isolates were used for morphological and physiological comparisons including a semi-papillate isolate from the VHS collection (DCE 236, WAC13205) previously isolated from fine roots of a recently dead *E. margarita* in the jarrah forest near Jarrahdale in 1981, and the ex-type isolate of *P. citricola* (IMI 021173) recovered from *Citrus sinensis* fruits in Taiwan (Sawada 1927) (Table 2).

Immediately prior to the present study, all isolates maintained in 90 mm Petri dishes on V8A media and as 9 mm V8A discs stored in 20 ml sterile water in McCartney bottles, were passaged through juvenile leaves of *Q. suber* used as baits on colonised agar discs flooded with sterile deionised water, and re-isolated using PARPNH selective medium.

**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, malt extract agar (MEA), corn-meal agar (CMA) and potato-dextrose agar (PDA) (all from BBL, Becton, Dickinson & Co, Sparks MD 21152 USA). Colony morphologies were described according to Brasier & Griffin (1979), Erwin & Ribeiro (1996) and Jung et al. (2003). Radial growth rate was recorded 5–7 d after the onset of linear growth along two lines intersecting the centre of the inoculum at right angles (Jung et al. 1999). The growth

| Culture no. | Identification | Host | Location | Reference | GenBank Accession No. |
|-------------|----------------|------|----------|-----------|-----------------------|
| WAC13201    | *P. multivora* (ex-type) | Eucalyptus marginata | Yalgornup, Western Australia (WA) | This study | FJ237521 FJ237508 |
| WAC13200    | *E. gomphocephala* | Yalgornup, WA | This study | FJ237522 FJ237509 |
| WAC13202    | *E. gomphocephala* | Yalgornup, WA | This study | FJ237520 |
| WAC13203    | *Agonis flexuosa* | Yalgornup, WA | This study | FJ237519 |
| WAC13204    | *E. gomphocephala* | Yalgornup, WA | This study | FJ237518 FJ237507 |
| WAC13205    | *E. marginata* | Jarrahdale, WA | This study | FJ237517 FJ237506 |
| DCE236      | *E. marginata* | Wanneroo, WA | This study | FJ237514 FJ237503 |
| WAC1618     | B. grandis | Pemberton, WA | This study | FJ237513 FJ237502 |
| DDS1450     | Soil | Walpole, WA | This study | FJ237515 FJ237504 |
| VHS16439    | B. littoralis | Mandararah, WA | This study | FJ237516 FJ237505 |
| P777        | *E. marginata* | Western Australia | This study | FJ237525 |
| P15945      | *P. citricola* | Citrus canariensis | Mallorca, Spain | Moralez et al. (2008) EUR44196 EUR44194 |
| 77a         | Quercus sp. | Hungary | Lakatos & Szabo (2008) | EU594606 |
| Citri-P1817 | Medicag sativa | South Africa | Knoo et al. (2004) | AY505197 |
| Ps-5        | Rhododendron sp. | Asturias, Spain | Moralez et al. (2008) | EU194425 |
| 83-185      | Antirrhinum majus | Switzerland | Letort et al. (unpubl.) | EU000083 |
| KACC4018    | Ziziphus jujuba | Korea | Hong et al. (unpubl.) | AF228080 |
| CH455       | Mangifer indica | Spain | Zee-Bonilla et al. (2007) | AM234209 |
| P16246      | – | Baleaeric Islands, Spain | Belbahri et al. (unpubl.) | EF153674 |
| P142        | Rhododendron | Switzerland | Belbahri et al. (unpubl.) | EF193230 |
| FR37016     | *P. sojae* | Japan | Villa et al. (2008) | AB217685 |
| BR514       | Phytophthora sp. | Canada | Rose et al. (unpubl.) | DQ821195 |
| IMI 021173  | *C. citrinum* fruit | Taiwan, 1927 | This study | FJ237512 FJ237512 |
| CBS 221.88  | *C. citricola* (ex-type) | *Citrus* sinensis | This study | FJ237526 FJ237508 |
| CBS 295.29  | *C. citricola* (authentic) | *C. sinensis* | This study | FJ237523 FJ237510 |
| CIT9        | Quercus robur | Pulling, Germany | This study | FJ237524 FJ237511 |
| CIT35       | *Q. petraea* | Tivoli, Slovenia | Schubert et al. (1999) | AJ007370 |
| CIT7        | *Q. robur* | Pulling, Germany | Schubert et al. (1999) | AJ007370 |
| MN21HH      | *Rhododendron* | USA | Schwingle et al. (2007) | DQ486661 |
| UASWS0208   | Soil from declining alder stand | Poland | Calmin et al. (unpubl.) | DQ396420 |
| 92-138      | Taxus sp. | Geneva, Switzerland | Belbahri et al. (unpubl.) | AF419946 |
| P131        | *Rhododendron* | Switzerland | Belbahri et al. (unpubl.) | EF193216 |
| 112         | – | Switzerland | Bragante et al. (unpubl.) | EU263906 |
| Citi-P0713  | – | Japan | Uddin et al. (unpubl.) | AB364792 |
| BR518       | Soil | Taipei, Taiwan | Rose et al. (unpubl.) | DQ821183 |
| IMI 013172  | *Rubus* secta | Ireland | Cooke et al. (2000) | AF267685 |
| 6f          | *P. inflata* | Poland | Cordeiro et al. (unpubl.) | EU240195 |
| P44         | – | Slovenia | Munda et al. (unpubl.) | EF423556 |
| InfGaul     | Gaultheria sharon | Scotland | Schlenzig et al. (2005) | AY597929 AY594685 |
| InfRhod2    | *Rhododendron* sp. | Scotland | Schlenzig et al. (2005) | AY597929 |
| InfVacc     | Vaccinium vitis-idea | Scotland | Schlenzig et al. (2005) | AY597929 AY594684 |
| 804         | Soil from declining alder stand | Poland | Cordeiro et al. (unpubl.) | EU240058 |
| IMI 342898  | *Syringa vulgaris* | UK | Cooke et al. (2000) – ITS | FJ237513 FJ237502 |
|             | – | – | Cooke et al. (2004) – cox1 | AF267685 AY564187 |

1 Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; IMI = CABI Bioscience (Imperial Mycological Institute), 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; DD8, DCE = earlier prefixes of VHS collection. Other isolate names and numbers are as given on GenBank.

2 Isolated used in the morphological and growth-temperature studies.

**Table 1** Isolates of *Phytophthora multivora*, *P. citricola* and *P. inflata* considered in the morphological, physiological and phylogenetic studies.
test was repeated once. For temperature growth studies, all isolates were subcultured onto V8A plates and incubated for 24 h at 20 °C to initiate growth. Three replicate plates for each isolate and temperature were then transferred to incubators set at 10, 15, 17.5, 20, 22.5, 25, 30 and 32.5 °C, and radial colony growth was measured as above after 5–7 d.

**Morphology of sporangia and gametangia**

Sporangia and gametangia were produced on V8A and measurements were made as described by Jung et al. (1999). Sporangia were obtained by flooding 5 × 5 mm square agar discs taken from growing margins of 3–5 d old colonies with non-sterile soil extract in 90 mm Petri dishes and incubating them in the dark at 18–22 °C for 12–16 h. The non-sterile soil extract was obtained by flooding 100 mL of commercial composted potting mix (Richgro, Jandakot, WA) with 1 L of deionised water. After 24 h at 10–25 °C, the soil extract was removed from the water surface with a pipette and diluted to 10 % with deionised water. Dimensions and characteristic features of 50 mature sporangia chosen at random were determined at ×400 magnification (BH-Olympus) for each isolate. For each isolate dimensions and characteristic features of 50 mature oogonia, oospores and antheridia, and diameters of 25 primary hyphae chosen at random were measured at ×400 magnification at the surface of 15 mm discs cut from the centre of 14–22 d old V8A cultures grown in the dark at 20 °C. For each isolate the oosporangial wall index was calculated as the ratio between the volume of the oospore wall and the volume of the entire oospore (Dick 1990).

**DNA isolation, amplification and sequencing**

The *Phytophthora* isolates were grown on half strength PDA (Becton, Dickinson and Company, Sparks, USA, 19.5g PDA, 7.5g of agar and 1L of distilled water) 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 AAG 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).

The mitochondrial gene *cox1* 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 AGAT G) (Martin & Tooley 2003). The clean-up of products and sequencing were the same as for the ITS region.

**Phylogenetic analysis**

In order to compare *Phytophthora* isolates used in this study with other closely related species (ITS clade 2, Cooke et al. 2000), additional sequences were obtained from GenBank (Table 1). Sequences were also obtained for species representing other ITS clades (Cooke et al. 2000). Sequence data for the ITS region were initially assembled using Sequence Navigator v. 1.01 (Perkin Elmer) and aligned in Clustal X (Thompson et al. 1997). Manual adjustments were made visually by inserting gaps where necessary in BioEdit v. 5.0.6 (Hall 2001). There were no gaps in the *cox1* alignment. All sequences derived in this study were deposited in GenBank and accession numbers are shown in Table 1.

 Parsimony analysis was performed in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (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 data set as that used in the parsimony analysis. First, MrModeltest v. 2.5 (Nylander J.A.A. 2004. Program distributed by the author. Evolutionary Biology Centre, Uppsala University) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v. 3.1 (Ronquist & Huelsenbeck 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 101 000 generations (i.e. 101 trees), well after the likelihood values converged to stationary, leaving 9 900 trees from which the consensus trees and posterior probabilities were calculated.

**Statistical analysis**

Analyses of Variances were carried out using Statistica v. 5.1 (Statsoft Inc., Tulsa, Oklahoma) to determine whether physiological and morphological measurements were different between isolates.

**RESULTS**

**Phylogenetic analysis**

The ITS dataset consisted of 894 characters of which 420 were parsimony informative. The dataset contained significant phylogenetic signal compared to 1 000 random trees (p < 0.01, g1 = -1.38). Heuristic searches resulted in 10 most parsimonious trees of 848 steps (CI = 0.72, RI = 0.89). The topology of the Bayesian tree was very similar (TreeBASE SN4153) (Fig. 2).

Several ITS sequences from GenBank are identical to *P. multivora* (AB217685, AB367494, AF228080, AM235209, DQ821185, EF153674, EF193230, EU000083, EU194425, EU244846, EU594606). These sequences were not all included in the phylogenetic analysis, but information on origin and studies they have derived from is given in Table 1 and the position of polymorphic nucleotides indicating their similarity to *P. multivora* in Table 2. In addition, on GenBank there are several isolates of *P. multivora*, originally designated as *P. sp.* 4 by Burgess et al. (2009) (EU301126–32 and EU0869149–99). All isolates of *P. multivora* reside in a strongly supported terminal clade clearly distinct from the ex-type and authentic type of *P. citricola* (IMI 021173 and CBS 295.29) within ITS clade 2 (Cooke et al. 2000). Two additional isolates listed on GenBank (Citri-P0713 and BR518) have identical sequence to the ex-type of *P. citricola* (IMI 021173) (Fig. 2). There are seven fixed polymorphisms that are different between *P. multivora* and IMI 021173 (Table 2). Isolates listed on GenBank as *P. citricola* from the Northern Hemisphere (CIT7, CIT9, CIT35, P44, MN211HH, UASWS0208, 92-198, P131, IMI031372, 112) differ by at least 10 bp from *P. multivora*. (Fig. 2, Table 2). Isolates listed on
Fig. 2 Bayesian inference tree using rDNA ITS sequences showing phylogenetic relationships between (A) clade 2 species and representative species from other clades and (B) isolates from the *P. citricola* complex. Numbers above branches represent posterior probability based on Bayesian analysis of the dataset. Both trees result from a single analysis as given in TreeBASE (SN4153). For tree A, clades were collapsed to show the relationship between isolates from *P. citricola* complex and other species in clade 2. Tree B shows the finer details within the *P. citricola* complex (node enclosed in circle on tree A) and the relationship between *P. multivora* and other *P. citricola* and *P. 'inflata'* isolates including the ex-type of *P. citricola* (IMI 021173).
Table 2 Positions of polymorphic nucleotides (bp) from aligned sequence data of the ITS gene region showing the variation between Phytophthora multivora, P. citricola and P. ‘inflata’ isolates. Polymorphisms that differ from the type of P. multivora (WAC13201) are in blue.

| Isolate no. | 4 | 15 | 20 | 43 | 54 | 67 | 154 | 397 | 412 | 485 | 543 | 633 | 650 | 704 | 736 |
|-------------|---|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| P. multivora | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| VHS16439    | A | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| WAC13205    | A | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| WAC13204    | A | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| WAC13203    | A | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| WAC13202    | A | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| WAC13200    | A | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| PT77        | A | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| VHS16168    | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| VHS16158    | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| DDS1450     | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| P15946      | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| P5-5        | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| 77a         | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| Citrip1817  | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| 83-185      | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| KACC40184   | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| Inf6f       | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| BR514       | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| CH455       | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| P16246      | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| P142        | A | – | C | C | – | T | T | A | C | G | A | G | C | T |   |
| P. citricola | A | C | C | – | T | T | A | C | G | A | G | C | T |   |
| IMI 021773* | A | – | C | T | T | T | T | T | G | G | G | G | T | T |
| CBS 295.29  | A | – | C | T | T | T | T | T | G | G | G | G | T | T |
| Citrip0713  | A | – | C | T | T | T | T | T | G | G | G | G | T | T |
| BR518       | A | – | C | T | T | T | T | T | G | G | G | G | T | T |
| CIT7        | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| CIT9        | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| CIT35       | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| P44         | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| MN211Hf     | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| UASWS0208   | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| 92-198      | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| P131        | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| IMI 031372  | A | – | T | T | T | – | T | T | G | T | A | G | T | T |
| 112         | A | – | T | T | T | – | T | T | G | T | A | G | T | T |
| P. ‘inflata’ | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| IMI 342898  | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| 804         | A | – | T | T | T | T | C | T | G | T | G | G | T | T |
| 8f          | A | – | T | T | T | T | C | C | G | T | G | G | T | T |
| InfGaul     | C | – | T | T | T | T | C | C | G | T | G | G | T | T |
| InfRhod2    | C | – | T | T | T | T | C | C | G | T | G | G | T | T |
| InfVac      | A | – | T | T | T | T | C | C | G | T | G | G | T | T |

GenBank as P. ‘inflata’ (6f, P44, InfGaul, InfRhod2, InfVacc, 804, IMI 342898) are dispersed among the northern hemisphere isolates of P. citricola (Fig. 2, Table 2) and it is unclear whether any of these isolates represent the original P. ‘inflata’.

The cox1 dataset consisted of 742 characters of which 107 were parsimony informative. The dataset contained significant phylogenetic signal compared to 1 000 random trees (p < 0.01, g1 = 0.66). Heuristic searches resulted in 12 most parsimonious trees of 301 steps (CI = 0.50, RI = 0.67). The topology of the Bayesian tree was very similar (TreeBASE SN4153) (Fig. 3).

Sporangia abundantia in cultura liquida, persistentia, terminalia, semi-papillata, ovoidea aut limoniforma, rare distorta vel bipapillata, 53 ± 10.1 × 31.8 ± 6.2 µm, ratio longitudo ad altitudinem 1.7 ± 0.2 µm. Systema sexus homothallicum; oogenia globosa vel rare subglobosa, 27.1 ± 2.1 µm. Oosporae fere plerocitaceae, 23.9 ± 2 µm, paries 2.6 ± 0.5 µm. Antheridia paragynosa, 13 ± 2.2 × 8.8 ± 1.1 µm. Chlamydosporae et inflationes hypharum non observarunt. Temperature crescentiae in agaro ‘V8A’, optima c. 25 °C et maxima 30–32.5 °C. Coloniae in agaro ‘V8A’ stellatae cum mycelio aerio restricto et margine submersa. Regiones ‘rDNA ITS’ et ‘cox1’ cum unica sequentia (GenBank FJ237508, FJ237521).

Etymology. Name refers to the wide host range (multi L. = many, -vora L. = feeding).

Sporangia were rarely observed on solid agar but were produced abundantly in non-sterile soil extract. The majority of sporangia for all P. multivora isolates and the ex-type of P. citricola (IMI 021173) were formed between 7–12 h after flooding with soil extract. Little variation in sporangial shapes was observed between the P. multivora isolates. The majority of sporangia were semipapillate and either ovoid, limoniform, ellipsoid or obpyriform (Fig. 4a–d, g–i), sometimes with just a very shallow apical thickening (Fig. 4f), non-cadu-
cous, occasionally forming a conspicuous basal plug (Fig. 4i) that protruded into the empty sporangium. Sporangia with two or three papillae or distorted shapes were occasionally formed by all isolates (Fig. 4e, f, j–l). Sporangia were typically borne terminally (Fig. 4a–f, j–l) but some were laterally attached (Fig. 4g) or intercalary (Fig. 4i). External proliferation was regularly observed (Fig. 4a–d, j, l), either irregular or in lax or dense sympodia. The majority of sporangia of each isolate had re-observed (Fig. 4a–d, j, l), either irregular or in lax or dense sympodia. The majority of sporangia of each isolate had released zoospores between 15–20 h after flooding. Compared to *P. citricola*, sporangia of *P. multivora* showed a higher proportion of abortion or direct germination (Fig. 4j–l) after 24–48 h within the same soil extract. After 24–48 h, bell-shaped sporangia were formed by all six isolates of *P. multivora* which germinated directly from two points without prior formation of papillae (Fig. 4k, l). The mean sporangial dimensions of the six *P. multivora* isolates were 51.0 ± 10.4 × 30.0 ± 5.1 µm (overall range of 25–97 × 13–63 µm) with a length/breadth ratio of 1.7 ± 0.22 (overall range 1.3–3.3). The mean sporangial dimensions of the ex-type of *P. citricola* (IMI 021173), at 50.9 ± 6.9 × 29.9 ± 5.1 µm (range 39–70 × 22–40 µm) and a length/breadth ratio of 1.7 ± 0.3 (overall range 1.3–2.6), were within the range of the *P. multivora* isolates (Table 3). In contrast to *P. multivora*, sporangia of *P. citricola* were generally more variable and often showed distorted shapes including: multiple papillae, curved apices and hyphal beaks. Twelve percent of sporangia of *P. citricola* were distorted compared to 5% in *P. multivora*. With 9% and 10%, respectively, *P. multivora* and *P. citricola* had a similar proportion of sporangia with lateral attachment to the sporangiosphere. Isolate WAC13204 was different from all other *P. multivora* isolates by forming significantly (p < 0.05) larger oospore walls significantly (p < 0.05) thicker oospore walls

![Bayesian inference tree using sequences of mitochondrial gene cox1 showing phylogenetic relationships between *P. multivora* and *P. citricola*, including the ex-type of *P. citricola* (IMI 021173).](image-url)
of *P. multivora* the oospore wall index was significantly higher (*p* < 0.0001) in *P. multivora* (0.52 ± 0.07) than in *P. citricola* (0.36 ± 0.05). Antheridia of both species were ovoid, club-shaped or irregular, almost exclusively paragynous, dichinous and typically attached close to the oogonial stalk. Intercalary and amphigynous antheridia were only rarely observed. After 4 wk in V8A at 20 °C, more than 90% of all *P. multivora* oospores had germinated directly. Since the thick inner oospore wall of *Phytophthora* species erodes during the germination process due to enzymatic digestion of its major components, the glucans, (Erwin & Ribeiro 1996) only the thin outer oospore wall surrounded by the thin oogonial wall was left (Fig. 5e). No direct germination was observed in cultures of the ex-type of *P. citricola* (IMI 021173) growing under the same conditions.

Colony morphology, growth rates and cardinal temperatures — Colony growth patterns of two isolates of *P. multivora* (WAC13201 and WAC13205) and the ex-type isolate of *P. citricola* (IMI 021173) growing under the same conditions.

Colony morphology, growth rates and cardinal temperatures. Colony growth patterns of two isolates of *P. multivora* (WAC13201 and WAC13205) and the ex-type isolate of *P. citricola* (IMI 021173) are shown in Fig. 6. All *P. multivora* isolates except isolate WAC13204 produced similar colony growth patterns on the four different types of media. On V8A, CMA and MEA *P. multivora* isolates produced limited aerial mycelium and distinct growth patterns, while isolate WAC13204 formed fluffy to felty, uniform colonies without distinct growth pattern. The colony morphology on V8A and MEA of all *P. multivora* isolates clearly differed from the colony morphology of the ex-type isolate of *P. citricola* (IMI 021173). *Phytophthora multivora* isolates produced stellate growth patterns with a clearly delimited, submerged margin on V8A and faintly stellate to dendroid patterns on MEA while *P. citricola* formed a typical chrysanthemum pattern on both media. On CMA, *P. multivora* isolates formed appressed to submerged colonies with a faintly stellate to petaloid pattern while *P. citricola* produced even sparser submerged colonies with a faintly stellate pattern. On PDA, the *P. multivora* isolates and *P. citricola* produced petaloid
Fig. 6 Colony morphology of isolates WAC13201 (ex-type) and WAC13205 of Phytophthora multivora, and the ex-type isolate of P. citricola (from top to bottom) after 6 d growth at 20 °C on V8 agar, malt extract agar, cornmeal agar and potato-dextrose agar (from left to right).
Morphological dimensions (µm) and temperature-growth relations of *Phytophthora multivora* and *P. citricola*. Isolate no.1 WAC13200 WAC13201 WAC13202 WAC13203 WAC13204 WAC13205 IMI 0211732

| Specimen | Sporangia | L × b mean | L × b range | Wall thickness | Oogonia | Mean diam | Mean diam | L × b range | Antheridia | L × b mean | L × b range | Growth rate on V8A at optimum (mm/d) | Growth rate on MEA (mm/d) | Growth rate on CMA (mm/d) | Growth rate on PDA (mm/d) |
|----------|-----------|------------|-------------|---------------|---------|-----------|-----------|-------------|------------|------------|-------------|---------------------------------|--------------------------|------------------------|------------------------|
| WAC13200 | 31.8 ± 4  | 53 ± 10.1  | 26–85       | 4.6 ± 0.4     | 25.7 ± 1.3 | 23.9 ± 2  | 21–21     | 9–11        | 13.1 ± 1.8 | 25 ± 1.2  | 38–70       | 5.7 ± 1.1                          | 4.5 ± 0.3                    | 4.9 ± 0.3                | 4.5 ± 0.3               |
| WAC13201 | 26.2 ± 3.1| 44.5 ± 7.8 | 21–28      | 4.9 ± 0.5     | 22.9 ± 1.5 | 22.3 ± 1.5 | 21–21     | 9–11        | 12.4 ± 1.4 | 25 ± 1.2  | 30–50      | 6.1 ± 1.2                          | 5.7 ± 0.3                    | 5.9 ± 0.3                | 5.7 ± 0.3               |
| WAC13202 | 31.8 ± 6.2| 44.2 ± 4.4 | 21–28      | 4.7 ± 0.4     | 22.9 ± 1.5 | 22.3 ± 1.5 | 21–21     | 9–11        | 12.4 ± 1.4 | 25 ± 1.2  | 30–50      | 6.1 ± 1.2                          | 5.7 ± 0.3                    | 5.9 ± 0.3                | 5.7 ± 0.3               |
| WAC13203 | 28.9 ± 4.2| 62.3 ± 10.8| 21–28      | 4.9 ± 0.5     | 22.9 ± 1.5 | 22.3 ± 1.5 | 21–21     | 9–11        | 12.4 ± 1.4 | 25 ± 1.2  | 30–50      | 6.1 ± 1.2                          | 5.7 ± 0.3                    | 5.9 ± 0.3                | 5.7 ± 0.3               |
| WAC13204 | 34.0 ± 4.9| 45.7 ± 5.2 | 21–28      | 4.9 ± 0.5     | 22.9 ± 1.5 | 22.3 ± 1.5 | 21–21     | 9–11        | 12.4 ± 1.4 | 25 ± 1.2  | 30–50      | 6.1 ± 1.2                          | 5.7 ± 0.3                    | 5.9 ± 0.3                | 5.7 ± 0.3               |
| WAC13205 | 27.9 ± 3.6| 50.9 ± 8.4 | 21–28      | 4.9 ± 0.5     | 22.9 ± 1.5 | 22.3 ± 1.5 | 21–21     | 9–11        | 12.4 ± 1.4 | 25 ± 1.2  | 30–50      | 6.1 ± 1.2                          | 5.7 ± 0.3                    | 5.9 ± 0.3                | 5.7 ± 0.3               |
| IMI 021173 | 50.0 ± 9.0| 69.6 ± 10.1| 21–28     | 4.9 ± 0.5     | 22.9 ± 1.5 | 22.3 ± 1.5 | 21–21     | 9–11        | 12.4 ± 1.4 | 25 ± 1.2  | 30–50      | 6.1 ± 1.2                          | 5.7 ± 0.3                    | 5.9 ± 0.3                | 5.7 ± 0.3               |

Temperature growth relations of *P. multivora* and the ex-type isolate of *P. citricola* are shown in Fig. 7. The maximum growth temperature for isolates of both *P. multivora* and the ex-type isolate of *P. citricola* (IMI 021173) on V8A was between 30–32.5 °C. All isolates of *P. multivora* except isolate WAC13204 were unable to grow at 32.5 °C, but started re-growth within 12 h when plates that were incubated for 7 d at 32.5 °C were transferred to 25 °C. All six *P. multivora* isolates had a growth optimum at 25 °C with growth rates ranging from 4.7–6.1 mm/d while *P. citricola* showed a broad growth optimum between 22.5 °C (5.7 mm/d) and 30 °C (5.5 mm/d). Compared to all *P. multivora* isolates the growth rate of *P. citricola* at 20 °C was higher on V8A and CMA and lower on PDA (Table 2). On V8A, over the whole temperature range except at 25 °C, all *P. multivora* isolates were markedly slower growing than *P. citricola* (Fig. 7).

Specimens examined. **Western Australia**, Yalgorup, from rhizosphere soil of declining *Eucalyptus marginata*, May 2007, P. Scott & T. Jung, holotype MURU 434 (dried culture on V8A, Herbarium of Murdoch University, Western Australia), culture ex-type WAC13201; from rhizosphere soil of declining *Eucalyptus gomphocephala*, May 2007, T. Jung & P. Scott, WAC13202; from rhizosphere soil of declining *Eucalyptus gomphocephala*, June 2007, P. Scott, WAC13203; from rhizosphere soil of declining *Agonis flexuosa*, June 2007, P. Scott, WAC13204; from rhizosphere soil of declining *Eucalyptus marginata*, 1980, unknown, WAC13205.

Notes — In previous studies *P. multivora* is referred to as *P. citricola* (Shearer et al. 1987, 1988, Shearer & Tippett 1989, Bunny 1995, Stukely et al. 1997), and more recently as *P. sp. 4* (Burgess et al. 2009). Many isolates from a wide range of host species in WA that had been identified as *P. citricola* in the past must be re-assigned to *P. multivora*. As indicated above, *P. multivora* has been isolated from the south-west of WA from rhizosphere soil of *E. gomphocephala*, *E. marginata* and *A. flexuosa* in Yalgorup National Park. It has also been recovered by the VHS from soil and root samples collected beneath dying, *Phytophthora*-sensitive ‘indicator species’ in native ecosystems in the south-west of WA by the VHS over the last 30 yr, which extends the host list to include *Banksia attenuata*, *B. grandis*, *B. littoralis*, *B. menziesii*, *B. prionotes*, *Conospermum* sp., *Leucopogon verticillatus*, *Xanthorrhoea gracilis*, *Podocarpus drouyniana*, *Patersonia* sp., *Bossiaea* sp., *Gastrolobium spinosum* and *Pinus radiata* (plantation) (Burgess et al. 2009). *Phytophthora multivora* has also recently been isolated from large girdling stem lesions of *B. attenuata* in Injidup, WA (G. Hardy unpubl. data, Fig. 1e, f), and from fine roots of...
**DISCUSSION**

Phytophthora multivora was previously identified as *P. citricola* in WA based solely on morphological characters including homothallic breeding behaviour, production of paragynous antheridia, semipapillate persistent sporangia and oogonia with dimensions in the correct range, absence of catenulate hyphal swellings in liquid culture, and similar growth rates at 25 °C. Phylogenetic analyses of the ITS and cox1 gene regions show that *P. multivora* 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*.

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), and that many of the differences are associated with host and geography (Oudemans et al. 1994, Bhat & Browne 2007). In the isozyme study of Oudemans et al. (1994) a global collection of 125 isolates of *P. citricola* clustered into five distinct subgroups suggesting *P. citricola* is a species complex instead of a single species which is to be expected considering the broad geographic and host range of *P. citricola* isolates (Fontaneto et al. 2008).

Even though multiple *P. citricola* sequences have been submitted to GenBank, sequence data for the ex-type of *P. citricola* (IMI 021173) from *Citrus sinensis* fruits in Taiwan (Sawada 1927) has not previously been available, and this has led to confusion in the phylogeny. Besides the ex-type culture, an authentic type of *P. citricola* (CBS 295.29) isolated from Citrus leaves in Japan was submitted to CBS in 1929 by Sawada. The present study is the first to provide sequence data of these isolates, and our results clearly demonstrate that the isolates from WA constitute a new species, *P. multivora*. Isolates designated as *P. inflata* were distributed through the *P. citricola* complex demonstrating the difficulty in distinguishing between *P. inflata* and *P. citricola*. The original *P. inflata* ex-type from elm trees in the United States (Caroselli & Tucker 1949) has been lost and it has been suggested that designated isolates of *P. inflata* from other hosts (Hall et al. 1992) are conspecific with *P. citricola* (Cooke et al. 2000).

Among isolates from the *P. citricola* complex, isolates now described as *P. multivora* are the most distant to the ex-type of *P. citricola*, differing in the ITS region by 7 bp. However, there appear to be many subclades within the *P. citricola* complex which may correspond to additional new taxa. Further study of this important species complex is required to elucidate the host and geographic range and phylogeny of isolates within the complex and to determine if they constitute new species.

In GenBank, 11 ITS sequences, designated as *P. citricola*, are identical to *P. multivora*. Seven are from unpublished studies in Hungary, Canada, Switzerland, Korea and Japan, and two sequences are from isolates of Moralejo et al. (2008) from ornamental nurseries in Spain, an isolate from *Mangifera indica* in Spain (Zea-Bonilla et al. 2007). In addition, an isolate designated as *P. sojae* in a study from Japan also has identical sequence (Villa et al. 2006). This low number of very recent submissions of *P. multivora* sequences as compared to the high number of other sequences from the *P. citricola* complex indicates that *P. multivora* may have been introduced to these countries. Due to the widespread distribution of *P. multivora* across natural ecosystems in WA, it is likely that WA may be a source of dispersal, possibly via the nursery trade (Brasier 2008).

In the cox1 analysis *P. multivora* and the ex-type isolate of *P. citricola* grouped together with other ITS clade 2 species, although the distance between *P. multivora* and *P. citricola* was greater in the cox1 analysis than in the ITS analysis. In the ITS sequence there was only 1bp difference between all isolates of *P. multivora*. In the cox1 analysis there were more differences resulting in the formation of subclades. The greater phylogenetic variation and presence of subclades in the cox1 analysis reflects the expected faster rate of mitochondrial than genomic DNA evolution (Kroon et al. 2004). The observed variability, however, strongly supports the hypothesis that *P. multivora* in WA is not a recent clonal introduction, but rather was introduced long ago or is endemic. This is also reflected by the phenotypic variability observed among isolates of *P. multivora*.

There was generally some variation in the colony growth patterns and growth rates, and in the dimensions of morphological structures of the different *P. multivora* isolates. However, isolate WAC13204 was particularly different from the other five isolates of *P. multivora*, having significantly larger sporangia, a higher maximum growth temperature and faster growth rates.

A cox1 sequence for *P. citricola* was available on GenBank from the study of Kroon et al. (2004). In their study, this putative *P. citricola* was closest to *P. cryptogea* and they discussed this incongruence, as it was one of the few species that did not fall into the same clades in both the mitochondrial and nuclear gene analysis. With our new sequences for *P. citricola*, including the ex-type isolate, it is clear that the sequence used for *P. citricola* by Kroon et al. (2004) was incorrect.

Morphological similarities between taxa, as observed between *P. multivora* and *P. citricola*, are increasingly found in the unravelling of different species complexes within the genus *Phytophthora* using molecular methods (Brasier et al. 2003, 2004, Jung et al. 2003). This study therefore highlights the importance of using ex-type cultures where available and the value of using molecular tools to unravel the ambiguity of species previously identified solely on morphological characteristics. Over the last 30 yr, in the absence of sufficient molecular techniques, *P. multivora* has been routinely identified in the south-west of WA as *P. citricola* using morphological characteristics. Similar misidentification of *Phytophthora* species has occurred with the identification of *P. pseudosyringae* isolates as *P. syringae* (Jung et al. 2003).

Despite the similarities, there are clear morphological and physiological differences between *P. multivora* and the ex-type isolate of *P. citricola*. If more isolates of *P. citricola* were to be examined the morphological differences between the two species may be less resolved. *Phytophthora multivora* and *P. citricola* produce different colony growth patterns on V8A, MEA and CMA with the most distinct variation observed on V8A. *Phytophthora multivora* has a clear optimum growth temperature of 25 °C while the optimum growth rate of *P. citricola* is at 22.5 °C and decreases by only 0.2 mm/d between 22.5–30 °C. Over the whole temperature range, except of the optimum temperature of 25 °C, *P. multivora* isolates are slower growing than *P. citricola*. Sporangial shapes of *P. multivora* are generally more uniform while in *P. citricola* sporangia are more variable and the frequency of distorted shapes is significantly higher. A high variability of sporangial shapes was also found by Zentmyer et al. (1974) studying *P. citricola* isolates from *Persea americana* in California. Although most morphological measurements of the ex-type isolate of *P. citricola* fell within the range of *P. multivora* isolates there were clear differences between both species. All six *P. multivora* isolates produced on average significantly smaller oogonia and oospores, and significantly thicker oospore walls than *P. citricola*. This is reflected by the oospore wall index, which is the ratio between the volume of
the oospore wall and the volume of the entire oospore (Dick 1990). The oospore wall index of P. multivora (0.52 ± 0.07) was almost 50% higher than that of the ex-type isolate of P. citricola (0.36 ± 0.05). A calculation of the oospore wall index using the original datasets of Jung et al. (1999, 2002, 2003) for P. europaea (0.37 ± 0.07), P. lilicis (0.41 ± 0.11), P. pseudo- syringae (0.27 ± 0.09), P. psychrophila (0.42 ± 0.06), P. quercina (0.45 ± 0.08), P. syringae (0.24 ± 0.07) and P. uliginosa (0.46 ± 0.09) demonstrated that P. multivora had the highest oospore wall index of all nine species examined. The thick oospore wall of P. multivora is most likely an adaptation to the seasonally extremely dry soil conditions in WA. This survival mechanism was also suggested for P. quercina in European oak forests (Jung et al. 1999, 2000). After 4 wk in V8A at 20 °C, in all six P. multivora isolates, more than 90% of the oospores had germinated directly. This lack of dormancy had previously been observed for oospor excs of P. medicaginis (Erwin & Ribeiro 1996), however, this does not preclude dormancy occurring under different conditions. No direct germination was observed in cultures of the ex-type isolate of P. citricola growing under the same conditions. This result corresponds to the low oospore germination rates observed in European isolates of the P. citri- cola complex (Delcan & Brasier 2001). Whether these differ- ences in germination rates reflect different survival mechanisms of the two species requires further investigation.

Phytophthora multivora can easily be distinguished from other homothallic Phytophthora species with paragynous antheridia and semi-papillate sporangia by its unique combination of morphological and physiological characters, and DNA sequences. Phytophthora multivora is separated from P. syringae by the absence of hyphal swellings, the occurrence of distorted and bipapillate sporangia, thicker oospore walls, different colony growth patterns on V8A, MEA and CMA, higher optimum and maximum temperatures for growth, and different ITS and cox1 sequences (Waterhouse & Waterston 1964, Erwin & Ribeiro 1996, Jung et al. 2003). Phytophthora multivora can be distin-

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