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

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Key words
- avocado
- buchu
- fynbos
- glucose-6-phosphate isomerase
- isozymes
- malate dehydrogenase
- pathogenicity
- root-rot
- taxonomy

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. cinnamomum var. cinnamomum, P. cinnamomum var. parviflora and P. cryptoea, 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. cinnamomum var. parviflora 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 cinnamomum is considered the most virulent and widespread species affecting Proteaceae (von Broembsen & Brits 1985, Cahill et al. 2008), but other species, namely P. citrica, P. cryptoea, P. drechsleri and P. nicotianae also cause disease on Proteaceous 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. citricula.
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 multifocus 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* CIT3 sensu Oudemans et al. 1994) as a distinct species, namely *P. menganai*, 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 β-tubulin (β-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. (2003) 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 *Agathosma* plantlets. 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 (β-tub, translation elongation factor 1α (EF-1α) 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 (Enwin & 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 comunal agar (CMA, Sigma-alridich, 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 mycelial 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 (*Penstemon 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 oo- 

**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
| Culture no. | Host | Morphological identification | Sequence identification | CIT group-ET | Isozyme genotype | Mating type | Nursery/Field | Location |
|------------|------|----------------------------|-------------------------|--------------|------------------|-------------|--------------|----------|
| 11821      | Stream water | P. citricola | P. capensis | 4–8 | EF CD AB | – | – | South Africa |
| 11823      | Olea capensis | P. citricola | P. capensis | 4–8 | EF CD AB | – | – | South Africa |
| 11819      | Curtisia dentate | P. citricola | P. capensis | 4–8 | EF CD AB | – | – | South Africa |
| STE-U 6255 | Agathosma crenulata | P. cinnamomi | P. cinnamomi var. cinnamomi | – | n.d. n.d. n.d. | A1 | Nursery | South Africa |
| STE-U 6256 | A. crenulata | P. cinnamomi | P. cinnamomi var. cinnamomi | – | n.d. n.d. n.d. | A1 | Nursery | South Africa |
| STE-U 6257 | A. crenulata | P. cinnamomi | P. cinnamomi var. cinnamomi | – | n.d. n.d. n.d. | A1 | Nursery | South Africa |
| STE-U 6258 | Agathosma betulina | P. cinnamomi | P. cinnamomi var. cinnamomi | – | n.d. n.d. n.d. | A1 | Field | South Africa |
| STE-U 6261 | A. betulina | P. cinnamomi | P. cinnamomi var. cinnamomi | – | n.d. n.d. n.d. | A1 | Field | South Africa |
| STE-U 6262 | A. betulina | P. cinnamomi | P. cinnamomi var. cinnamomi | – | n.d. n.d. n.d. | A1 | Field | South Africa |
| STE-U 6263 | A. betulina | P. cinnamomi | P. cinnamomi var. cinnamomi | – | n.d. n.d. n.d. | A1 | Field | South Africa |
| STE-U 6264 | A. betulina | P. cinnamomi | P. cinnamomi var. panispore | – | n.d. n.d. n.d. | A1 | Field | South Africa |
| STE-U 6265 | A. betulina | P. cinnamomi | P. cinnamomi var. panispore | – | n.d. n.d. n.d. | A1 | Field | South Africa |
| P1321      | Rubus idaeus | P. citricola | P. citricola | 2–4 | EF AA AA | – | – | USA |
| P6624      | Fragaria sp. | P. citricola | P. citricola | 2–4 | EF AA AA | – | – | Taiwan |
| P1814      | Citrus sp. | P. citricola | P. citricola s.str. | 1–3 | EF CC AA | – | – | South Africa |
| P1815      | Citrus sp. | P. citricola | P. citricola s.str. | 1–2 | EF CC AA | – | – | South Africa |
| STE-U 6262 | A. betulina | P. cryptogaea | P. cryptogaea | – | n.d. n.d. n.d. | No oogonia | Field | South Africa |
| STE-U 6263 | A. betulina | P. cryptogaea | P. cryptogaea | – | n.d. n.d. n.d. | A1 | Field | South Africa |
| STE-U 6261 | A. betulina | P. cryptogaea | P. cryptogaea | – | n.d. n.d. n.d. | A1 | Field | South Africa |
| STE-U 6264 | A. betulina | P. drechleri | P. drechleri | – | n.d. n.d. n.d. | A2 | Field | South Africa |
| P0513      | Persea americana | P. citricola | P. mengei | 5–9 | AB CD AA | – | – | Mexico |
| P1165      | P. americana | P. citricola | P. mengei | 5–10 | CD CD AA | – | – | Guatemala |
| STE-U 6267 | A. betulina | P. citricola | P. multivora | 3 | EF BB AA | Homothallic | Nursery | South Africa |
| STE-U 6268 | A. crenulata | P. citricola | P. multivora | 3 | EF BB AA | Homothallic | Nursery | South Africa |
| STE-U 6270 | A. crenulata | P. citricola | P. multivora | 3 | EF BB AA | Homothallic | Nursery | South Africa |
| STE-U 6558 | A. betulina | P. citricola | P. multivora | 3 | EF BB AA | Homothallic | Nursery | South Africa |
| STE-U 6271 | A. betulina | P. citricola | P. multivora | 3 | EF BB AA | Homothallic | Nursery | South Africa |
| P1818      | Medicago sativa | P. citricola | P. multivora | 3–6 | – BB – | – | – | South Africa |
| P1817      | M. sativa | P. citricola | P. multivora | 3–7 | EF BB AA | – | – | South Africa |
| P1821      | Ocotea bullata | P. citricola | P. multivora | 3–6 | EF BB AA | – | – | South Africa |
| STE-U 6273 | A. betulina | P. Nicotiana | P. nicotiana | – | n.d. n.d. n.d. | A2 | Nursery | South Africa |
| P1611      | Malus sp. | P. citricola | P. plurivora | 1–1 | EF CC AA | – | – | USA |
| STE-U 6272 | A. betulina | P. citricola | P. taxon emzansi | New | EF CC AA | Homothallic | Field | South Africa |
| STE-U 6269 | A. betulina | P. citricola | P. taxon emzansi | New | EF CC AA | Homothallic | Field | South Africa |

1 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).
2 Sequence based identifications were conducted using a combined β-tubulin / cytochrome oxidase I / translation elongation factor 1 phylogeny.
3 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).
4 Glucose-6-phosphate isomerase (synonym of phosphoglucose isomerase (PGI)) alleles (A to F).
5 Malate dehydrogenase (MDH) loci MDH-1 and MDH-2 alleles (A to D).
6 s.str. = sensu stricto.
\textit{P. citricola} were studied comparatively using 12 \textit{P. citricola} CIT groups (1–5 sensu Oudemans et al. (1994) reference isolates (P1165, P1321, P1611, P1814, P1815, P1817–P1819 and P1821–P6224). 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 \textit{Agathosma} and the 12 references 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 \textit{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 \textit{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 x200, x400 and x1000 magnification.

Mating type determination of heterothallic species

The mating type of heterothallic isolates obtained from \textit{Agathosma} plants was determined by pairing each isolate with a known A1 and A2 tester strain of the corresponding species or closely related species (\textit{P. cinnamomi} isolates P2425 and P2160, \textit{P. cryptogea} isolates P1088 and P1704 and \textit{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 x100 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.

\textbf{DNA isolation, amplification and sequencing}

DNA was extracted from 33 isolates (20 Phytophthora isolates obtained from \textit{Agathosma} and 13 \textit{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 \textit{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 ENLGF1 and ENLGR1 primers (Kroon et al. 2004); iv) cox1 with primers FM83 and FM84 (Martin & Tooley 2003); and v) NADH with NADH1 and NADHR1 primers (Kroon et al. 2004). Each PCR amplification consisted of 1x 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, Randall, South Africa) (except for cox1), 2 \(\mu\)L genomic DNA and MgCl\(_2\) and primers specific for each primer pair, in a total reaction volume of 40 \(\mu\)L. The MgCl\(_2\) 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 PCRacpse clean-up kit (Invitek, 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.

\textbf{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 \textit{Phytophthora} isolates that were similar to \textit{P. citricola} and \textit{P. cinnamomi} required further phylogenetic analyses, since it is known that \textit{P. citricola} contains cryptic species (Jung & Burgess 2009) and it was not clear whether the \textit{P. cinnamomi} isolates were \textit{P. cinnamomi} or \textit{P. cinnamomi} var. parvispora. Since \textit{P. cinnamomi} and \textit{P. cinnamomi} belong to \textit{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 \textit{P. citricola} clade 2 analyses isolates of \textit{P. multivora}, \textit{P. plurivora}, the type and authentic type strains of \textit{P. citricola} submitted by Jung & Burgess (2009) were also included. In the ITS phylogeny, the sequences of \textit{P. citricola} type (GenBank FJ237526: IMI 021173 = CBS 221.88) and of \textit{P. citricola} authentic type (GenBank FJ560913; CBS 295.29) have been included, which represents \textit{P. citricola} s.str. The sequences of these type and authentic types were identical to P1814 and P1815, which therefore also represent \textit{P. citricola} s.str. Only the group I \textit{P. citricola} sequences of \textit{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 | GenBank accession no. |
|-------------|------------------------|-----------------------|
| P1822       | P. capensis            | GU191219 GU191325 GU191196 GU191267 GU191277 |
| P1823       | P. capensis            | GU191231 GU191327 GU191198 GU191256 GU191298 |
| P1819       | P. capensis (ex-type)  | GU191232 GU191328 GU191199 GU191257 GU191275 |
| STE-U 6255  | P. cinnamomi var. cinnamomi | GU191204 GU191304 GU191175 GU902245 GU191273 |
| STE-U 6256  | P. cinnamomi var. cinnamomi | GU191206 GU191305 GU191176 GU191239 GU191262 |
| STE-U 6257  | P. cinnamomi var. cinnamomi | GU191207 GU191300 GU191171 GU191240 GU191281 |
| STE-U 6258  | P. cinnamomi var. cinnamomi | GU191208 GU191306 GU191177 GU191236 GU191289 |
| STE-U 6259  | P. cinnamomi var. cinnamomi | GU191209 GU191303 GU191174 GU191237 GU191283 |
| STE-U 6260  | P. cinnamomi var. cinnamomi | GU191210 GU191302 GU191173 GU191241 GU191291 |
| STE-U 6266  | P. cinnamomi var. cinnamomi | GU191205 GU191301 GU191172 GU191238 GU191290 |
| STE-U 6265  | P. cinnamomi var. parvispora | GU191211 GU191319 GU191190 GU191246 GU191286 |
| P1814       | P. citricola s.str.    | GU191217 GU191321 GU191192 GU191263 GU191278 |
| P1815       | P. citricola s.str.    | GU191218 GU191322 GU191193 GU191264 GU191274 |
| P1321       | P. citricola CIT2     | GU191216 GU191320 GU191191 GU191262 GU191296 |
| P1615       | P. citricola s.str.    | GU191218 GU191322 GU191193 GU191264 GU191274 |
| STE-U 6261  | P. cinnamomi          | GU191214 GU191309 GU191180 GU191243 GU191287 |
| STE-U 6262  | P. cinnamomi          | GU191213 GU191307 GU191178 GU191242 GU191268 |
| STE-U 6263  | P. cinnamomi          | GU191212 GU191308 GU191179 GU191244 GU191288 |
| STE-U 6264  | P. drechsleri         | GU191215 GU191310 GU191181 GU191247 GU191271 |
| P0513       | P. mengei             | GU191234 GU191332 GU191203 GU191261 GU191295 |
| P1165       | P. mengei             | GU191235 GU191331 GU191202 GU191260 GU191280 |
| P1816       | P. multiflora         | GU191212 GU191323 GU191194 GU191265 GU191297 |
| P1817       | P. multiflora         | GU191229 GU191329 GU191200 GU191258 – |
| P1821       | P. multiflora         | GU191222 GU191324 GU191195 GU191266 GU191276 |
| STE-U 6267  | P. multiflora         | GU191225 GU191315 GU191186 GU191251 GU191294 |
| STE-U 6268  | P. multiflora         | GU191226 GU191312 GU191183 GU191254 GU191285 |
| STE-U 6270  | P. multiflora         | GU191227 GU191311 GU191182 GU191253 GU191284 |
| STE-U 6271  | P. multiflora         | GU191224 GU191313 GU191184 GU191252 GU191293 |
| STE-U 6558  | P. multiflora         | GU191227 GU191314 GU191185 GU191248 GU191292 |
| STE-U 6273  | P. cinnamomopsis      | GU902246 GU191318 GU191189 GU191245 GU191272 |
| P1611       | P. plurivora          | GU191233 GU191330 GU191201 GU191259 GU191279 |
| STE-U 6269  | P. taxon emzansi      | GU191228 GU191317 GU191188 GU191250 GU191270 |
| STE-U 6272  | P. taxon emzansi      | GU191220 GU191316 GU191187 GU191249 GU191269 |

1 Sequence based identifications were conducted using a combined β-tubulin (β-tub) / cytochrome oxidase I (cox1) / translation elongation factor 1α (EF-1α) phylogeny.
2 Internal transcribed spacer regions (ITS1 and ITS2) and 5.8S nuclear ribosomal RNA gene.
3 NADH dehydrogenase subunit I.

Bayesian analyses were conducted using MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). The optimal model of sequence 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 (β-tub, EF-1α 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 140 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 CaCO₃ 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 an 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 phar-malyte 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 am-photolyte 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. xaxon emzansi (STE-U 6269), P. plurivora (STE-U 6558, 6268), P. cryptogea (STE-U 6262, 6263) and P. drechsléri (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 iso-lates 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. cryptogea, P. drechsléri and P. nicotianae (Table 1). All species except P. nicotianae were identified in field plants, whereas only P. cin-namomi, P. cryptogea 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. drechsléri. Differentiation between P. cryptogea and P. drechsléri isolates was based on growth at 35 °C. Eight iso-lates were identified as P. cinnamomi and seven as P. cryptogea.

Two distinct morphological types were evident among the P. cin namomi 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 av-erage 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 felly, 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. 26.8 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. cito-rica complex, a total of seven isolates. The remaining 13 isolates were heterothallic; only the single isolates each of P. drechsléri 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. citr icola complex

Below, the taxon designation of the P. citricola isolates as de-termined 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 representa-tive 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 charac-teristics 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 differ-ences 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.* mengei (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.* mengei 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. 2Pi), the second group had a radially-striate with central woolliness pattern (e.g. STE-U 6270, Fig. 2Pi), 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 mengei* 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...
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. mengei 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. mengei 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, β-tub, NADH, EF-1α 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 (Mostowfizadeh-Ghalamfarsa 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-1α 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. cinnam-
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Parasporal features of *P. taxon emzansi*. a. Ovoid sporangium; b. loose sympodium 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 µm; b = 50 µm; g = 10 µm.

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 β-tub / EF-1α / 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 β-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. mengei* since isolate P0513 (CIT5, ET 9) had 100 % ITS sequence identity with the newly described *P. mengei* (GenBank EU748545, EU748546) sequences, whereas P1165 (CIT5, ET 10) only differed by one base pair from this sequence. The two *P. mengei* 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.
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 sequences 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. 5 Phytophthora capensis. a–e. Mature oogonia, thick-walled, round oospores and paragynous antheridia; f. branched hyphae; g–l. semi-papillate, limoniform sporangia with central attachment. — Scale bars: a, b, d–i = 20 µm; c = 10 µm.](image)

![Fig. 6 Morphological features of *P. mengei*. 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 µm;](image)
The combined β-tub / EF-1α / 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 β-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 6265 & 6266); 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 6265 and 6266) 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 heterogeneous at the MDH-2 locus and shared one allele with the other isolates (Table 1).
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, β-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

*Phytophthora multivorae* morpholigice valde similis, sed oogoniis minoribus, 20–27.5 µm diam (plus minusve 24 µm), oosporis 20–27.5 µm diam (plus minusve 22.7 µm) et sporangiiis minoribus, 27.5–50 µm longis (plus minusve 39.1 µm) et 17.5–32.5 µm latis (plus minusve 24 µm) distinguuntur. Differt a *P. plurivora* oogoniis, oosporis et sporangiiis distincte minoribus. Regiones ‘rDNA ITS’, ‘cox1’, ‘NADH’, ‘β-tub’ et ‘EF-1α’ 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 µm. 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, diclinous in origin, single-celled and more or less square ranging from 5–12.5 µm (mean L × B 9.0–9.1 µm; SD L×B 1.8 × 1.7 µm) (Fig. 2k, 5a–d). Oogonia are round, the diameter ranges from 20–27.5 µm (mean
and 22.5–32.5 µm. 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 of variability in size and shape ranging from 10–15 µm. No chlamydospores 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.

**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. citrulina* 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 in log phase is an ecologically established population.

**Pathogenicity**

During the pathogenicity trials, symptoms associated with root rot (wilting, yellowing and leaf blight) developed 10–20 d 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) 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, I), to ovoid (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 is 27.9 µm (SD 2.6 µm) covering a range between 26.7–31.5 µm.

**Fig. 10** Percentage mortality of potted *Agathosma betulina* seedlings inoculated with selected *Phytophthora* isolates and grown under controlled conditions (* cin = cinnamomi).
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 genes 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 triplid 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-1α 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) heterozygoty 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 taxon in the *P. citricola* cluster, and a distinct colony morphology.

In our study a combined (α-tub/EF-1α/cox1) phylogeny was sufficiently 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. mengei* 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); iv) *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 the *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 & Rossello 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 taxon in the *P. citricola* cluster (in ii) high proportion (17 %) of distinctive bi- or tri-lobed sporangia; and ii) oogonia with a distinctive ‘beaked’-shaped form of aplotherium. 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 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-1α and β-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 & 6); *P. taxon emzansi*; *P. capsensis* (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 SSCP 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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