



# *Phytophthora* taxa associated with cultivated *Agathosma*, with emphasis on the *P. citricola* complex and *P. capensis* sp. nov.

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## Key words

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**Abstract** *Agathosma* species, which are indigenous to South Africa, are also cultivated for commercial use. Recently growers experienced severe plant loss, and symptoms shown by affected plants suggested that a soilborne disease could be the cause of death. A number of *Phytophthora* taxa were isolated from diseased plants, and this paper reports their identity, mating type, and pathogenicity to young *Agathosma* plants. Using morphological and sequence data seven *Phytophthora* taxa were identified: the A1 mating type of *P. cinnamomi* var. *cinnamomi*, *P. cinnamomi* var. *parvispora* and *P. cryptogea*, the A2 mating type of *P. drechsleri* and *P. nicotianae*, and two homothallic taxa from the *P. citricola* complex. The identity of isolates in the *P. citricola* complex was resolved using reference isolates of *P. citricola* CIT groups 1 to 5 sensu Oudemans et al. (1994) along with multi-locus phylogenies (three nuclear and two mitochondrial regions), isozyme analyses, morphological characteristics and temperature-growth studies. These analyses revealed the isolates from *Agathosma* to include *P. multivora* and a putative novel species, *P. taxon emzansi*. Furthermore, among the *P. citricola* reference isolates the presence of a new species was revealed, described here as *P. capensis*. Findings of our study, along with some recent other studies, have contributed to resolving some of the species complexity within the *P. citricola* complex, resulting in the identification of a number of phylogenetically distinct taxa. The pathogenicity of representative isolates of the taxa from *Agathosma* was tested on *A. betulina* seedlings. The putative novel species, *P. taxon emzansi*, and *P. cinnamomi* var. *parvispora* were non-pathogenic, whereas the other species were pathogenic to this host.

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## INTRODUCTION

*Agathosma* species (*Rutaceae*) are indigenous to South Africa, and are slow-growing, sclerophyllous shrubs. The local common name given to these plants is 'buchu'. They form part of the Cape Floral Kingdom, which occupies the smallest land area of all plant kingdoms, but has the greatest species richness known to mankind, even surpassing that of the tropical rain forests (Cowling & Richardson 1995). The Cape Floral Kingdom, often referred to as 'Fynbos' (meaning fine-leaved shrub), is found in the Western and Southern Cape provinces of South Africa. The Western Cape enjoys a Mediterranean climate but has very poor, sandy, acidic soils to which native flora are well adapted. Many indigenous plants in South Africa are grown for commercial gain. Of the naturally occurring *Agathosma* species only *A. betulina* and *A. crenulata* are cultivated locally to produce traditional medicines, cosmetic products, aromatic oils and food flavourants (Lubbe et al. 2003).

Although commercial production of *Agathosma* is hampered by soilborne disease (Lubbe et al. 2003), little is known about pathogens that cause disease on this host. So far the only soilborne disease reported on *Agathosma* is Fusarium wilt caused by *Fusarium oxysporum* (Lubbe et al. 2003). By contrast both Fusarium wilt and *Phytophthora* have been reported on other Cape Floral Kingdom plants in South Africa, notably

the *Proteaceae* (Crous et al. 2004). *Phytophthora* diseases of *Proteaceae* occur both in the wild and commercial production stands in Australia and South Africa (Boersma et al. 2000, Crous et al. 2004). Symptoms shown by infected plants include wilting, chlorosis, root- and collar-rot, and widespread death is a consequence in both commercial fields and the wild (von Broembsen & Brits 1985, von Broembsen & Kruger 1985, Wills 1993, Boersma et al. 2000). *Phytophthora cinnamomi* is considered the most virulent and widespread species affecting *Proteaceae* (von Broembsen & Brits 1985, Cahill et al. 2008), but other species, namely *P. citricola*, *P. cryptogea*, *P. drechsleri* and *P. nicotianae* also cause disease on *Proteaceae* plants in Australia (Hardy & Sivasithamparam 1988, Tynan et al. 1998, Boersma et al. 2000).

Identification of *Phytophthora* species and determining their role in limiting plant production is important, as it will influence disease management strategies. Correct identification of *Phytophthora* requires the use of both morphological and adaptive (i.e. temperature-growth) characteristics as well as molecular data, since morphological criteria alone are too variable and cannot be used reliably to identify species (Boersma et al. 2000). Traditionally, sequences of the internal transcribed spacer (ITS) region of the nuclear rDNA operon are used to identify *Phytophthora* species. This gene region was also used by Cooke et al. (2000) to sub-divide the genus *Phytophthora* into seven clades. Although the ITS region is useful, sequence data from other gene regions may be required to distinguish cryptic species, i.e. those that cannot be differentiated morphologically, but that are phylogenetically distinct (Schena & Cooke 2006, Burgess et al. 2009). This is an especially important tool for resolving species in *Phytophthora* species complexes such as *P. citricola*.

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In the *P. citricola* complex, Oudemans et al. (1994) were the first to contribute significantly to our understanding of the phenotypic and genotypic variation within the complex. Using an unweighted pair group method with arithmetic mean (UPGMA) analysis of multi-locus isozyme data, Oudemans et al. (1994) identified five distinct groups described as CIT1 through to CIT5 from a global collection of 125 *P. citricola* isolates. The five *P. citricola* CIT groups, hereafter referred to as *P. citricola* CIT groups sensu Oudemans et al. (1994), could be further subdivided into 10 electrophoretic types (ETs). Some CIT groups contained more than one ET, for example CIT1 consisted of three ETs (1–3); CIT3 also contained three ETs (5–7), but only two ETs (9 and 10) were identified in CIT5. Among the isolates investigated by Oudemans et al. (1994), the CIT4 group was hypothesised to be unique to indigenous plants in South Africa. Subsequent to the study of Oudemans et al. (1994), a few DNA-based studies have investigated the diversity within the *P. citricola* complex. Kong et al. (2003) used ITS single-strand-conformation polymorphisms (SSCPs) to identify four genetically different *P. citricola* subgroups (I–IV), of which groups I–III have been published as distinct *P. citricola* subgroups (Gallegly & Hong 2008). Recently, Hong et al. (2009) described the subgroup from avocado (synonym *P. citricola* CIT5 sensu Oudemans et al. 1994) as a distinct species, namely *P. menzei*, based on morphology and sequence analyses of the ITS region and three other gene regions. The aforementioned studies did not include any isolates of the *P. citricola* CIT sensu Oudemans et al. (1994) groups. Scott et al. (2009) and Jung & Burgess (2009) used sequence data of the ITS, cytochrome oxidase 1 (*cox1*) and  $\beta$ -tubulin ( $\beta$ -tub) regions to describe two new species, *P. multivora* (synonym CIT3, *Phytophthora* sp. 4) and *P. plurivora* (synonym CIT1), within the *P. citricola* complex, using only three of the CIT groups (CIT1 (ET1 and 2), CIT2 and CIT3 (ET7)) as reference sequences. Jung & Burgess (2009) further identified a new putative ITS clade in the *P. citricola* complex, named *P. citricola* clade E, which includes the *P. citricola* isolate (IMI 1031372) used in the phylogeny of Cooke et al. (2000) and one *P. citricola* CIT2 sensu Oudemans et al. (1994) isolate (P1321).

In South Africa, *Agathosma* growers experienced plant losses associated with symptoms of root rots including wilt and chlorosis. Because *Phytophthora* species are well-known pathogens of native flora in this country, the first aim of our study was to determine whether *Phytophthora* species were associated with symptomatic *Agathosma* plants. Secondly, we wanted to identify all *Phytophthora* isolates obtained in the survey to species level using morphological features and sequence data of three nuclear regions ( $\beta$ -tub, translation elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) and ITS) and two mitochondrial regions (NADH dehydrogenase subunit I (NADH) and *cox1*). Since isolates initially identified as *P. citricola* were among those found on diseased *Agathosma* plants, multi-locus phylogenetic analyses were required to determine their position in the *P. citricola* complex. The multi-locus phylogenetic analyses and morphological studies included 13 representative isolates of the *P. citricola* CIT groups 1 to 5 sensu Oudemans et al. (1994). Lastly, the mating type and pathogenicity towards *A. betulina* seedlings were determined for the different *Phytophthora* taxa from *Agathosma*.

## MATERIALS AND METHODS

### Isolates

A survey of seven nurseries and nine fields in different *Agathosma* plantations was conducted during 2004–2005 in the Western Cape Province of South Africa. Symptomatic plants of varying ages were sampled, of which some exhibited typical symptoms associated with root rot, viz leaf chlorosis, wilt and rot of the outer root tissue. Prior to isolation, plant parts

were surface disinfected by first submerging in 1 % NaOCl for 2 min, followed by 70 % alcohol for 1 min and then rinsing in distilled water. Pieces of tissue from the dead-live margins of discoloured roots and the root collar region were plated onto 2 % potato-dextrose agar (PDA, Biolab, Midrand, South Africa) and PARPH medium (Erwin & Ribeiro 1996). Mycelial growth that developed on the isolation media was transferred to PDA. Plates were incubated under near-ultraviolet and cool white light with a 12 h photoperiod for 3 wk. Preliminary identifications to genus level were made using microscopy.

### Reference isolates

Reference isolates representing the A1 and A2 mating types of *P. cinnamomi* (P2425, P2160), *P. cryptogea* (P1088, P1704) and *P. nicotianae* (P1452, P1325) were obtained from the World *Phytophthora* Genetic Resource Collection (WOC) (<http://phytophthora.ucr.edu/default.html>). Thirteen *P. citricola* isolates were also obtained from this collection and included all five *P. citricola* CIT groups sensu Oudemans et al. (1994) and almost all of the ETs, with the exception of CIT3 (ET 5) within the groups, i.e. three CIT1 isolates (ETs 1–3), two CIT2 isolates (ET 4), three CIT3 isolates (ETs 6 and 7), three CIT4 isolates (ET 8) and two CIT5 isolates (ETs 9 and 10) (Table 1).

All isolates, including those obtained in the survey were cultured on cornmeal agar (CMA, Sigma-aldrich, St Louis, USA) and V8-agar (Galindo & Gallegly 1960). Blocks of culture (5 × 5 mm) were stored at 15–18 °C in sterile distilled water with and without sterile citrus leaf disks, as well as on slants of potato-carrot agar (Dhingra & Sinclair 1985).

### Morphological, culture and mating type characterisation

#### Morphology and cardinal temperature studies

Isolates of *Phytophthora* from *Agathosma* plants were grouped based on phenotypic traits in a similar way to that described by Brasier et al. (2003), using gross colony morphology.

Mean radial growth rate (mm per day) of isolates was then determined on CMA at 5 °C intervals between 5–35 °C. A 5 mm diam mycelium plug was placed at the centre of a 9 cm diam Petri plate and the average growth rate (mm per day) was calculated after 5 d. Three replicate plates were used for each isolate. At the end of the study, plates that had been incubated at 35 °C were transferred to 25 °C to assess whether mycelial re-growth occurred thereby obtaining an estimate of upper lethal temperature.

In order to produce morphological structures, isolates were cultured on CMA and incubated at 25 °C in the dark. Soil extract was prepared by filtering a soil suspension (20 g sandy soil/L distilled water) through Whatman no.1 filter paper and the filtrate was autoclaved for 20 min at 121 °C and 15 kpa. Five agar plugs (5 mm diam) of each isolate cut from the actively growing colony margins were placed in sterile 9 cm Petri dishes containing 5 mL sterile soil-water extract plus two 1 cm long freshly picked, unsterilised blades of grass (*Pennisetum clandestinum*). Cultures were incubated at 25 °C under cool white fluorescent light for 12 h until sporulation was observed. Sporangia, hyphal bodies, chlamydospores, oogonia and oospores were measured using a compound light microscope with differential interference contrast illumination at ×60, ×400 and ×1000 magnifications. The tabulated key of Erwin & Ribeiro (1996) and descriptions by Gallegly & Hong (2008) were used to place isolates into putative species groups.

#### Comparative examination of isolates in the *P. citricola* complex

At the start of the study, seven isolates from *Agathosma* plants (STE-U 6558, 6267–6272) that were provisionally identified as

Table 1 Phytophthora isolates collected from *Agathosma* species in South Africa as well as reference *P. citricola* complex isolates used in the study of Oudemans et al. (1994).

Culture no. <sup>1</sup>	Host	Morphological identification	Sequence identification <sup>2</sup>	CIT group-ET <sup>3</sup>	Isozyme genotype			Mating type	Nursery/Field	Location
					GPI <sup>4</sup>	MDH-1 <sup>5</sup>	MDH-2 <sup>5</sup>			
P1822	Stream water	<i>P. citricola</i>	<i>P. capensis</i>	4–8	EF	CD	AB	–	–	South Africa
P1823	<i>Olea capensis</i>	<i>P. citricola</i>	<i>P. capensis</i>	4–8	EF	CD	AB	–	–	South Africa
P1819	<i>Curtisia dentate</i>	<i>P. citricola</i>	<i>P. capensis</i>	4–8	EF	CD	AB	–	–	South Africa
STE-U 6255	<i>Agathosma crenulata</i>	<i>P. cinnamomi</i>	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	–	n.d.	n.d.	n.d.	A1	Nursery	South Africa
STE-U 6256	<i>A. crenulata</i>	<i>P. cinnamomi</i>	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	–	n.d.	n.d.	n.d.	A1	Nursery	South Africa
STE-U 6257	<i>A. crenulata</i>	<i>P. cinnamomi</i>	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	–	n.d.	n.d.	n.d.	A1	Nursery	South Africa
STE-U 6258	<i>Agathosma betulina</i>	<i>P. cinnamomi</i>	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	–	n.d.	n.d.	n.d.	A1	Field	South Africa
STE-U 6259	<i>A. betulina</i>	<i>P. cinnamomi</i>	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	–	n.d.	n.d.	n.d.	A1	Field	South Africa
STE-U 6260	<i>A. betulina</i>	<i>P. cinnamomi</i>	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	–	n.d.	n.d.	n.d.	A1	Field	South Africa
STE-U 6266	<i>A. betulina</i>	<i>P. cinnamomi</i>	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	–	n.d.	n.d.	n.d.	A1	Field	South Africa
STE-U 6265	<i>A. betulina</i>	<i>P. cinnamomi</i>	<i>P. cinnamomi</i> var. <i>parvispora</i>	–	n.d.	n.d.	n.d.	A1	Field	South Africa
P1321	<i>Rubus idaeus</i>	<i>P. citricola</i>	<i>P. citricola</i>	2–4	EF	AA	AA	–	–	USA
P6624	<i>Fragaria</i> sp.	<i>P. citricola</i>	<i>P. citricola</i>	2–4	EF	AA	AA	–	–	Taiwan
P1814	<i>Citrus</i> sp.	<i>P. citricola</i>	<i>P. citricola</i> s.str. <sup>6</sup>	1–3	EF	CC	AA	–	–	South Africa
P1815	<i>Citrus</i> sp.	<i>P. citricola</i>	<i>P. citricola</i> s.str.	1–2	EF	CC	AA	–	–	South Africa
STE-U 6262	<i>A. betulina</i>	<i>P. cryptogea</i>	<i>P. cryptogea</i>	–	n.d.	n.d.	n.d.	No oogonia	Field	South Africa
STE-U 6263	<i>A. betulina</i>	<i>P. cryptogea</i>	<i>P. cryptogea</i>	–	n.d.	n.d.	n.d.	A1	Field	South Africa
STE-U 6261	<i>A. betulina</i>	<i>P. cryptogea</i>	<i>P. cryptogea</i>	–	n.d.	n.d.	n.d.	A1	Field	South Africa
STE-U 6264	<i>A. betulina</i>	<i>P. drechsleri</i>	<i>P. drechsleri</i>	–	n.d.	n.d.	n.d.	A2	Field	South Africa
P0513	<i>Persea americana</i>	<i>P. citricola</i>	<i>P. mingei</i>	5–9	AB	CD	AA	–	–	Mexico
P1165	<i>P. americana</i>	<i>P. citricola</i>	<i>P. mingei</i>	5–10	CD	CD	AA	–	–	Guatemala
STE-U 6267	<i>A. betulina</i>	<i>P. citricola</i>	<i>P. multivora</i>	3	EF	BB	AA	Homothallic	Nursery	South Africa
STE-U 6268	<i>A. crenulata</i>	<i>P. citricola</i>	<i>P. multivora</i>	3	EF	BB	AA	Homothallic	Nursery	South Africa
STE-U 6270	<i>A. crenulata</i>	<i>P. citricola</i>	<i>P. multivora</i>	3	EF	BB	AA	Homothallic	Nursery	South Africa
STE-U 6558	<i>A. betulina</i>	<i>P. citricola</i>	<i>P. multivora</i>	3	EF	BB	AA	Homothallic	Nursery	South Africa
STE-U 6271	<i>A. betulina</i>	<i>P. citricola</i>	<i>P. multivora</i>	3	EF	BB	AA	Homothallic	Nursery	South Africa
P1818	<i>Medicago sativa</i>	<i>P. citricola</i>	<i>P. multivora</i>	3–6	–	BB	–	–	–	South Africa
P1817	<i>M. sativa</i>	<i>P. citricola</i>	<i>P. multivora</i>	3–7	EF	BB	AA	–	–	South Africa
P1821	<i>Ocotea bullata</i>	<i>P. citricola</i>	<i>P. multivora</i>	3–6	EF	BB	AA	–	–	South Africa
STE-U 6273	<i>A. betulina</i>	<i>P. nicotianae</i>	<i>P. nicotianae</i>	–	n.d.	n.d.	n.d.	A2	Nursery	South Africa
P1611	<i>Malus</i> sp.	<i>P. citricola</i>	<i>P. plurivora</i>	1–1	EF	CC	AA	–	–	USA
STE-U 6272	<i>A. betulina</i>	<i>P. citricola</i>	<i>P. taxon emzansi</i>	New	EF	CC	AA	Homothallic	Field	South Africa
STE-U 6269	<i>A. betulina</i>	<i>P. citricola</i>	<i>P. taxon emzansi</i>	New	EF	CC	AA	Homothallic	Field	South Africa

<sup>1</sup> Isolates with STE-U codes were isolated during the current study, whereas those with P-codes are from the study of Oudemans et al. (1994).

<sup>2</sup> Sequence based identifications were conducted using a combined  $\beta$ -tubulin / cytochrome oxidase I / translation elongation factor 1 $\alpha$  phylogeny.

<sup>3</sup> The *Phytophthora citricola* multi-locus isozyme group (CIT) of *P. citricola* isolates are followed by the electrophoretic group (ET) as reported by Oudemans et al. (1994).

<sup>4</sup> Glucose-6-phosphate isomerase (synonym of phosphoglucose isomerase (PGI)) alleles (A to F).

<sup>5</sup> Malate dehydrogenase (MDH) loci MDH-1 and MDH-2 alleles (A to D).

<sup>6</sup> s.str. = sensu stricto.

*P. citricola* were studied comparatively using 12 *P. citricola* CIT groups (1–5) sensu Oudemans et al. (1994) reference isolates (P1165, P1321, P1611, P1814, P1815, P1817–P1819 and P1821–P6624). Isolate P0513 (CIT5, ET9) was not included in these studies, since the isolate has become unstable (i.e. growth rate and colony morphology were variable across tests, and oospores were not produced). As the study progressed representative isolates were selected for detailed investigation as indicated in the text.

The methods used for the growth studies and induction of morphological structures were the same as those described by Brasier & Kirk (2001). For the growth studies all seven isolates from *Agathosma* and the 12 reference isolates were used for comparison. Eleven temperatures were tested (1, 5, 10, 15, 20, 22.5, 25, 27.5, 30, 35 and 36 °C) with two replicates per isolate-temperature combination. Perpendicular diameters were measured 4 d after plates were inoculated and the mean growth rates (mm per day) calculated for each taxon in the *P. citricola* complex. Once these measurements had been made plates incubated at 20 °C were returned to the 20 °C incubator for a further 3 d, after which the colony morphologies were recorded i.e. after 7 d incubation.

A representative subset of the above isolates was then selected for detailed morphological study (measurement of structures). Isolates from *Agathosma* included STE-U 6269, 6270, 6272 and 6558 and from the reference collection P1165, P1321, P1611, P1814, P1817, P1819 and P1822. To compare morphological features, isolates were plated onto carrot agar (CA, Brasier 1969) and incubated at 20 °C in the dark for 11 or 20 d to produce sexual reproductive structures. To induce sporangial formation, mycelial discs were cut from the margin of 3–5 day old colonies on CA, placed in fresh pond water, incubated at 20 °C in the dark and examined after 24 h onwards. Mycelia supporting morphological structures were mounted in 5 % cotton blue in lactic acid and examined using brightfield microscopy with  $\times 200$ ,  $\times 400$  and  $\times 1000$  magnification.

#### Mating type determination of heterothallic species

The mating type of heterothallic isolates obtained from *Agathosma* plants was determined by pairing each isolate with a known A1 and A2 tester strain of the corresponding species or closely related species (*P. cinnamomi* isolates P2425 and P2160, *P. cryptogea* isolates P1088 and P1704 and *P. nicotianae* isolates P1452 and P1325). Duplicate pairings were carried out on CA (Brasier & Kirk 2001) and plates were incubated at 20 °C in the dark for 3 wk. Plates were inverted and examined for the presence of oospores using a light microscope with  $\times 100$  magnification. Isolates were designated as A1 mating type if oospores were formed with the A2 tester isolate, and vice versa for the designation of A2 mating type isolates.

#### DNA isolation, amplification and sequencing

DNA was extracted from 33 isolates (20 *Phytophthora* isolates obtained from *Agathosma* and 13 *P. citricola* CIT groups sensu Oudemans et al. (1994) reference isolates). Isolates were cultured on V8-agar for 2 wk. DNA was extracted from these cultures using the GenElute Plant genomic DNA miniprep kit (Sigma-Aldrich, St Louis, USA) according to manufacturer's instructions, with the exception that mycelia scraped from culture plates were first macerated in micro centrifuge tubes in the extraction buffer for 5 min in a Mixer Mill type MM 301 beater (Retsch GmbH & Co, Haan, Germany).

Three nuclear gene regions ( $\beta$ -tub, ITS and EF-1 $\alpha$ ) and two mitochondrial gene regions (*cox1* and NADH) were PCR amplified and sequenced for all the *Phytophthora* isolates. The DNA regions and primers used for amplification included: i) the ITS region with primers ITS4 (White et al. 1990) and

ITS6 (Cooke & Duncan 1997); ii)  $\beta$ -tub with primers TUBF1 and TUBR1 (Kroon et al. 2004); iii) EF-1 $\alpha$  with ELONGF1 and ELONGR1 primers (Kroon et al. 2004); iv) *cox1* with primers FM83 and FM84 (Martin & Tooley 2003); and v) NADH with NADHF1 and NADHR1 primers (Kroon et al. 2004). Each PCR amplification consisted of 1 $\times$  BIOTAQ™ PCR buffer (Bioline, London, UK), 0.2 mM dNTPs, 1 U BIOTAQ™ polymerase (Bioline), 0.05  $\mu$ g/ $\mu$ L bovine serum albumin (BSA) Fraction V (Roche Diagnostics, Randburg, South Africa) (except for *cox1*), 2  $\mu$ L genomic DNA and MgCl<sub>2</sub> and primers specific for each primer pair, in a total reaction volume of 40  $\mu$ L. The MgCl<sub>2</sub> concentrations were 2.5 mM for ITS and  $\beta$ -tub, 1.5 mM for EF-1 $\alpha$  and NADH and 1.5 to 4 mM for *cox1* (different species required different concentrations). The primer concentrations for each gene region were 0.2  $\mu$ M for ITS, 0.4  $\mu$ M for EF-1 $\alpha$  and NADH, 0.313  $\mu$ M for *cox1* and 1  $\mu$ M for  $\beta$ -tub. The PCR amplifications were conducted in a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA) thermal cycler. Amplification conditions consisted of 1 cycle of 94 °C for 7 min followed by 40 cycles of 94 °C for 30 s, 58 °C ( $\beta$ -tub, EF-1 $\alpha$ , NADH), 52 °C (*cox1*) or 50 °C (ITS) for 30 s, 72 °C for 90 s and a final extension step of 7 min at 72 °C.

Amplification products were purified using the MSB® Spin PCR-purification clean-up kit (Invitex, Berlin, Germany) according to manufacturer's instructions. Sequencing reactions of the PCR products were conducted in both directions using the ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Ready reaction Kit (Applied Biosystems) according to manufacturer's instructions. Sequence reaction products were analysed by the Central Analytical Sequencing Facility at Stellenbosch University on an ABI PRISM® 3130xl DNA Sequencer (Applied Biosystems). Geneious Pro 3.0.6 (Biomatters Ltd., Auckland, New Zealand) was used for obtaining double strand consensus sequence data.

#### Molecular phylogenies

Isolates were identified using BLAST analyses of consensus gene sequence data (Table 2) to those in NCBI's GenBank sequence database. Only GenBank sequences deposited and published by recognised authorities including Cooke et al. (2000), Martin & Tooley (2003), Kroon et al. (2004), Hong et al. (2009), Jung & Burgess (2009) and Scott et al. (2009) were used for species identification. The sequences of *Phytophthora* isolates that were similar to *P. citricola* and *P. cinnamomi* required further phylogenetic analyses, since it is known that *P. citricola* contains cryptic species (Jung & Burgess 2009) and it was not clear whether the *P. cinnamomi* isolates were *P. cinnamomi* or *P. cinnamomi* var. *parvispora*. Since *P. citricola* and *P. cinnamomi* belong to *Phytophthora* clade 2 and 7 (Cooke et al. 2000) respectively, phylogenies were constructed separately for these clades. Reference sequences used in clades 2 and 7 phylogenies were from Cooke et al. (2000), Martin & Tooley (2003), Kroon et al. (2004) and Abad et al. (2008). Additionally, for *P. citricola* clade 2 analyses isolates of *P. multivora*, *P. plurivora*, the type and authentic type strains of *P. citricola* submitted by Jung & Burgess (2009) were also included. In the ITS phylogeny, the sequences of *P. citricola* type (GenBank FJ237526; IMI 021173 = CBS 221.88) and of *P. citricola* authentic type (GenBank FJ560913; CBS 295.29) have been included, which represents *P. citricola* s.str. The sequences of these type and authentic types were identical to P1814 and P1815, which therefore also represent *P. citricola* s.str. Only the group I *P. citricola* sequences of *P. citricola* groups I to III of Gallegly & Hong (2008) were included, since these were published by Jung & Burgess (2009). The group II and III GenBank ITS sequences are unpublished and were therefore not used in our phylogenetic study. ITS GenBank sequences that had a similarity of more than 98 %

**Table 2** GenBank accession numbers for five regions that were sequenced from *Phytophthora* isolates used in the study.

Culture no.	Sequence identification <sup>1</sup>	GenBank accession no.				
		ITS <sup>2</sup>	$\beta$ -tub	EF-1 $\alpha$	NADH <sup>3</sup>	cox1
P1822	<i>P. capensis</i>	GU191219	GU191325	GU191196	GU191267	GU191277
P1823	<i>P. capensis</i>	GU191231	GU191327	GU191198	GU191256	GU191298
P1819	<i>P. capensis</i> (ex-type)	GU191232	GU191328	GU191199	GU191257	GU191275
STE-U 6255	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	GU191204	GU191304	GU191175	GU902245	GU191273
STE-U 6256	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	GU191206	GU191305	GU191176	GU191239	GU191282
STE-U 6257	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	GU191207	GU191300	GU191171	GU191240	GU191281
STE-U 6258	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	GU191208	GU191306	GU191177	GU191236	GU191289
STE-U 6259	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	GU191209	GU191303	GU191174	GU191237	GU191283
STE-U 6260	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	GU191210	GU191302	GU191173	GU191241	GU191291
STE-U 6266	<i>P. cinnamomi</i> var. <i>cinnamomi</i>	GU191205	GU191301	GU191172	GU191238	GU191290
STE-U 6265	<i>P. cinnamomi</i> var. <i>parvispora</i>	GU191211	GU191319	GU191190	GU191246	GU191286
P1814	<i>P. citricola</i> s.str.	GU191217	GU191321	GU191192	GU191263	GU191278
P1815	<i>P. citricola</i> s.str.	GU191218	GU191322	GU191193	GU191264	GU191274
P1321	<i>P. citricola</i> CIT2	GU191216	GU191320	GU191191	GU191262	GU191296
P6624	<i>P. citricola</i> CIT2	GU191230	GU191326	GU191197	GU191255	GU191280
STE-U 6261	<i>P. cryptogea</i>	GU191214	GU191309	GU191180	GU191243	GU191287
STE-U 6262	<i>P. cryptogea</i>	GU191213	GU191307	GU191178	GU191242	GU191268
STE-U 6263	<i>P. cryptogea</i>	GU191212	GU191308	GU191179	GU191244	GU191288
STE-U 6264	<i>P. drechsleri</i>	GU191215	GU191310	GU191181	GU191247	GU191271
P0513	<i>P. menzei</i>	GU191234	GU191332	GU191203	GU191261	GU191295
P1165	<i>P. menzei</i>	GU191235	GU191331	GU191202	GU191260	GU191299
P1817	<i>P. multivora</i>	GU191221	GU191323	GU191194	GU191265	GU191297
P1818	<i>P. multivora</i>	GU191229	GU191329	GU191200	GU191258	–
P1821	<i>P. multivora</i>	GU191222	GU191324	GU191195	GU191266	GU191276
STE-U 6267	<i>P. multivora</i>	GU191225	GU191315	GU191186	GU191251	GU191294
STE-U 6268	<i>P. multivora</i>	GU191226	GU191312	GU191183	GU191254	GU191285
STE-U 6270	<i>P. multivora</i>	GU191227	GU191311	GU191182	GU191253	GU191284
STE-U 6271	<i>P. multivora</i>	GU191224	GU191313	GU191184	GU191252	GU191293
STE-U 6558	<i>P. multivora</i>	GU191223	GU191314	GU191185	GU191248	GU191292
STE-U 6273	<i>P. nicotianae</i>	GU902246	GU191318	GU191189	GU191245	GU191272
P1611	<i>P. plurivora</i>	GU191233	GU191330	GU191201	GU191259	GU191279
STE-U 6269	<i>P. taxon emzansi</i>	GU191228	GU191317	GU191188	GU191250	GU191270
STE-U 6272	<i>P. taxon emzansi</i>	GU191220	GU191316	GU191187	GU191249	GU191269

<sup>1</sup> Sequence based identifications were conducted using a combined  $\beta$ -tubulin ( $\beta$ -tub) / cytochrome oxidase I (cox1) / translation elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) phylogeny.

<sup>2</sup> Internal transcribed spacer regions (ITS1 and ITS2) and 5.8S nuclear ribosomal RNA gene.

<sup>3</sup> NADH dehydrogenase subunit I.

to the CIT4 group sequences and sequences of STE-U 6272 and 6269, were also used in the clade 2 phylogeny. Additional sequences used for the *P. cinnamomi* clade 7 analyses included GenBank sequences that had more than 98 % similarity to isolate STE-U 6265 that showed highest sequence similarity to a GenBank accession of *P. cinnamomi* var. *parvispora*.

The sequences of each clade were aligned using MAFFT sequence alignment programme v6 (Kato & Toh 2008), and were manually adjusted in Sequence Alignment Editor v2.0a11 (Rambaut 2002). For each clade, maximum parsimony analysis was conducted as described below. Additionally, for phylogenies presented in this paper, Bayesian analysis was also conducted. The alignments and trees were lodged in TreeBASE (S10489).

Maximum parsimony analysis was conducted using PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford 2003), with the heuristic search option with 100 random taxon additions. Tree bisection and reconstruction (TBR) was used as the branch swapping algorithm with the option of saving no more than 10 trees with a score greater than or equal to five (Harrison & Langdale 2006). Gaps were treated as missing data and all characters were unordered and of equal weight. Bootstrap support values were calculated from 1 000 heuristic search replicates and 10 random taxon additions. Other measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and the rescaled consistency index (RC) values. Partition homogeneity tests were conducted in PAUP to test the pairwise congruency between the various mitochondrial and nuclear gene regions.

Bayesian analyses were conducted using MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). The optimal model of se-

quence evolution for each clade alignment was selected using the program MrModeltest (J.J.A. Nylander, available from the internet: <http://www.abc.se/~nylander/>) and the likelihood and prior settings were changed according to the best models found for each partition. Markov chains were initiated from a random tree and run for 1 million generations, keeping one out of every 100th generation. Convergence among chains was monitored by examining plots of log-likelihood values and observing when the values of the four chains reached a plateau. The average deviation of split frequencies were 0.00304, 0.00607 and 0.00373 for the *P. cinnamomi* ('clade 2'), *P. citricola* ITS ('clade 7'), and *P. citricola* combined ( $\beta$ -tub, EF-1 $\alpha$  and cox1) phylogenies, respectively. The potential scale reduction factors (PSRF) were one for each of the parameters for all of the three analyses. For the ITS phylogenies the first 28 500 and 40 000 generations (burn-in) were discarded for the analyses of clades 2 and 7 respectively, and the remaining samples were used to calculate the 50 % majority-rule tree and the posterior probability for the individual branches. The first 14 200 generations were discarded for the analyses of the combined phylogeny of the *P. citricola* complex.

### Isozyme analyses

All 20 *P. citricola* complex isolates (seven *Agathosma* isolates and 13 *P. citricola* CIT groups sensu Oudemans et al. (1994) reference isolates) were analysed for glucose-6-phosphate isomerase (GPI) (synonym = phosphoglucose isomerase) and malate dehydrogenase (MDH) banding patterns. These two isozymes include two of the three isozymes that were identified by Oudemans et al. (1994) as being able to differentiate the five CIT groups. Isolates were grown for seven days in pea broth (120 g of frozen peas, boiled in 1 L distilled water,

strained and 2 g CaCO<sub>3</sub> added) at 25 °C in the dark. Harvested mycelia (0.5 g) were added to 300 µL extraction buffer (Man in 't Veld et al. 2007) in 1.5 mL centrifuge tubes. After the addition of 0.5 g glass beads (2 mm diam), the tubes were shaken for 1 min on a Mixer Mill type MM 301 beater. The tubes were centrifuged for 10 min at 14 000 rpm at 4 °C in a Eppendorf 5810 R centrifuge (Eppendorf AG, Hamburg, Germany), and the supernatant was used for gel electrophoresis. Isozyme analyses were conducted using a Mini-Vertical unit SE 260 (GE Healthcare Bio-Sciences Corp, Piscataway, USA) and 1 mm gel spacers. GPI was resolved on 5 % polyacrylamide gels using a pharmalyte with pH 5 to 6 (Sigma-Adrich) and buffer systems as previously described (McLeod et al. 2001). Gels were run at 10 °C for 1.5 h at 200 V followed by 1.5 h at 400 V. GPI staining was carried out according to McLeod et al. (2001). MDH analyses were conducted similarly to the GPI analyses except that 8 % acrylamide gels were used that contained an ampholyte with pH range 8 to 10 (Fluka, St Louis, USA). MDH gels were run at 4 °C for 1 h at 100 V, 1.5 h at 200 V and 1.5 hrs at 400 V. Staining for MDH activity was done according to Man in 't Veld et al. (2007).

### Pathogenicity tests

Koch's postulates were conducted on *A. betulina* seedlings to determine pathogenicity of the isolated *Phytophthora* taxa. Seeds were planted in pasteurised potting mix (Hygrotech mix, Hygrotech SA) and after 5 mo transplanted into a new potting mix inoculated with 2 % w/v sandbran inoculum (Lamprecht et al. 1988). Pots were inoculated separately with *P. nicotianae* (STE-U 6273), *P. cinnamomi* var. *parvispora* (STE-U 6265), *P. cinnamomi* var. *cinnamomi* (STE-U 6258), *P. taxon emzansi* (STE-U 6269), *P. multivora* (STE-U 6558, 6268), *P. cryptogea* (STE-U 6262, 6263) and *P. drechsleri* (STE-U 6264). The control treatment received only sterile sandbran. Plants were incubated under natural light in the laboratory at 22 ± 2 °C, watered twice weekly and evaluated for symptom development after 36 d. After this period the number of dead seedlings was recorded and isolations were made from roots as described above. In total, four trials were conducted; two during 2007 and two during 2008, which each contained seven replicates per treatment.

## RESULTS

### Isolates

In total 20 *Phytophthora* isolates were obtained from diseased *Agathosma* plants sampled during the survey. Nine of the isolates were from nursery seedlings, whereas 11 isolates were obtained from commercial field plants (Table 1). Four out of the seven nurseries and seven out of the nine field sites tested positive for *Phytophthora*.

### Morphological, culture and mating type characterisation

Based on morphological and temperature growth studies, the *Phytophthora* isolates from *Agathosma* were initially grouped into five species: *P. cinnamomi*, *P. citricola*, *P. cryptogea*, *P. drechsleri* and *P. nicotianae* (Table 1). All species except *P. nicotianae* were identified in field plants, whereas only *P. cinnamomi*, *P. citricola* and *P. nicotianae* were found in nurseries (Table 1). Only a single isolate (STE-U 6273) of *P. nicotianae* was obtained. Three isolates, STE-U 6261–6263, resembled *P. cryptogea* and a single isolate, STE-U 6264, was similar to *P. drechsleri*. Differentiation between *P. cryptogea* and *P. drechsleri* isolates was based on growth at 35 °C. Eight isolates were identified as *P. cinnamomi* and seven as *P. citricola*. Two distinct morphological types were evident among the *P. cinnamomi* group of isolates and growth studies further confirmed

this separation. Isolates STE-U 6255–6260 and 6266 fitted the morphological description of *P. cinnamomi* var. *cinnamomi* (Erwin & Ribeiro 1996), having a rosaceous or petaloid colony growth pattern on CMA with coralloid main hyphae (6.8 µm wide) and nodose side branches (2.5–3.4 µm wide). The cultures formed abundant (sub)globose chlamydospores (av. 45 µm diam) in terminal grapelike clusters, and sporangia with average dimensions of 34–50 × 20–34 µm and a L/B ratio of ± 1.6. Isolate STE-U 6265 was morphologically different from the above isolates having features similar to *P. cinnamomi* var. *parvispora* (Erwin & Ribeiro 1996). The isolate formed colonies with felty, thick aerial mycelium that formed a coarse growth pattern on CMA with an uneven margin. Main hyphae were coralloid (5–7 µm wide) with lateral branches, some of those having thin-walled hyphal swellings (av. 26 × 28 µm), others with distorted shapes or lobulate, digitate inflated elements. Mean sporangial dimensions were 14–23 × 25–45 µm, the L : B ratio was 2.17 and chlamydospores were not observed. There were no differences in cardinal growth temperatures between *P. cinnamomi* var. *cinnamomi* and *P. cinnamomi* var. *parvispora* isolates i.e. minimum (no-growth at < 10 °C but culture still viable), optimum (25 °C) and maximum (32 °C). However, *P. cinnamomi* var. *cinnamomi* had a significantly faster daily radial growth rate (26 mm/d) at 25 °C compared to *P. cinnamomi* var. *parvispora* (15 mm/d).

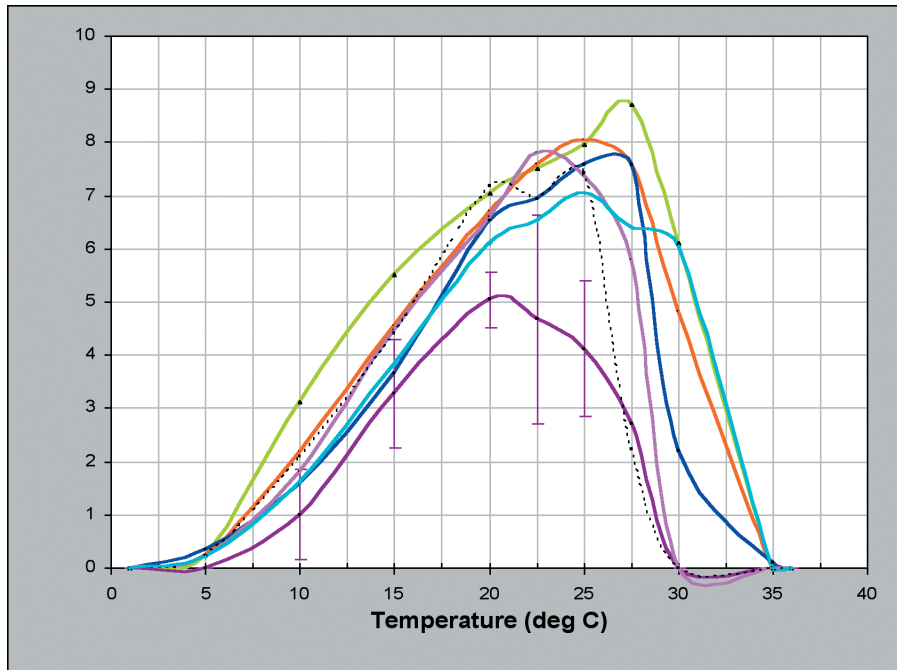
Both homothallic and heterothallic isolates were obtained from *Agathosma*. All the homothallic isolates belonged to the *P. citricola* complex, a total of seven isolates. The remaining 13 isolates were heterothallic; only the single isolates each of *P. drechsleri* and *P. nicotianae* were of the A2 mating type, the others (*P. cinnamomi* and *P. cryptogea*) were A1 mating type (Table 1).

### Comparative examination of isolates in the *P. citricola* complex

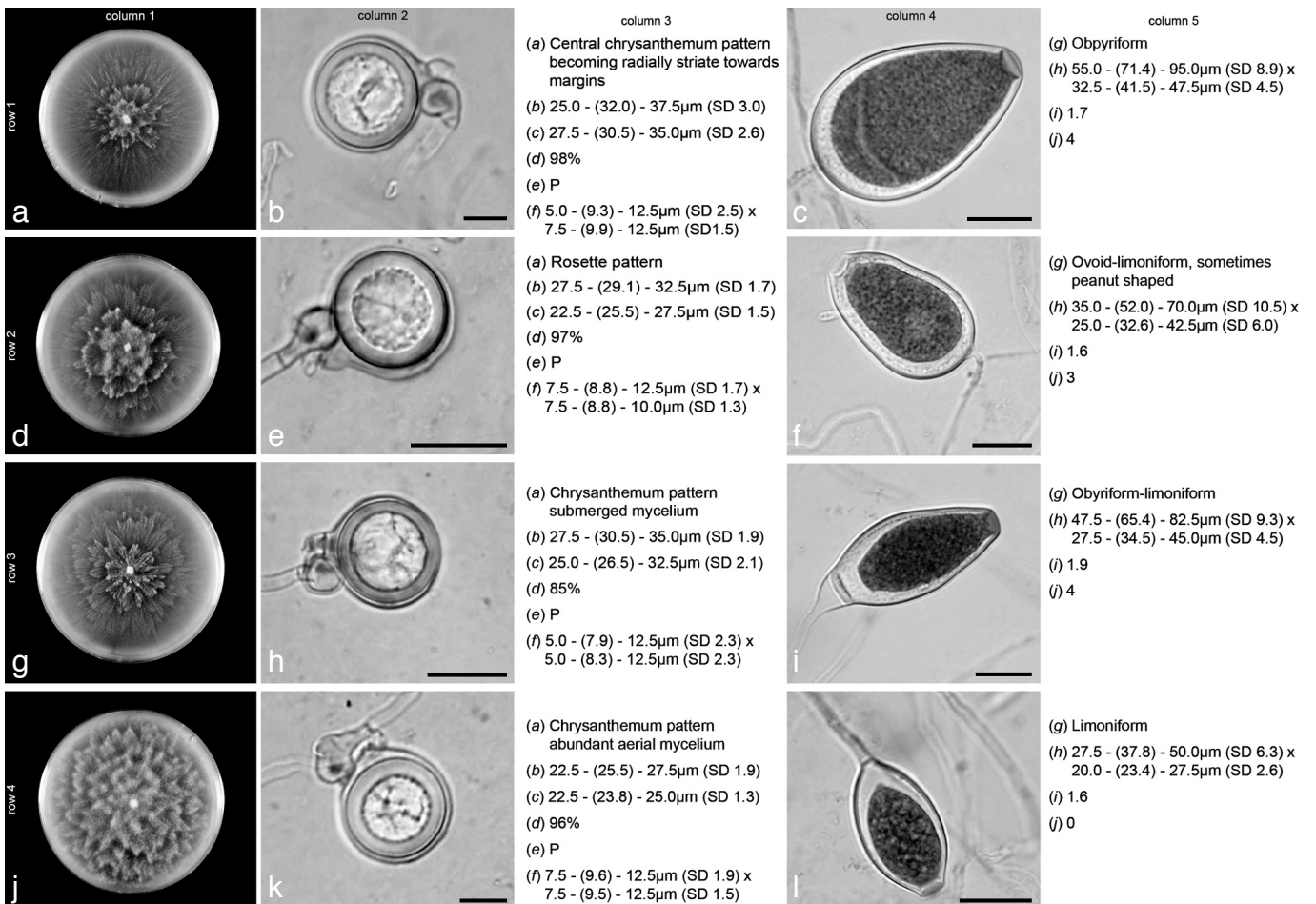
Below, the taxon designation of the *P. citricola* isolates as determined by phylogenetic analyses (see phylogenetic results section; Table 1 and Fig. 8, 9) will be used. Seven taxa were identified among the *P. citricola* complex reference and *Agathosma* isolates (Table 1 and Fig. 8, 9); *P. capensis* is proposed as a new species (see below) and *P. taxon emzansi* as a new taxon.

Temperature-growth rate relationship was one parameter that revealed differences between taxa in the *P. citricola* complex, although it is acknowledged that only small numbers of isolates were used for tests (Fig. 1). *Phytophthora multivora* (synonym CIT3, ETs 6 & 7) which included both reference and *Agathosma* isolates, showed similarities in growth trends with *P. plurivora* (synonym CIT1, ET1), the fastest growing of all taxa in the complex (Fig. 1). Although the data is representative of only one *P. plurivora* isolate this trend was also reported by Jung & Burgess (2009). However, they reported optimum and maximum temperatures of 25 °C and 32 °C respectively for both these species (Jung & Burgess 2009), as opposed to 27.5 °C and 35 °C obtained for *P. multivora* and *P. plurivora* in our study (Fig. 1).

*Phytophthora taxon emzansi* (STE-U 6269 and 6272 from *Agathosma*) showed quite different temperature growth characteristics when compared to the other species in the complex. The growth rate of *P. taxon emzansi* was significantly lower than the other taxa (Fig. 1). It should be noted, however, that there were differences in the growth rates of the two *P. taxon emzansi* isolates – growth of STE-U 6272 was much slower than STE-U 6269. Since there were such noticeable differences between the two isolates, trends for this taxon cannot be regarded as definitive. Optimal growth in *P. taxon emzansi* occurred at 20–22.5 °C, which was different to the other species



**Fig. 1** Mean radial growth rates (mm per day) on carrot agar of *Phytophthora* taxa in the *Phytophthora citricola* complex. The standard error (SE) is included only for *P. taxon emzansi* where there were significant differences in growth rate between isolates. All other species in the *P. citricola* complex had less than 10 % SE at each temperature and therefore error bars are not shown. Green line – *P. plurivora* (CIT1) (N = 1); orange line – *P. citricola* clade E sensu Jung & Burgess (2009) (syn CIT2) (N = 2); dark blue line – *P. multivora* (CIT3) (N = 8); purple line – *P. taxon emzansi* (CIT4) (N = 2); pink line – *P. capensis* (CIT4) (N = 3); dashed black line – *P. menzei* (CIT5) (N = 1), light blue line – *P. citricola* sensu Oudemans et al. (1994) (CIT1, ET 2 & 3) (N = 2)



**Fig. 2** Morphological features of taxa in the *Phytophthora citricola* cluster. **Column 1:** Colony morphology on carrot agar after 7d @ 20 °C; **Column 2:** Sexual reproductive structures; **Column 3:** Colony morphology descriptions and measurements of sexual reproductive structures (see below\*); **Column 4:** Sporangia; **Column 5:** Descriptions and measurements of sporangia (see below\*); **Row 1:** *P. citricola* clade E sensu Jung & Burgess (2009) (Synonym CIT2 sensu Oudemans et al. 1994) isolate P1321; **Row 2:** *P. plurivora* isolate P1611; **Row 3:** *P. citricola* s.s. isolate P1814; **Row 4:** *P. capensis* isolate P1819; **Row 5:** *P. taxon emzansi* isolate STE-U 6269; **Row 6:** *P. multivora* (Pi) isolate STE-U 6558; (Pii) isolate P1817; (Piii) isolate STE-U 6270; **Row 7:** *P. menzei* isolate P1165.

in the complex having optimal growth temperatures of 25 °C or more (Fig. 1). The maximum temperature at which growth of *P. taxon emzansi* was measured was 27.5 °C, with 30 °C being lethal.

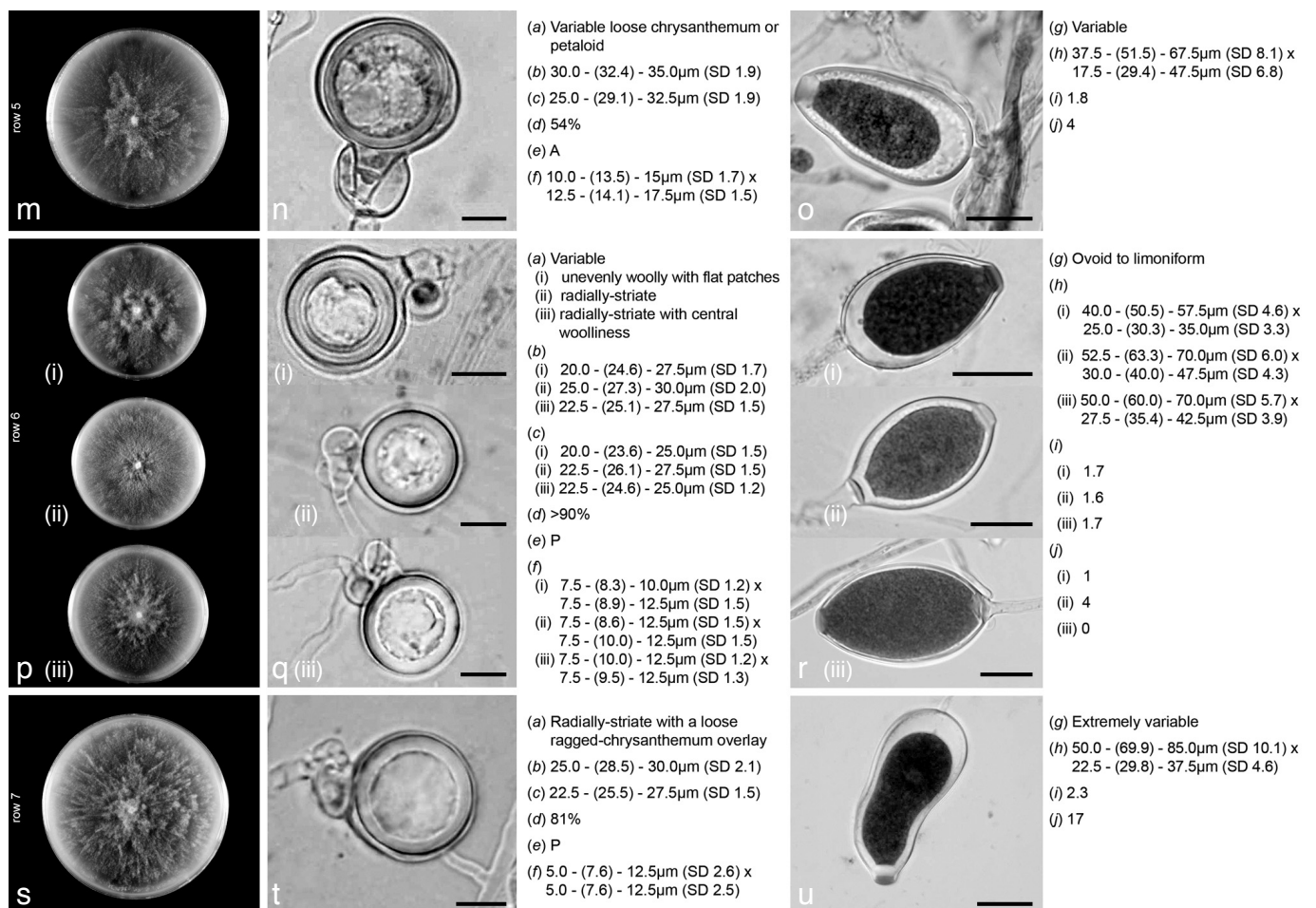
The growth of *P. capensis* (synonym CIT4) was fast and comparable to *P. citricola* clade E sensu Jung & Burgess (2009) (synonym CIT2). However, *P. capensis* had an optimal growth temperature of 22.5 °C as opposed to 25 °C for *P. citricola* clade E (Fig. 1). Furthermore, at temperatures above 22.5 °C, the growth of *P. capensis* tailed off in comparison to *P. citricola* clade E. The lethal temperature for *P. capensis* was 30 °C, but was 35 °C for *P. citricola* clade E.

Colony morphology of the various taxa in the *P. citricola* complex differed (see Fig. 2, column 1). Even though only a small number of isolates were represented, distinctive colony morphology patterns were identifiable. The morphology of *P. capensis* was striking by having abundant aerial mycelium forming a clear chrysanthemum pattern (van der Plaats-Niterink 1986) (Fig. 2j). A similar morphology was evident in *P. citricola* s.str. (synonym CIT1, ETs 2 & 3) with the notable difference of colonies having little aerial mycelium (Fig. 2g). The chrysanthemum pattern was formed by mycelium mostly submerged in agar. *Phytophthora citricola* clade E sensu Jung & Burgess (2009) also had similar colony morphology to the above but the chrysanthemum pattern only formed close to the inoculum plug and changed to a radially-striate pattern as the colony advanced to the margin of the Petri dish (Fig. 2a).

Variable colony morphologies were present in both *P. multivora* and *P. taxon emzansi*. Within *P. multivora* three broad groups could be distinguished, all had a moderate amount of aerial mycelium: one group formed a radially-striate colony pattern (e.g. P1817, Fig. 2Pii), the second group had a radially-striate with central woolliness pattern (e.g. STE-U 6270, Fig. 2Piii) and in the third group the pattern was unevenly woolly with flat patches (e.g. STE-U 6558, Fig. 2Pi). In *P. taxon emzansi* colony morphology was variable but isolate STE-U 6269 had a loose chrysanthemum pattern (Fig. 2m) whereas STE-U 6272 had much less growth but showed a woolly slightly petaloid colony morphology.

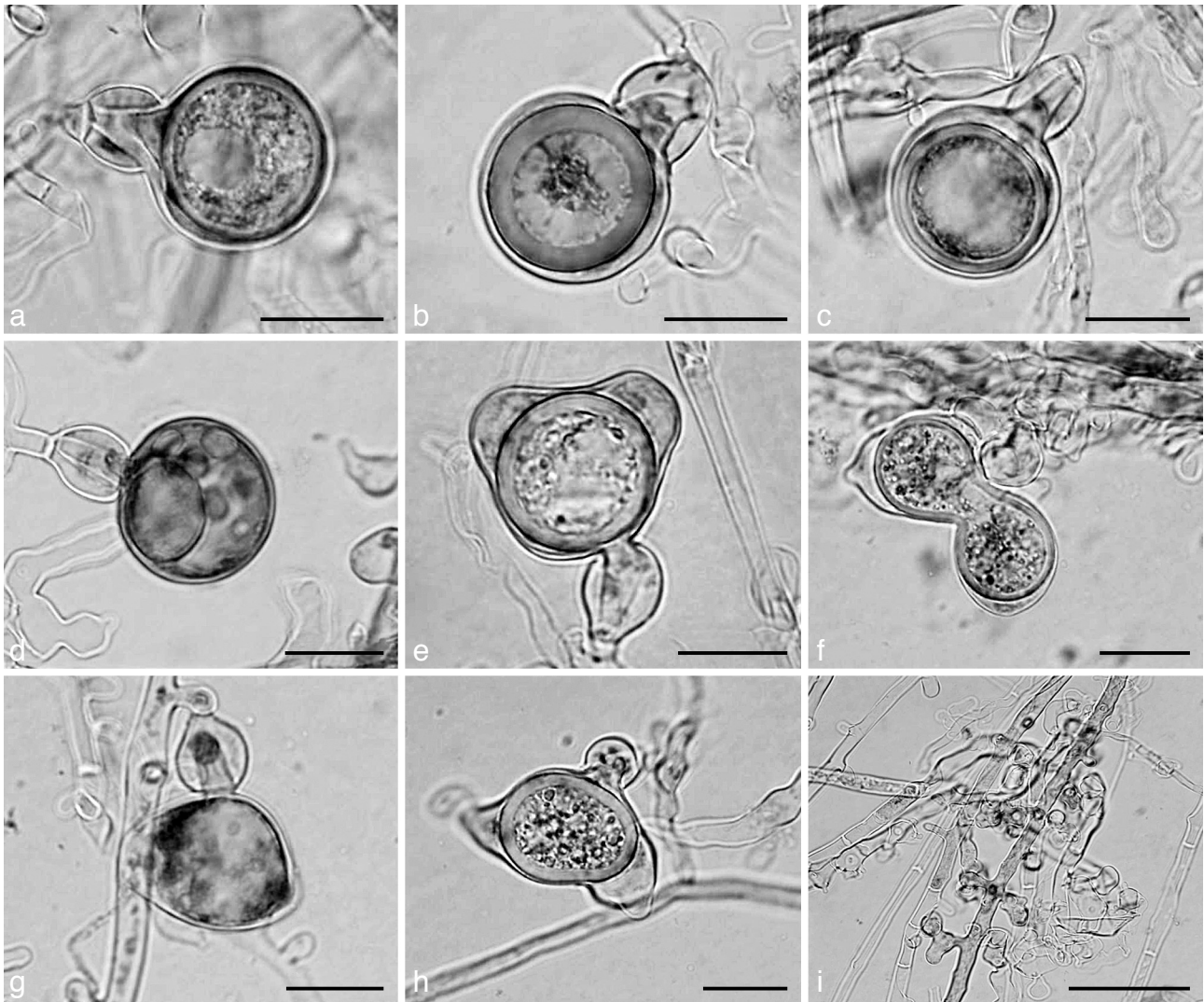
*Phytophthora plurivora* showed a rosette colony morphology (van der Plaats-Niterink 1986) with little aerial mycelium (Fig. 2d). *Phytophthora menzei* had a moderate amount of aerial mycelium that demonstrated what we describe as: a radially-striate with a loose ragged-chrysanthemum overlay pattern (Fig. 2s).

Sexual reproductive features distinguished species in the *P. citricola* complex in only two cases (Fig. 2, column 2). *Phytophthora taxon emzansi* was clearly different to the other taxa because the attachment of the antheridia to the oogonia was amphigynous (Fig. 2n and 3a–c), whereas all other taxa had paragynous antheridia. Antheridia of *P. taxon emzansi* were also significantly bigger (on average 14 × 14.5 µm) (Fig. 2, column 3) than those of the other species in the complex and they had longer antheridial stalks. Oogonia of *P. taxon emzansi* also had high levels of zygotic abortion (42–46 %) (Fig. 3d, f, g); low oogonia maturity (54–58 %) and high levels of



**Fig. 2** (cont.) \*Column 3 descriptions and measurements of sexual reproductive structures: (a): Colony morphology description; (b): Oogonium diameter (lower – (mean) – upper range) (N = 20); (c): Oospore diameter (lower – (mean) – upper range) (N = 20); (d): % Oospore maturity; (e): Antheridial attachment (P = paragynous; A = amphigynous); (f): Antheridial dimensions (L × B) (lower – (mean) – upper range). ♦ Column 5 description and measurements of sporangia; (g): Sporangial shape; (h): Sporangial dimensions (L × B) (lower – (mean) – upper range) (N = 20); (i): L : B ratio; (j): % Bi- or tri-lobed sporangia. — Scale bars: b, k, n, q, t = 10 µm; c, e, f, h, i, l, o, r, u = 20 µm.





**Fig. 3** Morphological features of *P. taxon emzansi*. a–c. Normal mature oogonia, oospores and antheridia; d, g. aborted oospores; e, f, h. abnormal oogonia; i. hyphal branching. — Scale bars: a–h = 20  $\mu$ m; i = 50  $\mu$ m.

deformed oogonia (Fig. 3e–h) compared with the other species in the *P. citricola* complex where percentage mature oogonia ranged from 80–98 % dependent upon species (Fig. 2, column 3). Oogonia of *P. menzei* were distinctive with a proportion of oospores showing a 'beaked'-shaped form of aplerotism (Fig. 6a), whereas the other species in the *P. citricola* complex had plerotic oospores. The term 'beaked' is derived from a plan-view of a duck's head (Fig. 6a).

Sporangia formed by all taxa in the *P. citricola* complex are semi-papillate (Fig. 2, column 4). Sporangia of *P. capensis* are remarkable in that they are very uniform in shape although variable in size (Fig. 2l, 5g–i). They also have the smallest dimensions of all species in the *P. citricola* complex (Fig. 2, column 5). By contrast the sporangia of *P. taxon emzansi* sporangia are highly variable in size and shape (Fig. 4a–l). Sporangia of *P. menzei* are distinctive in the abnormal forms that are prominently bi- or tri-lobed (Fig. 6b–d).

#### Molecular characterisation

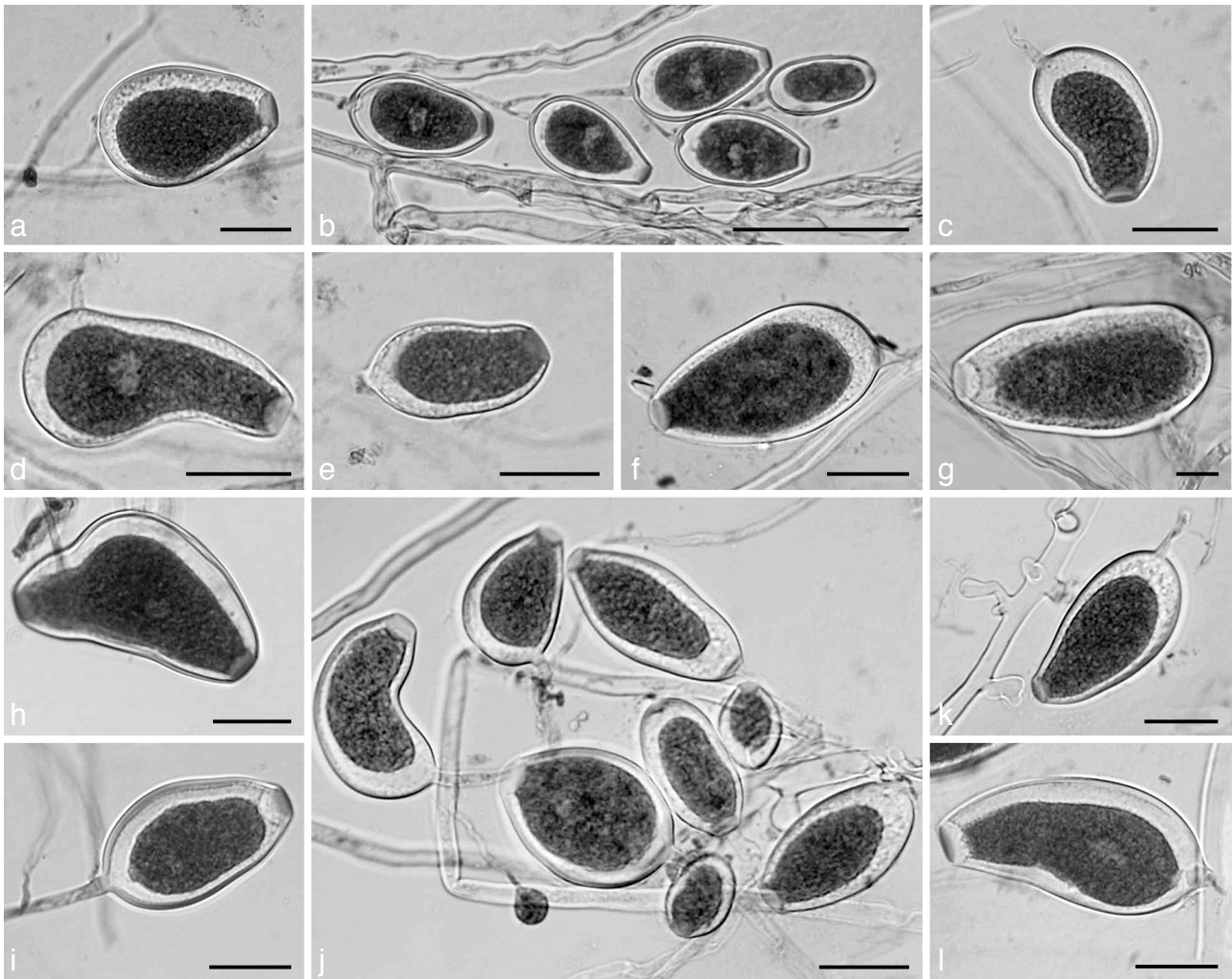
The morphological identification of isolate STE-U 6273 as *P. nicotianae* was confirmed by BLAST analyses since the sequences of this isolate had 99.7 %, 99.4 %, 99.9 %, 99.7 % and 99.5 % similarity to the published ITS,  $\beta$ -tub, NADH, EF-1 $\alpha$  and *cox1* gene region sequences respectively of Cooke et al. (2000), Martin & Tooley (2003) and Kroon et al. (2004). BLAST analyses of sequence data of the morphologically identified *P. drechsleri* (STE-U 6264) and *P. cryptogea* (STE-U 6261–

6263) isolates could not discriminate between these two species which are taxonomically challenging (Mostowfzadeh-Ghalmfarsa et al. 2009), and therefore growth rate data was used to separate these species. BLAST analyses of the sequences of these two species showed that the ITS and *cox1* sequences of *P. drechsleri*, and the NADH, EF-1 $\alpha$  and *cox1* sequences of *P. cryptogea* had at least 97 % or more similarity to the reference sequences (Cooke et al. 2000, Martin & Tooley 2003, Kroon et al. 2004), whereas the remaining sequences of these two species had 98 % or more similarity to the reference sequences (Cooke et al. 2000, Martin & Tooley 2003, Kroon et al. 2004).

BLAST analyses showed that the sequences of the morphologically identified *P. citricola* isolates as defined by Erwin & Ribeiro (1996), and the *P. cinnamomi* isolates (both varieties) had similarity to these species and other related taxa in *Phytophthora* clade 2 and 7, respectively. The identities of the sequences of these isolates were investigated further using phylogenetic analyses.

#### Phylogenetic analyses of *Phytophthora* clade 7

The ITS sequences of the seven isolates that were morphologically identified as *P. cinnamomi* var. *cinnamomi* (Table 1) were identical, and formed a monophyletic clade (100 % bootstrap support; 1.00 posterior probability) that included the sequence of the *P. cinnamomi* isolate of Cooke et al. (2000) and three *P. cinnamomi* GenBank sequences (AY302181, AY302176, AY964101) (Fig. 7). The sequence of the morphologically identified *P. cinnamomi*



**Fig. 4** Sporangial features of *P. taxon emzansi*. a. Ovoid sporangium; b. loose symposium of sporangia; c, k, l. elongated wine-glass shaped sporangia with bent top and slightly off-set attachment; d. sac-shaped sporangium with off-set attachment; e. peanut-shaped sporangium; f, g, i. wine-glass shaped sporangium with wavy walls (g has off-set attachment); h. bi-lobed sporangium; j. variable shapes and attachments. — Scale bars: a, c–f, h–l = 20  $\mu$ m; b = 50  $\mu$ m; g = 10  $\mu$ m.

*moni* var. *parvispora* isolate (STE-U 6265) was distinct from *P. cinnamomi* var. *cinnamomi*, and grouped with high bootstrap and posterior probability support (100 %, 1.00) in a clade basal to the *P. cinnamomi* var. *cinnamomi* clade. This basal clade also included GenBank sequences submitted as *P. cinnamomi* var. *parvispora* (AY302184, FJ802006) and *P. cinnamomi* (AY45752) (Fig. 7).

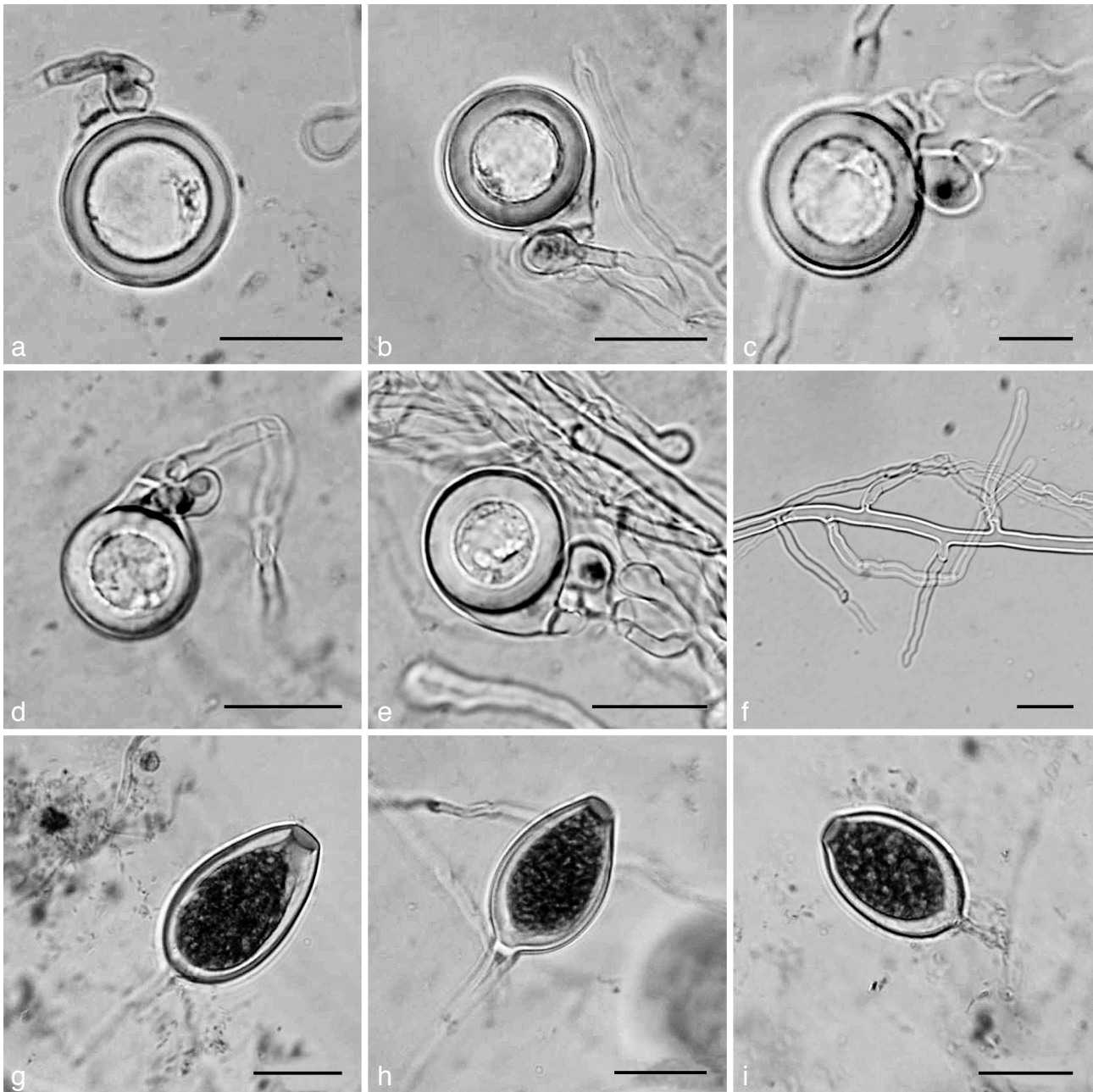
The  $\beta$ -tub, EF-1 $\alpha$ , NADH and *cox1* datasets were combined into one analysis, since results of the partition homogeneity test showed that the datasets were congruent ( $P = 0.096$ ). This combined analysis supported the findings of the ITS phylogeny, and also clustered the *P. cinnamomi* varieties into two distinct clades, each with high bootstrap support (100 %), with the *P. cinnamomi* var. *parvispora* sequence also being ancestral (data not shown).

#### Phylogenetic analyses of *Phytophthora* clade 2

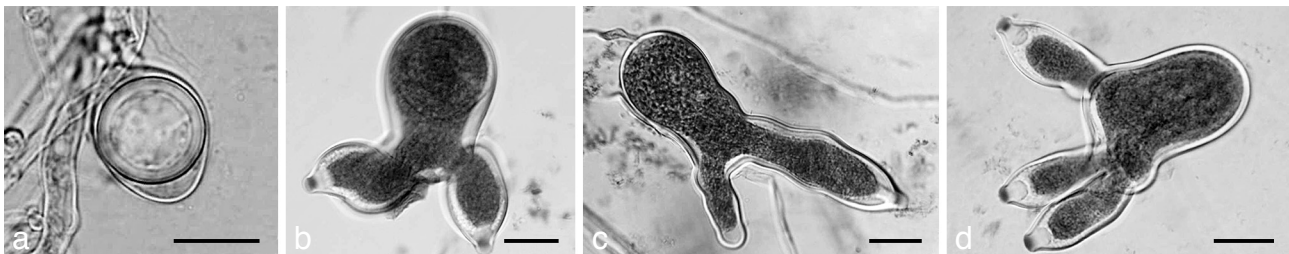
Partition homogeneity testing showed that most of the nuclear gene datasets could be combined with each other, as well as with the mitochondrial gene datasets. The NADH and ITS datasets were the only datasets that were incongruent with some of the other gene datasets. The  $P$ -values for the different dataset tests were as follows: for ITS and  $\beta$ -tub dataset  $P = 0.139$ , ITS and EF-1 $\alpha$  dataset  $P = 0.098$ , ITS and *cox1* dataset  $P = 0.001$ , *cox1* and  $\beta$ -tub dataset  $P = 0.732$ , *cox1* and EF-1 $\alpha$  dataset  $P = 0.235$ , NADH and  $\beta$ -tub dataset  $P = 0.015$ , NADH and EF-1 $\alpha$  dataset  $P = 0.001$ , and NADH and *cox1* dataset  $P = 0.084$ , and ITS and NADH dataset  $P = 0.995$ .

Although most of the gene regions were congruent and several single gene and combined phylogenies were constructed, only two phylogenies will be discussed namely the ITS phylogeny (Fig. 8), and a combined  $\beta$ -tub / EF-1 $\alpha$  / *cox1* phylogeny (Fig. 9). The ITS phylogeny is important since many GenBank ITS sequences are available for *Phytophthora* and specifically *P. citricola* complex isolates, which were required to determine if any other known sequences were present that were related to the CIT4 group (subsequently described as *P. capensis*) and STE-U 6272 and 6269 (subsequently referred to as *P. taxon emzansi*). For most *P. citricola* isolates in GenBank, only ITS sequence data is available. The exception is the  $\beta$ -tub and *cox1* sequences submitted for some isolates that were used in the study by Jung & Burgess (2009).

The ITS phylogeny grouped almost all of the *P. citricola* isolates, with the exception of the two CIT5 isolates (P0513, P1165), into one large clade with good bootstrap (84 %) and high posterior probability support (0.99) (Fig. 8). The *P. citricola* CIT5 isolates were identified as *P. menzei* since isolate P0513 (CIT5, ET 9) had 100 % ITS sequence identity with the newly described *P. menzei* (GenBank EU748545, EU748546) sequences, whereas P1165 (CIT5, ET 10) only differed by one base pair from this sequence. The two *P. menzei* isolates (P0513, P1165) grouped basal to the large *P. citricola* clade in a clade with high bootstrap and posterior probability support (89 %, 1.00) that also contained *P. tropicalis* and *P. capsici* sequences.



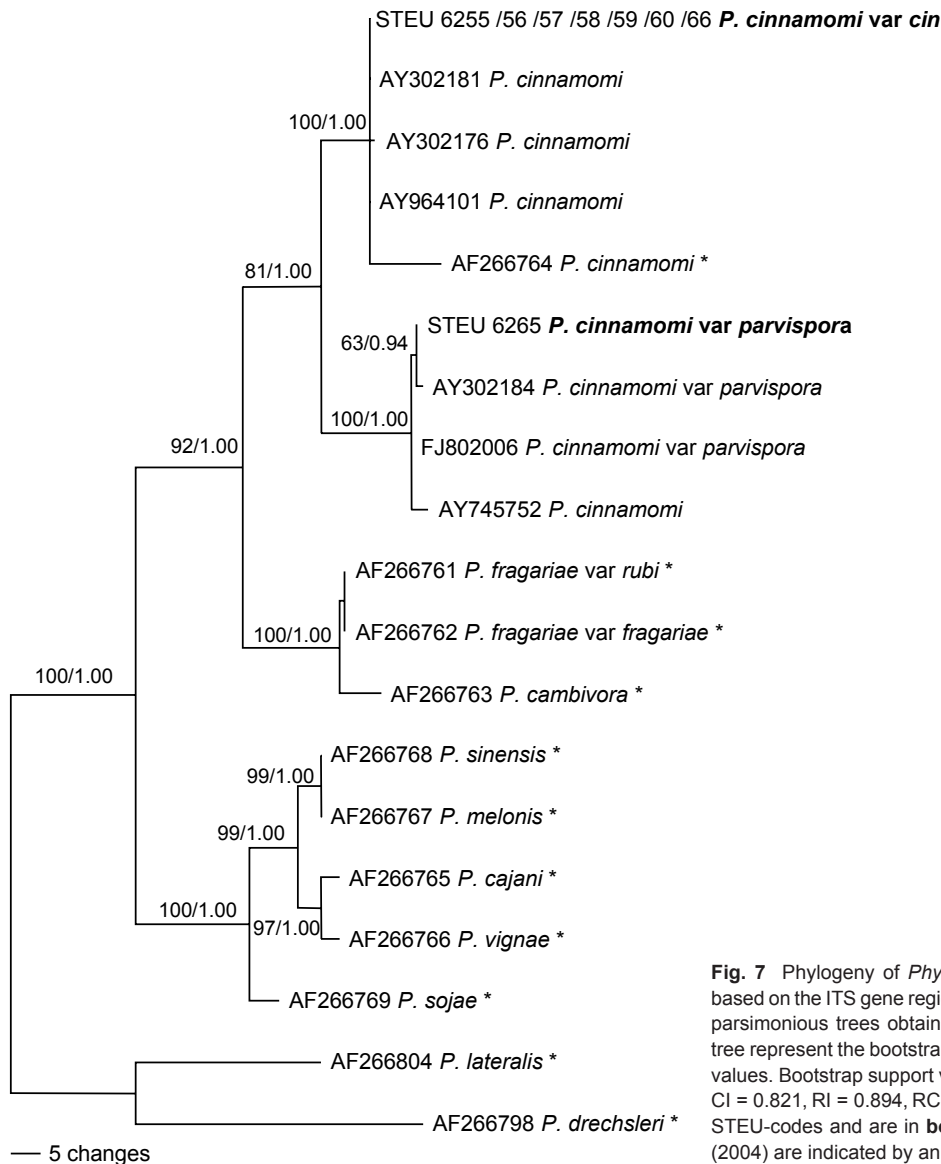
**Fig. 5** *Phytophthora capensis*. a–e. Mature oogonia, thick-walled, round oospores and paragynous antheridia; f. branched hyphae; g–i. semi-papillate, limoniform sporangia with central attachment. — Scale bars: a, b, d–i = 20  $\mu$ m; c = 10  $\mu$ m.



**Fig. 6** Morphological features of *P. menzei*. a. Mature oospore with 'beaked' aplerotic oogonium; b–c. bi-lobed irregular shaped sporangia; d. tri-lobed irregular shaped sporangium. — Scale bars: a–d = 20  $\mu$ m;

In the large *P. citricola* ITS clade, which contained all the *Agathosma* isolates, there was only good bootstrap and posterior probability support for the *P. multivora*/CIT3-clade (100 %, 1.00), the CIT2-clade/*P. citricola* clade E (88 %, 1.00), and a clade (97 %, 1.00) containing the two possible new species *P. taxon emzansi* (STE-U 6272 and 6269) and *P. capensis*/CIT4 (P1822, P1819 and P1823). There were no GenBank ITS se-

quences that clustered within the latter clade. The *Agathosma* isolates STE-U 6271, 6558, 6267, 6270 and 6268 clustered into the *P. multivora*/CIT3-clade. The *P. plurivora*/CIT1 (ET 1)-clade and *P. citricola* I (Gallegly & Hong 2008)-clade, had less than 64 % bootstrap and 0.91 posterior probability support, whereas the *P. citricola* s.str./CIT1 (ETs 2 & 3)-clade was unresolved (Fig. 8).



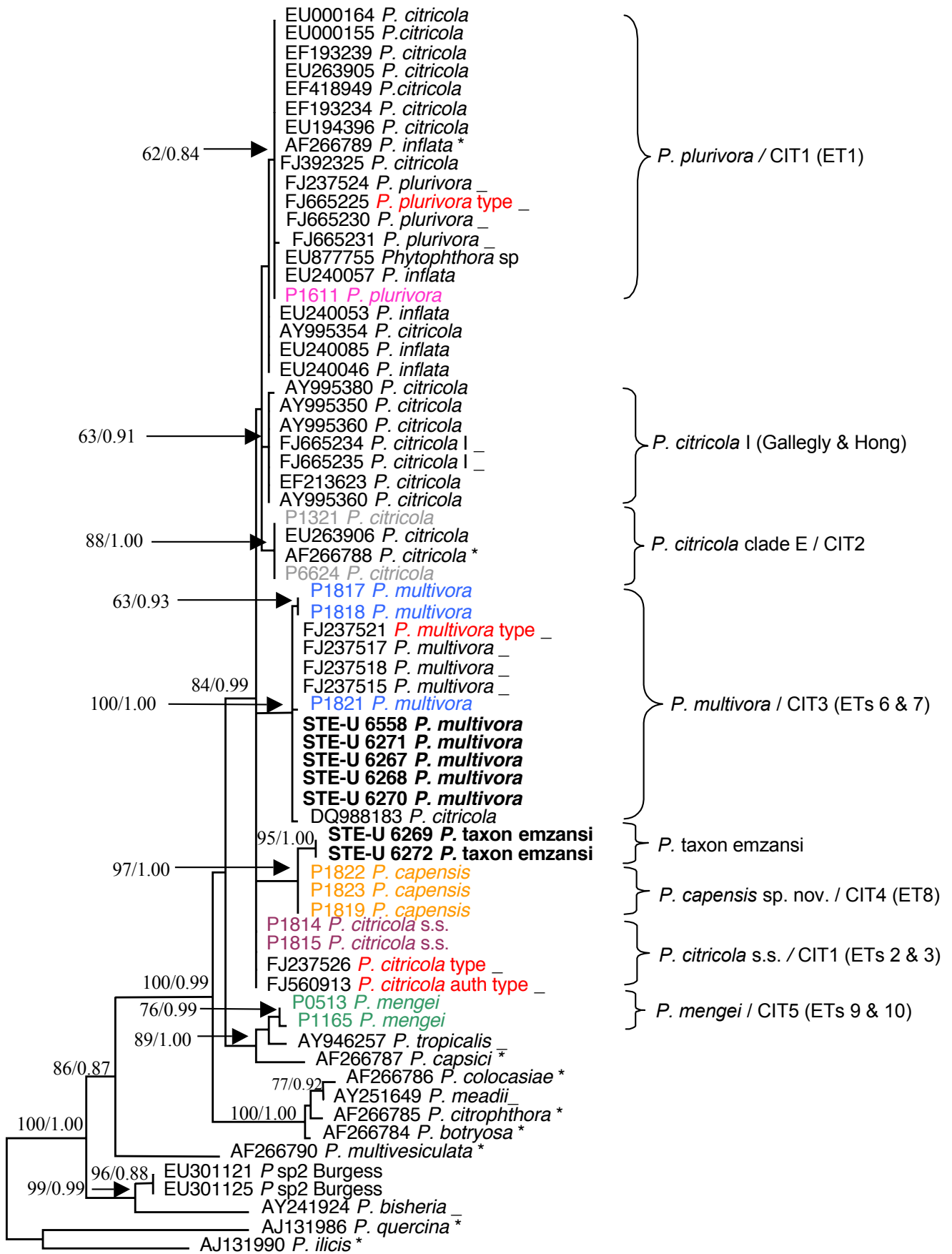
**Fig. 7** Phylogeny of *Phytophthora* clade 7 species (Cooke et al. 2000) based on the ITS gene region. The tree presents one of the four equally most parsimonious trees obtained from a heuristic search. Numbers within the tree represent the bootstrap support values followed by posterior probability values. Bootstrap support values below 60 % are not shown. Length = 263, CI = 0.821, RI = 0.894, RC = 0.734. Isolates obtained from *Agathosma* have STEU-codes and are in **bold**. Sequences of species used by Kroon et al. (2004) are indicated by an asterisk (\*).

The combined  $\beta$ -tub / EF-1 $\alpha$  / *cox1* phylogeny (Fig. 9) gave better resolution of taxa than the ITS phylogeny. In the combined phylogeny the relatedness of the CIT groups to known species such as *P. plurivora*, *P. multivora* and *P. citricola* s.str., was deduced based on the grouping of these isolates in the individual  $\beta$ -tub and *cox1* trees that contained the reference sequences of these taxa used in the studies of Jung & Burgess (2009) and Scott et al. (2009). The combined phylogeny clustered the *Agathosma P. citricola* isolates and the *P. citricola* CIT groups sensu Oudemans et al. (1994) reference isolates into seven clades: 1) *P. plurivora*/CIT1 (ET 1) clade; 2) *P. citricola* s.str./CIT1 (ETs 2 & 3) clade; 3) *P. citricola* clade E/CIT2 clade; 4) the putative new species *P. taxon emzansi* clade (STE-U 6272 and 6269); 5) the putative new species *P. capensis* clade (P1822, P1819 and P1823); 6) *P. multivora*/CIT3 (ETs 6 & 7) clade; and 7) *P. mengei*/CIT5 (ETs 9 & 10) clade. As expected the *P. mengei* sequences clustered basal and separate from the other *P. citricola* isolates. The *P. multivora*/CIT3 clade had good bootstrap and posterior probability support (100 %, 1.00) as did the *P. taxon emzansi* (100 %, 1.00) and *P. capensis* (100 %, 1.00) clades. Good bootstrap and posterior probability support were also found for the *P. citricola* clade E/CIT2 clade (89 %, 1.00) and *P. citricola* s.str./CIT1 (ET1) (99 %, 1.00) clades. Only one isolate of *P. plurivora* was included and it grouped basal to *P. citricola* clade E s.str. Jung & Burgess (2009) with no support.

#### Isozyme analyses of *P. citricola* complex isolates

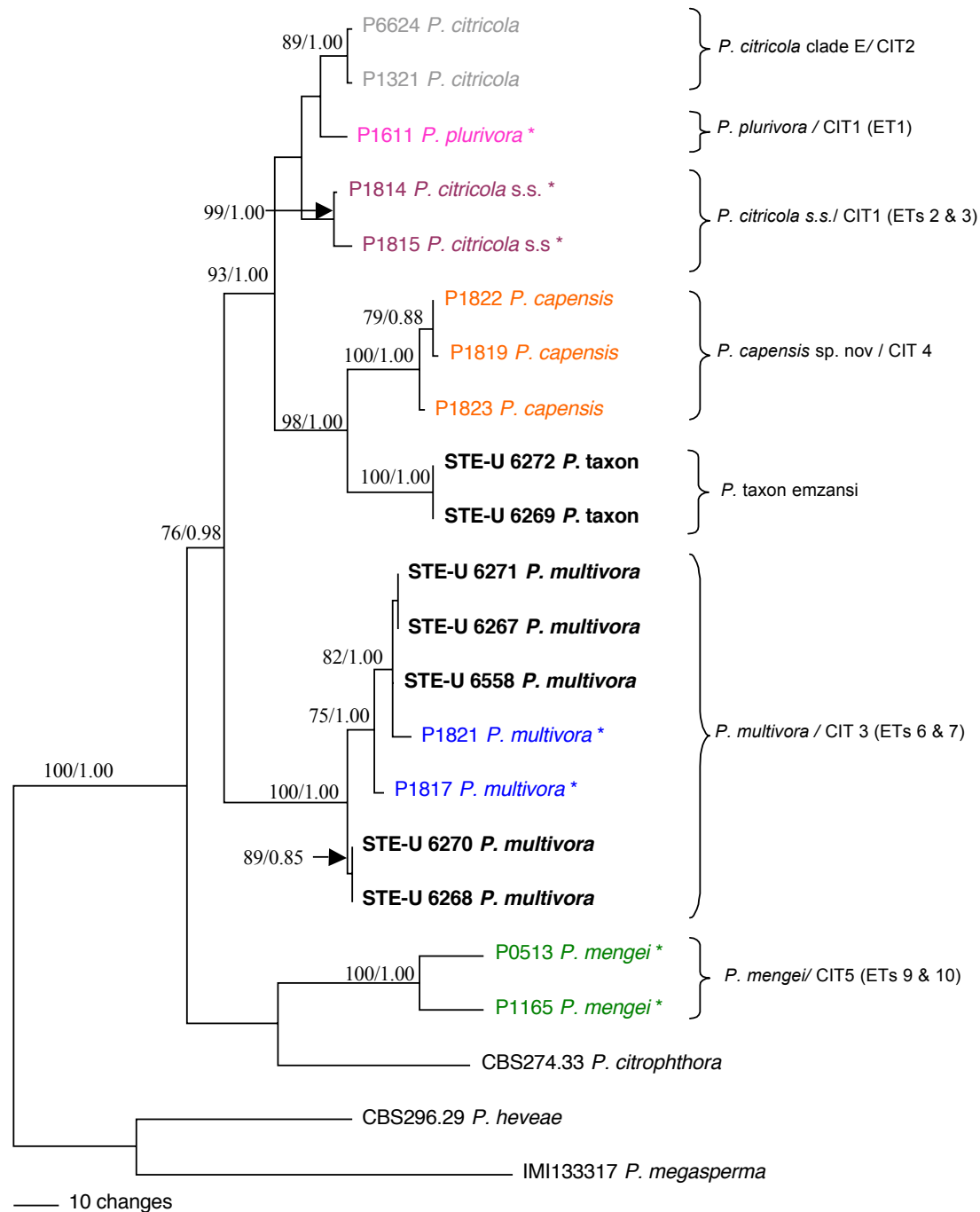
The GPI analyses revealed the presence of six alleles (A to F) among the isolates. The two *P. mengei* isolates each had a pair of private alleles at the GPI locus. The remaining isolates were monomorphic at the GPI locus, each containing two alleles (Table 1).

The MDH analyses revealed, as expected, the presence of two MDH loci, MDH-1 and MDH-2. At the MDH-1 locus four alleles (A to D) were resolved. The *P. multivora* (CIT3) isolates (*Agathosma* and reference isolates) were all homozygous for allele C, whereas the *P. citricola* clade E sensu Jung & Burgess (2009) (CIT2) were homozygous for allele A. The *P. taxon emzansi* isolates (STE-U 6269 and 6272) had the same homozygous genotype as the *P. plurivora* and *P. citricola* s.str. reference isolates. The three *P. capensis* and *P. mengei* reference isolates had the same MDH-1 genotype. At the MDH-2 locus, most of the isolates, except the *P. capensis* isolates, were monomorphic. The *P. capensis* isolates were heterozygous at the MDH-2 locus and shared one allele with the other isolates (Table 1).



- 1 change

**Fig. 8** Phylogeny of *Phytophthora* clade 2 species (Cooke et al. 2000) based on the ITS gene region. The tree presents one of the 52 equally most parsimonious trees obtained from a heuristic search. Numbers within the tree represent the bootstrap support values followed by posterior probability values. Length = 223, CI = 0.789, RI = 0.900, RC = 0.710. Isolates obtained from *Agathosma* have STEU-codes and are in **bold**. Sequences of species used by Cooke et al. (2000) are indicated by \* and those of Abad et al. (2008), Jung et al. (2009) and Scott et al. (2009) by \_. Sequences of the study of Oudemans et al. (1994) that represent the different *P. citricola* multi-locus isozyme groups all have P-codes. These isolates were all received as *P. citricola*, but are re-named here as different taxa based on their phylogenetic clustering (Fig. 9).



**Fig. 9** Phylogeny of *Phytophthora* clade 2 species (Cooke et al. 2000) based on the combined elongation factor,  $\beta$ -tubulin and cytochrome oxidase I gene regions. The tree presents one of the 42 equally most parsimonious trees of a heuristic search. Numbers within the tree represent the bootstrap support values followed by posterior probability values. Bootstrap support values below 60 % are not shown. Length = 471, CI = 0.779, RI = 0.820, RC = 0.639. Isolates obtained from *Agathosma* have STEU-codes and are in **bold**. Isolates followed by a \* were all received as *P. citricola* and are from the study of Oudemans et al. (1994), but are re-named here as different taxa based on their phylogenetic clustering.

## Taxonomy

***Phytophthora capensis*** C.M. Bezuidenhout, Denman, A. McLeod & S.A. Kirk, *sp. nov.* — MycoBank MB518805; Fig. 2j–l, 5a–i

*Phytophthorae multivorae* morphologica valde similis, sed oogoniis minoribus, 20–27.5  $\mu\text{m}$  diam (plus minusve 24  $\mu\text{m}$ ), oosporis 20–27.5  $\mu\text{m}$  diam (plus minusve 22.7  $\mu\text{m}$ ) et sporangiis minoribus, 27.5–50  $\mu\text{m}$  longis (plus minusve 39.1  $\mu\text{m}$ ) et 17.5–32.5  $\mu\text{m}$  latis (plus minusve 24  $\mu\text{m}$ ) distinguitur. Differt a *P. plurivora* oogoniis, oosporis et sporangiis distincte minoribus. Regiones 'rDNA ITS', 'cox1', 'NADH', ' $\beta$ -tub' et 'EF-1 $\alpha$ ' cum unica sequential (GenBank GU191232, GU191275, GU191257, GU191328, GU191199).

**Etymology.** From 'The Cape' – the historic and generic name given to the area it was isolated from.

*Phytophthora capensis* has a distinctive uniform, chrysanthemum-pattern colony morphology on carrot agar (CA) (Fig. 2j) and forms abundant aerial mycelium after four days at temperatures between 15–27.5 °C. The mycelium is hyaline, with a low level of branching (similar to that of *P. cactorum* as reported in Erwin & Ribeiro 1996) (Fig. 5f) and on average hyphal diameter is 6  $\mu\text{m}$ . It is homothallic, forming numerous oospores after 8 d when incubated in the dark on CA at temperatures ranging from 15–22.5 °C. The number of oospores produced in culture decline sharply at temperatures above 22.5 °C and ceased at 27.5 °C. Antheridia are paragynous, declinuous in origin, single-celled and more or less square ranging from 5–12.5  $\mu\text{m}$  (mean L  $\times$  B 9.0–9.1  $\mu\text{m}$ ; SD L  $\times$  B 1.8  $\times$  1.7  $\mu\text{m}$ ) (Fig. 2k, 5a–d). Oogonia are round, the diameter ranges from 20–27.5  $\mu\text{m}$  (mean

24 µm, SD 2.5 µm) and they are formed on the terminal ends of hyphae. Oospores are plerotic and thick-walled (> 2.5 µm (mean 2.7 µm) (Fig. 2k, 5a–e). The average oospore diameter is 22.7 µm (SD 2 µm) covering a range between 20–27.5 µm, and > 90 % of oospores reach maturity. Sporangia are semi-papillate, non-caducous, non-proliferating and single, formed on the terminal ends of sporangiophores (Fig. 5g–i). There is a high degree in uniformity of shape: limoniform, occasionally ovoid and they are single lobed. They are attached centrally with no basal plug and only small to moderate numbers of sporangia are formed in pond water. The size of sporangia is variable ranging in length from 27.5–50 µm (av. 39.1 µm; SD 6 µm) and in width from 17.5–32.5 µm (av. 24 µm; SD 3.3 µm). The length : breadth ratio is 1.6–1.7, exit pore width ranges from 5–(5.5)–7.5 µm and the diameter of encysted zoospores is 10–(12.7)–15 µm. No chlamydozoospores or hyphal swellings observed. Cardinal temperatures: minimum 5 °C, optimum 22.5 °C, maximum 27.5 °C and lethal temperature 30 °C (Fig. 1). Daily growth rate at 20 °C was 6.6 mm per day.

*Specimen examined.* SOUTH AFRICA, Western Cape Province, on roots of *Curtisia dentata*, date?, S. von Broembsen, P1819 holotype (World *Phytophthora* Collection), maintained in metabolically inactive state, also deposited at CBS.

### *Phytophthora* taxon emzansi

This taxon is not described here as a species, even though the molecular data support it as such. The isolates are morphologically unstable, and there is presently no evidence that this taxon is an ecologically established population.

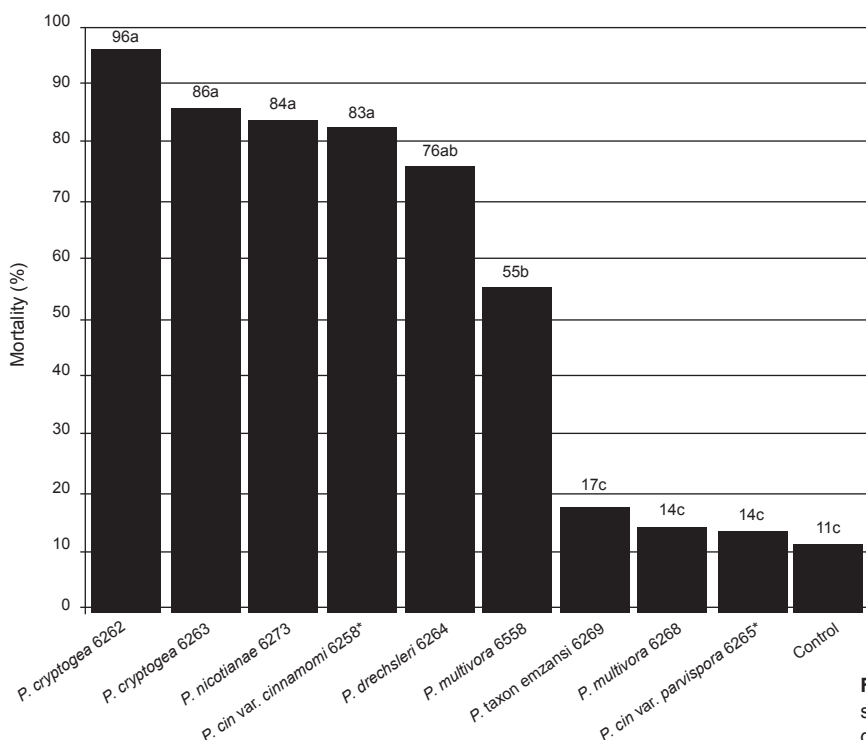
*Etymology.* From the Zulu word meaning south or from the south, indicating that it was isolated from the south (of Africa).

*Phytophthora* taxon emzansi is distinctive within the *P. citricola* complex by its slow growth, amphigynous antheridia and high level of thin-walled, abortive oospores and multi-shaped sporangia. Only two isolates represent this taxon at this stage, and there was a high degree of variability in colony morphology and growth rates between isolates. The colony morphology on CA included a loose chrysanthemum pattern and woolly petaloid pattern (Fig. 2m). The hyphae are on average 6–7.5 µm diam and have developed a moderate number of short branches (a similar

degree of branching to that depicted for *P. syringae* in Erwin & Ribeiro 1996) (Fig. 3i). Low levels of aerial mycelium were produced at the lower temperature spectrum (5–22.5 °C) but this increased to a moderate amount at 25–27.5 °C. Antheridia are amphigynous (Fig. 2n, 3a–e). Paragyny is very rare. Antheridia are single-celled, large and range from ball to block-shaped, the length ranges from 10–20 µm (av. 14.5 µm; SD 2.1 µm) and width between 12.5–17.5 µm (av. 14 µm; SD 1.4 µm). Oogonia are round, the diameter ranges from 25–37.5 µm (mean 30.7 µm, SD 3.1 µm) and they are formed on the terminal ends of hyphae. Frequently a tapering oogonial stalk can be seen through empty antheridia (Fig. 2n, 3a–d). Between 54–58 % of oogonia mature to form oospores, the other 42–46 % of oospores aborted or did not mature (Fig. 3d, f–h). Aborted oogonia often developed abnormal forms (Fig. 3f, h). A mixture of plerotic and aplerotic oospores form in mature oogonia (53 : 47 plerotic : aplerotic) and mature oospores tend towards being thin-walled (< 2.5 µm). The average oospore diameter is 27.9 µm (SD 2.6 µm) covering a range between 22.5–32.5 µm. Sporangia are semi-papillate, non-caducous, non-proliferating and single (Fig. 4) or occasionally formed in loose sympodia (Fig. 4b). They are very variable in size and shape ranging from an elongated wine-glass form (Fig. 2o), with smooth to slightly wavy sides or bent top (Fig. 4c, i, k, l), to ovoid sometimes having a ‘pinched’ area above the broadest part of the base resulting in a sac-like shape, or occasionally slightly peanut- or kidney-shaped (Fig. 4d, j). Most were single lobed (rarely bi-lobed) (Fig. 4h) and attached centrally but a proportion (37.5 %) had an eccentric (off set) attachment (Fig. 4d, f, g). The dimensions of sporangia range from (length) 35–67.5 µm (mean 49.6 µm; SD 8.6 µm) by (breadth) 17.5–47.5 µm (mean 27.4 µm; SD 5.7 µm). The length : breadth ratio is 1.8–1.9, exit pore width ranges from 5–7.5 µm and the diameter of encysted zoospores ranges from 10–15 µm. No chlamydozoospores or hyphal swellings observed. Cardinal temperatures: minimum 10 °C, optimum 20 °C, maximum 27.5 °C and lethal temperature 30 °C (Fig. 1). Daily growth rate at 20 °C was 5 mm per day.

### Pathogenicity

During the pathogenicity trials, symptoms associated with root rot (wilting, yellowing and leaf blight) developed 10–20 d



**Fig. 10** Percentage mortality of potted *Agathosma betulina* seedlings inoculated with selected *Phytophthora* isolates and grown under controlled conditions (\* cin = *cinnamomi*).

after inoculation. At the end of the trial some of the inoculated plants had very few roots, of which some showed sloughing-off of the root epidermis and cortex. Both *P. cryptogea* isolates (STE-U 6262, 6263) and *P. nicotianae* isolate (STE-U 6273) were pathogenic, causing more than 84 % mortality (Fig. 10). The *P. drechsleri* isolate (STE-U 6264) and one of the *P. multivora* isolates (STE-U 6558) were pathogenic but caused less seedling mortality than the former, differing significantly from the control. Non-pathogenic isolates (i.e. those that did not differ significantly from the control in percentage dead seedlings) included the other *P. multivora* isolate (STE-U 6268), *P. taxon emzansi* (STE-U 6269) and *P. cinnamomi* var. *parvispora* (STE-U 6265) isolates (Fig. 10). Control plants remained healthy throughout the experiments, except for three plants that died during the first trial due to transplant shock (no *Phytophthora* was isolated from these plants). Back isolations from symptomatic plants yielded only the respective inoculated *Phytophthora* species.

## DISCUSSION

The research conducted in our study was initiated in 2006 as an investigation into the threat of *Phytophthora* to commercial *Agathosma* growers in the Western Cape Province of South Africa. The study led us in particular to a morphological and phylogenetic comparison of the taxa in the *P. citricola* complex within *Phytophthora* ITS clade 2 (Cooke et al. 2000). Our work not only provides an important link with the comprehensive work of Oudemans et al. (1994), but also complements the studies of Jung & Burgess (2009) and Scott et al. (2009), whom recently described two new species, *P. multivora* and *P. plurivora* within the *P. citricola* complex. Results from the latter two studies and the current study revealed the presence of several phylogenetic taxa within the *P. citricola* complex. The specific taxa will be discussed in subsequent sections.

Overall our study has increased knowledge on *Phytophthora* species associated with commercially produced *Agathosma* in South Africa. It is the first to reveal multiple *Phytophthora* taxa on *Agathosma*, including *P. cinnamomi* var. *cinnamomi*, *P. cryptogea*, *P. multivora*, *P. drechsleri*, *P. nicotianae*, *P. cinnamomi* var. *parvispora* and a new taxon, *P. taxon emzansi*. The latter taxon and *P. multivora* falls within the *P. citricola* complex, with *P. taxon emzansi* being phylogenetically related to a new species in the complex, *P. capensis*. Isolates of *P. capensis* were previously isolated from stream water and indigenous hosts other than *Agathosma* in South Africa (Oudemans et al. 1994), and were assigned to *P. citricola* CIT4 by Oudemans et al. (1994).

For the present, *P. taxon emzansi* has been designated only as an informal taxon and not as a new species. In our study only two isolates were available, both from the same host plant. Furthermore, while the properties of the taxon appear to be unique, the morphological characteristics of the two *P. taxon emzansi* isolates demonstrated a number of instabilities and inconsistencies, including variable growth rate and colony morphology, a high degree of plasticity in sporangial morphology, high levels of zygotic abortion (42 %) and poorly developed gametangia. Similar aberrant features were noted in the hybrid species *P. alni* and its variants by Brasier et al. (2004). The aberrant morphological features in *P. taxon emzansi* suggest some genetic instability. Evidence of this genetic instability was not found in any of the three nuclear or two mitochondrial gene regions that were sequenced, since the sequences of these gene regions were identical in both isolates. These gene regions, however, only represent a small fraction of the complete genome that in other clade 2 species such as *P. capsici* is approximately 65 Mb, potentially representing more than 15 000 genes in other

oomycete species (Govers & Gijzen 2006). Furthermore, although the absence of ITS dimorphic sites and triploid isozyme genotypes in the two *P. taxon emzansi* isolates suggest that they might not be hybrids, the presence of seven heterozygous sites in the EF-1 $\alpha$  sequences of the isolates may provide some evidence of the fact that the isolates are still stabilising after a hybridisation or introgression event. This however, would require further investigation. Whether *P. taxon emzansi* should be designated at the species level should await i) investigation of more isolates; ii) demonstration of a greater consistency of characters; and iii) investigations into whether or not it may be a hybrid or introgressant.

*Phytophthora capensis* and *Phytophthora taxon emzansi* are related phylogenetically, but distinct taxa within the *P. citricola* cluster in *Phytophthora* clade 2, and have only been reported from South Africa. Indeed, no available GenBank ITS sequences matched either taxon. Their distinctiveness was further supported by i) the clade structure of a combined phylogeny (Fig. 9); ii) heterozygosity at MDH and GPI loci in *P. capensis*, compared to the homozygosity in *P. taxon emzansi*; and iii) *P. capensis* containing private alleles at the MDH and GPI loci. Furthermore, based on our specific study *P. capensis* was morphologically distinct, exhibiting the smallest (in terms of length) sporangia of the taxa in the *P. citricola* cluster, and a distinct colony morphology.

In our study a combined  $\beta$ -tub / EF-1 $\alpha$  / *cox1* phylogeny was sufficient for resolving not only the two new taxa, but also the other taxa within the *P. citricola* cluster. The multi-gene phylogeny revealed six distinct clades (*P. mingei* excluded) including i) *P. multivora* (synonyms *P. citricola* CIT3, ETs 6 & 7 sensu Oudemans et al. 1994); ii) *P. taxon emzansi*; iii) *P. capensis* (synonym *P. citricola* CIT4 sensu Oudemans et al. 1994); vi) *P. citricola* s.str. (synonyms *P. citricola* CIT1, ETs 2 & 3 sensu Oudemans et al. 1994); v) *P. plurivora* (synonym *P. citricola* CIT1, ET 1 sensu Oudemans et al. 1994); and vi) *P. citricola* clade E sensu Jung & Burgess (2009) (synonym *P. citricola* CIT2 sensu Oudemans et al. 1994). However, in our study, although the individual clades are well supported, there is a lack of bootstrap and posterior probability support for the phylogenetic backbone of *P. citricola* s.str. (CIT2, ETs 2 & 3), *P. plurivora* (CIT1, ET 1) and *P. citricola* clade E sensu Jung & Burgess (2009) (CIT2) clades. Therefore, the relationship of these three clades to one another and to the remaining *P. citricola* taxa is still uncertain. Incorporating only one isolate of *P. plurivora* in our phylogeny, due to lack of available sequence data, may have contributed to this. Therefore, the analyses of more isolates from these three clades could add resolution of these clades and establish their phylogenetic relationships.

The recent designation of *P. multivora* and *P. plurivora* as new species within the *P. citricola* cluster relied mainly on the use of the ITS and *cox1* regions (Jung & Burgess 2009, Scott et al. 2009). However, the exclusive use of these two regions for species identification and phylogenetic inference may not be sufficiently reliable for differentiating phylogenetic species. The *cox1* region should not be used in isolation, since it is inherited maternally and will therefore not reflect some evolutionary forces such as hybridisation events (Martin & Tooley 2003), which are now well known in *Phytophthora* (Brasier et al. 2004, Man in 't Veld et al. 2007, Ersek & Nagy 2008). Although ITS sequences are the most well-represented among GenBank sequences of the *P. citricola* cluster, this region does not unambiguously resolve *P. citricola* complex taxa (Jung & Burgess 2009, and this study). Other problems associated with the ITS region include polymorphic ITS copies within the same isolate and difficulties in obtaining unambiguous alignments (Martin & Tooley 2003, Brasier et al. 2004, Drenth et al. 2006, Kageyama et al. 2007). The latter caveats of the ITS region are also well



known for plants (Álvarez & Wendel 2003, Bailey et al. 2003, Feliner & Rosselló 2007). One advantage of ITS sequences is that they can sometimes reveal the presence of hybrids (Man in 't Veld et al. 2007), should these be present.

Analyses of morphological and physiological features of taxa in the *P. citricola* complex may complement and support phylogenetic analyses and taxon identification, but distinguishing features may vary within taxa. In the current study, even though only a small number of isolates were examined, the analyses revealed distinct differences within and between some taxa in the *P. citricola* cluster. The most useful criteria in our study were temperature-growth relationships, colony type, antheridial attachment and dimensions, and gametangial features such as frequency of oospore abortion and developmental abnormalities. The gametangial abnormalities are, however, more related to genetic characteristics. In some cases (*P. capensis*, *P. taxon emzansi* and *P. mengei*) sporangial features, including abnormalities, were also diagnostic. In this, we are broadly in agreement with Jung & Burgess (2009). Although some of our results (for example temperature growth studies and reproductive structure size ranges) differ from those of Jung & Burgess (2009), these discrepancies are probably largely attributable to using different growth media, temperatures and each group using only a few isolates of each taxon. Furthermore, comparison of morphological data from several studies underpins the problem of defining the range of variation within and between species. Therefore, in practice, for taxa within the *P. citricola* cluster, if several isolates are investigated, morphological criteria may often overlap between taxa.

*Phytophthora mengei* (synonym *P. citricola* CIT5 sensu Oudemans et al. 1994), recently described by Hong et al. (2009) should no longer be considered as a component of the *P. citricola* complex due to its morphological distinctiveness and phylogenetic relatedness to *P. capsici* (Oudemans et al. 1994, Cooke & Duncan 1997, Hong et al. 2009, Jung & Burgess 2009, current study). Our results, however, indicated that *P. mengei* may be genetically more diverse than previously reported (Hong et al. 2009). Hong et al. (2009) did not include any of Oudemans et al. (1994) reference isolates in their study, but based on our sequence data, they likely only included representatives of the *P. citricola* CIT5 (ET 9) sensu Oudemans et al. (1994) group, and not the one known CIT5 (ET 10) isolate that was isolated from Guatemala (Oudemans et al. 1994). In our study both phylogenetic and isozyme analyses showed that CIT5, ET 9 and ET 10 are genetically diverse, but are conspecific. Our study has also shown that *P. mengei* differ from the taxa in the *P. citricola* cluster in its i) high proportion (17 %) of distinctive bi- or tri-lobed sporangia; and ii) oogonia with a distinctive 'beaked'-shaped form of apertotism. Hong et al. (2009) also reported that *P. mengei* produced 'bizarre-shaped' sporangia. The sporangial L : B ratio (2.3) of our *P. mengei* was the largest of all our investigated taxa, but differed from the ratios (1.6 to 1.78) reported by Hong et al. (2009) and Oudemans et al. (1994). Reasons for this could be differences in growth conditions, or the use of only the *P. mengei* CIT5 (ET 10) isolate in our morphological study, as opposed to the CIT5 (ET 9) isolates used in other studies. This emphasises the difficulty of comparing results across different studies, and dealing with the possible large within taxon variation.

Apart from *P. taxon emzansi*, most of the *P. citricola* cluster isolates from *Agathosma* were identified as *P. multivora* (synonym CIT3 (ETs 6 & 7) sensu Oudemans et al. 1994). *Phytophthora multivora* has a wide host range and is also an important widespread pathogen in natural ecosystems in Western Australia (WA) (Scott et al. 2009). In our study, *P. multivora* isolates (*Agathosma* and reference isolates) were shown to be morphologically and genetically rather diverse, which is in agreement

with Scott et al. 2009. Scott et al. (2009) hypothesized that *P. multivora* is native to WA, and was dispersed to other countries via the nursery trade. Alternatively, *P. multivora* may be native to WA and South Africa, since these two continents previously formed part of the southern precursor supercontinent (Gondwanaland), as is still evident today by their sharing of unique flora such as the *Proteaceae*. Whether our South African *P. multivora* isolates from *Agathosma*, and isolates from other hosts obtained more than 16 yr ago (Oudemans et al. 1994) were introduced from WA or are native to South Africa, may be revealed by studying more isolates, using markers that reveal greater genomic polymorphism.

The current study constitutes the first report of *P. multivora*, *P. taxon emzansi* and *P. cinnamomi* var. *parvispora* from *Agathosma*, or indeed any genus from the Cape Floral Kingdom. The other *Phytophthora* species associated with *Agathosma* have been reported from other Cape Floral families, mainly the *Proteaceae* (Hardy & Sivasithamparam 1988, Tynan et al. 1998, Boersma et al. 2000). *Phytophthora* taxon emzansi and *P. cinnamomi* var. *parvispora* were found to be non-pathogenic towards *A. betulina*, whereas *P. multivora* isolates varied in their aggressiveness. In contrast, *P. cinnamomi* var. *cinnamomi* was among the four most pathogenic taxa on this host, the others being *P. cryptogea*, *P. drechsleri* and *P. nicotianae*. The two *P. cinnamomi* varieties not only differed markedly in their pathogenic ability, but were also found here to be both morphologically and phylogenetically distinct. This is in agreement with other studies that have also suggested that *P. cinnamomi* var. *parvispora* should be considered a separate species (Blair et al. 2008, Gallegly & Hong 2008). Only the A1 mating type of *P. cinnamomi* var. *cinnamomi* was obtained from commercially produced *Agathosma* plants. In contrast, on commercial *Proteaceae* the predominant mating type observed is A2 mating type, the A1 being more prevalent on native vegetation (Linde et al. 1997, Denman & Sadie 2001).

It is clear that the *P. citricola* cluster requires further study. Our study has both identified new *Phytophthora* taxa associated with *Agathosma*, and shown that a multi-gene sequencing approach is important for resolving cryptic taxa within the cluster. Gene regions found here to be most useful for delineating clades within the cluster were the *cox1*, EF-1 $\alpha$  and  $\beta$ -tub regions. In future investigations it is thus recommended that these gene regions be included, together with reference isolates or sequences used in our current study viz. *P. multivora* (CIT3 (ETs 5 & 6)); *P. taxon emzansi*; *P. capensis* (CIT4); *P. citricola* s.str. (CIT1 (ETs 2 & 3)); *P. plurivora* (CIT1 (ET1)) and *P. citricola* clade E sensu Jung & Burgess (2009) (CIT2). Isolates of *P. citricola* CIT3 (ET 5) sensu Oudemans et al. (1994) and *P. citricola* subgroups I (possible synonym of the currently invalid species *P. pini*) to III (Gallegly & Hong 2008, Gallegly et al. 2010), which have been reported as being distinct based on SSCPs of the ITS region, should also be included to clarify their status. Considerable further behaviour and morphological analyses of the *P. citricola* cluster taxa should also be carried out to identify any unique features and to help understand the ecology and adaptive behaviour of the taxa. Morphological and adaptive behaviour could give insights into the origins and evolution of species.

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