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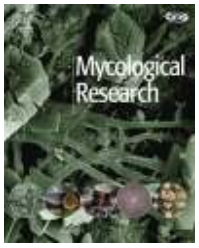


# Two new *Phytophthora* species from South African *Eucalyptus* plantations

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ARTICLE INFO

*Article history:*
Received 11 October 2006
Received in revised form
1 July 2007
Accepted 22 August 2007
Published online 1 September 2007
*Corresponding Editor:*
David E. L. Cooke

*Keywords:*
Oomycetes
Phylogeny
Root and collar rot
Taxonomy

ABSTRACT

A recent study to determine the cause of collar and root rot disease outbreaks of cold tolerant Eucalyptus species in South Africa resulted in the isolation of two putative new *Phytophthora* species. Based on phylogenetic comparisons using the ITS and β-tubulin gene regions, these species were shown to be distinct from known species. These differences were also supported by robust morphological characteristics. The names, *Phytophthora frigida* sp. nov. and *Phytophthora alticola* sp. nov. are thus provided for these taxa, which are phylogenetically closely related to species within the ITS clade 2 (*P. citricola*, *P. tropicali* and *P.multivesiculata*) and 4 (*P. arecae* and *P. megakarya*), respectively. *Phytophthora frigida* is heterothallic, and produces stellate to rosaceous growth patterns on growth medium, corraloid hyphae, sporangia with a variety of distorted shapes and has the ability to grow at low temperatures. *Phytophthora alticola* is homothallic and has a slower growth rate in culture. Both *P. frigida* and *P. alticola* are pathogenic to *Eucalyptus dunnii*. In pathogenicity tests, they were, however, less pathogenic than *P. cinnamomi*, which is a well-known pathogen of Eucalyptus in South Africa.



## Introduction

Cold-tolerant *Eucalyptus* spp. are grown extensively for pulpwood production in summer rainfall areas of South Africa with an altitude above 1150 m (Swain & Gardner 2003). During the mid-1980s, an increased demand for pulpwood led to the expansion of cold-tolerant *Eucalyptus* plantations (Darrow 1996). This period also marked the beginning of breeding programmes for cold-tolerant *Eucalyptus* spp. and the introduction of several alternative *Eucalyptus* spp. from seeds collected in natural stands in Australia (Darrow 1994; Swain & Gardner 2003). Several cold-tolerant species with high commercial potential have since been reported (Clarke *et al.* 1999; Little & Gardner 2003). However, some species such as *E. fastigata* and *E. fraxinoides* are well known for their susceptibility to infection by *Phytophthora cinnamomi* (Wingfield & Kemp 1994), which is considered to be an introduced pathogen in South Africa (Linde *et al.* 1999).

*Phytophthora* collar and root rot is a widespread disease affecting a number of cold-tolerant *Eucalyptus* spp. in South Africa (Linde *et al.* 1994a,b). This disease hampers progress towards introducing alternative *Eucalyptus* species yielding high pulp volumes. The most common disease symptom is progressive wilting of the leaves due to the girdling of the root collars. When the bark is removed, brown lesions extending from the roots are typically observed. Other disease symptoms include root disease, bleeding lesions from diseased stem tissue, and the formation of epicormic shoots on the

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doi:10.1016/j.mycres.2007.08.011

stems of dying trees. Dying trees are usually present in small patches throughout the plantations, especially in areas prone to water-logging during the rainy seasons.

*Phytophthora* spp. known to be associated with collar and root rot of *Eucalyptus* spp. in South Africa include *P. boehmeriae*, *P. cinnamomi*, and *P. nicotianae* (Linde *et al.* 1994b). In 2001, *P. nicotianae* caused disease outbreaks on several cold-tolerant *Eucalyptus* spp. in South Africa (Maseko *et al.* 2001). This was particularly interesting as *P. cinnamomi,* rather than *P. nicotianae,* has typically been associated with mortality of cold-tolerant *Eucalyptus* spp. (Linde *et al.* 1994b). During the same period, new and invasive *Phytophthora* spp. such as *P. ramorum* and *P. quercina* were emerging as important pathogens in Europe and North America (Jung *et al.* 1999; Werres *et al.* 2001; Rizzo *et al.* 2002). This prompted extensive surveys of cold-tolerant *Eucalyptus* stands to assess the presence of *P. nicotianae* and other possible invasive *Phytophthora* spp. that might be present on *Eucalyptus* spp. in South Africa.

Isolations of *Phytophthora* spp. during surveys of cold-tolerant *Eucalyptus* spp. yielded two groups of isolates that could not be assigned to known species. The aim of this study was to characterise these new *Phytophthora* spp. based on comparisons of DNA sequence data and morphology. Pathogenicity tests were also conducted with isolates representing the two unknown species, as well as *P. cinnamomi*, which was included for comparative purposes.

## Material and methods

### *Sampling and isolation of isolates*

Between 2000 and 2004, *Phytophthora* root rot was recorded in several plantations of cold-tolerant *Eucalyptus* spp. in KwaZulu-Natal Province (Swain *et al.* 2000). In particular, three areas severely affected by this disease were located in Sutton plantation near Ixopo (29° 58′S, 30° 08′E), Mid-Illovo (29° 53′S, 30° 24′E), and Paulpietersburg (27° 31′S, 30° 47′E) provenance/progeny trials. Four soil samples from the top 10 cm at the bases of dying trees were pooled in a single plastic bag. In addition, plant tissue was collected from infected root collars. Isolation from soil and diseased plant samples was performed within 48 h of collection. A total of 368 diseased trees and 240 soil samples were collected and assayed for the presence of *Phytophthora* spp.

Soil samples were flooded with distilled water and baited using citrus leaf discs (5 mm, diam) or *Eucalyptus sieberi* cotyledons as described by Grimm & Alexander (1973) and Marks & Kassaby (1974), respectively. After incubation at room temperature in the dark for 2–3 d, the leaf discs or cotyledons were plated on modified selective NARPH agar [Difco, Detroit, MI, corn meal agar (CMA), 17 g $l^{-1}$ amended with 50 μg $ml^{-1}$ nystatin, 200 μg $ml^{-1}$ ampicillin, 10 μg $ml^{-1}$ rifampicin, 25 μg$ml^{-1}$ pentacloronitrobenzene (PCNB), and 50 μg$ml^{-1}$ hymexazol 3 hydroxy-5-methylisoxazole, Sigma-Aldrich, St. Louis] (Hüberli *et al.* 2000). Small pieces of diseased plant tissue were plated directly on NARPH. Petri dishes were incubated at room temperature in the dark and examined after 2–3 d using a compound microscope. Hyphal tips were cut from the edges of growing colonies and subcultured onto clarified V8 juice agar (V8A, Campbell's V8 juice 340 ml, 5 g $CaCO_3$, 15 g agar and 900 ml distilled water) and CMA for further study and storage. Isolates could be divided into four groups based on colony morphology. These corresponded to *P. cinnamomi* or *P. nicotianae* and two unknown groups (unpublished data). Single zoospore cultures for isolates residing in each of these two groups, tentatively treated as *Phytophthora* sp. A, and *Phytophthora* sp. B, were made using the method described by Wang-Ching & Wen-Hsiung (1997). Ten isolates were randomly selected from each of the unknown *Phytophthora* spp. for more detailed study.

All but one of the isolates of *Phytophthora* sp. A were from dying *Eucalyptus smithii* in Sutton plantation after extensive sampling during 2000 and 2001 (unpubl. data). The only exception was an isolate (CMW 19428) from *Acacia decurrens,* which was received by the diagnostic clinic of the Tree Protection Co-operative Programme (http://www.fabinet.up.ac.za/tpcp). Six isolates representing *Phytophthora* sp. B were from diseased *E. bajensis* (CMW19416–21) in provenance/progeny trials at Mid-Illovo and Paulpietersburg in the KwaZulu-Natal Province. Four additional isolates included in this study were from diseased *E. dunnii* (CMW19422–24) and *E. macarthurii* (CMW20393) samples submitted to the diagnostic clinic. Cultures of *P. arecae*, *P. colocasiae*, *P. multivesiculata*, and *P. nicotianae,* included in this study for comparative purposes, were obtained from the Centraalbureau voor Schimmelcultures (CBS; Table 1). In addition, isolates of several *Phytophthora* spp. found in South Africa were also included (Table 1). All isolates used are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) and representative isolates of the new taxa have been deposited with the Centraalbureau voor Schimmelcutlures (CBS), Utrecht, The Netherlands.

### *DNA isolation, PCR reactions, and sequencing*

An agar block derived from a single zoospore culture for each of the 20 isolates representing the two unknown *Phytophthora* spp. was grown in 50 ml of 25 % clarified V8 broth at room temperature for 3–5 d. After harvesting, mycelium was freeze-dried and stored in Eppendorf tubes at room temperature. DNA was extracted using a phenol–chloroform DNA extraction method slightly modified from that described by Al-Samarrai & Schmid (2000). The ITS regions of the rDNA gene repeat for the unknown *Phytophthora* spp. was amplified using the forward ITS 6 (5′GAA GGT GAA GTC TAA CAA GG 3′) and reverse ITS 4 (5′TCC TCC GCT TAT TGA TAT GC 3′) primers (Cooke & Duncan 1997). Amplification of the β-tubulin gene was done using the Oom-Btub-up415 F (5′ CGCATCAACGTG TACTACAA 3′) and Oom-Btub1o1401 R (5′ CGC TTG AAC ATC TCC TGG 3′) universal primers and PCR protocol of Bilodeau *et al.* (2007). The PCR reaction mixture (50 μl) contained DNA template (50–90 ng) 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM of each deoxynucleotide triphosphate, 150 nM of each primer and 1.25 U of Taq polymerase (Fermentas, UAB, Lithuania). The PCR conditions included an initial DNA template denaturation at 96 °C for 2 min, fol'owed by 30 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 30 s, extension 72 °C for 1 min, and final cycle at 72 °C for 10 min. A negative control consisting of all ingredients

**Table 1 – Species and isolates of *Phytophthora* species examined in this study**

| Isolate number | Species | [a]Group | [b]Clade | Host | Location | ITS | β-tubulin |
|---|---|---|---|---|---|---|---|
| CMW21922 | *Phytophthora cactorum* | I | 1a | Apple rootstock | Stellenbosch, W. Cape | DQ988205 | DQ988244 |
| CMW19445 | *P. cactorum* | I | 1a | Apple rootstock | Stellenbosch, W. Cape | DQ988206 | DQ988245 |
| CMW19442 | *P. nicotianae* | II | 1b | *Citrus jambhiri* | Tzaneen, Limpopo | DQ988174 | DQ988213 |
| CMW19443 | *P. nicotianae* | II | 1b | *Acacia mearnsii* | Lions River, KZN | DQ988175 | DQ988214 |
| CMW19444 | *P. nicotianae* | II | 1b | *Eucalyptus smithii* | Hodgsons, KwaZulu-Natal | DQ988176 | DQ988215 |
| CMW19441 | *P. boehmeriae* | II | 9 & 10 | *E. smithii* | Ixopo, KwaZulu-Natal | DQ988207 | DQ988246 |
| CMW19440 | *P. boehmeriae* | II | 9 &10 | *E. smithii* | Howick, KwaZulu-Natal | DQ988208 | DQ988247 |
| CMW19439 | *P. boehmeriae* | II | 9 &10 | *E. smithii* | Ixopo, KwaZulu-Natal | DQ988209 | DQ988248 |
| CBS 305.62 | *P. arecae* | II | 4 | *Areca catechu* | India | DQ988202 | DQ988241 |
| CMW19437 | *P. arecae* | II | 4 | Unknown | Stellenbosch, W. Cape | DQ988203 | DQ988242 |
| CMW19436 | *P. arecae* | II | 4 | Unknown | Stellenbosch, W. Cape | DQ988204 | DQ988243 |
| CMW19425 | *P. alticola* | II | 4 | *E. dunnii* | PaulPetersburg, KZN | DQ988196 | DQ988235 |
| CMW19424 | *P. alticola* | II | 4 | *E. macarthurii* | Midillovo, KwaZulu-Natal | DQ988197 | DQ988236 |
| CMW19423 | *P. alticola* | II | 4 | *E. dunnii* | Paulpetersburg, KZN | DQ988198 | DQ988237 |
| CMW19422 | *P. alticola* | II | 4 | *E. dunnii* | Paulpetersburg, KZN | DQ988199 | DQ988238 |
| CMW19421 | *P. alticola* | II | 4 | *E. dunnii* | Paulpetersburg, KZN | DQ988200 | DQ988239 |
| CMW19420 | *P. alticola* | II | 4 | *E. badjensis* | Midillovo, KwaZulu-Natal | | |
| CMW19419 | *P. alticola* | II | 4 | *E. badjensis* | Paulpetersburg, KZN | DQ988201 | DQ988240 |
| CMW19418 | *P. alticola* | II | 4 | *E. badjensis* | Midillovo, KwaZulu-Natal | | |
| CMW19417 | *P. alticola* | II | 4 | *E. badjensis* | Midillovo, KwaZulu-Natal | | |
| CMW19416 | *P. alticola* | II | 4 | *E. badjensis* | PaulPetersburg, KZN | | |
| CMW20206 | *P. citrophthora* | II | 2 | *Citrus* rootstock | W. Cape | DQ988186 | DQ988225 |
| CMW20204 | *P. citrophthora* | II | 2 | *Citrus* rootstock | W. Cape | DQ988187 | DQ988226 |
| CMW20198 | *P. citrophthora* | II | 2 | *Citrus* rootstock | W. Cape | DQ988188 | DQ988227 |
| CMW19415 | *P. citricola* | III | 2 | *C. limonia* | W. Cape | DQ988183 | DQ988222 |
| CMW19414 | *P. citricola* | III | 2 | *C. limonia* | W. Cape | DQ988185 | DQ988224 |
| CMW19413 | *P. citricola* | III | 2 | *C. limonia* | W. Cape | DQ988184 | DQ988223 |
| CMW19435 | *P. frigida* | IV | 2 | *E. smithii* | Ixopo, KwaZulu-Natal | DQ988177 | DQ988216 |
| CMW19434 | *P. frigida* | IV | 2 | *E. smithii* | Pietermaritzburg, KZN | DQ988178 | DQ988217 |
| CMW19433 | *P. frigida* | IV | 2 | *E. smithii* | Lions River, KZN | DQ988179 | DQ988218 |
| CMW19432 | *P. frigida* | IV | 2 | *E. smithii* | Ixopo, KwaZulu-Natal | DQ988180 | DQ988219 |
| CMW19431 | *P. frigida* | IV | 2 | *E. smithii* | Pietermaritzburg, KZN | DQ988181 | DQ988220 |
| CMW20311 | *P. frigida* (ex-type) | IV | 2 | *E. smithii* | Ixopo, KwaZulu-Natal | DQ988182 | DQ988221 |
| CMW19429 | *P. frigida* | IV | 2 | *E. smithii* | Ixopo, KwaZulu-Natal | | |
| CMW19428 | *P. frigida* | IV | 2 | *Acacia decurrens* | Seven Oaks, KZN | | |
| CMW19427 | *P. frigida* | IV | 2 | *E. smithii* | Bloemendal, KZN | | |
| CMW19426 | *P. frigida* | IV | 2 | *E. smithii* | Shafton, KwaZulu-Natal | | |
| CBS545.96 | *P. multivesiculata* | IV | 2 | *Cymbidium sp* | Netherlands | DQ988192 | DQ988231 |
| CBS 955.87 | *P. colocasiae* | IV | 2 | *Colocasia esculenta* | India | | |
| CMW22018 | *P. colocasiae* | IV | 2 | *Protea sp* | W. Cape | DQ988191 | DQ988230 |
| CMW20195 | *P. colocasiae* | IV | 2 | *Protea sp* | W. Cape | DQ988190 | DQ988229 |
| CMW19410 | *P. cryptogea* | VI | 8a | *Vitis vinifera* | W. Cape | DQ988194 | DQ988233 |
| CMW19411 | *P. cryptogea* | VI | 8a | *Vitis vinifera* | W. Cape | DQ988193 | DQ988232 |
| CMW20393 | *P. cryptogea* | VI | 8a | *Vitis vinifera* | W. Cape | DQ988195 | DQ988234 |
| CMW19408 | *P. cinnamomi* | VI | 7a | *E. fraxinoides* | Hodgsons, KwaZulu-Natal | DQ988171 | DQ988210 |
| CMW19406 | *P. cinnamomi* | VI | 7a | *E. dunnii* | Ixopo, KwaZulu-Natal | | |
| CMW19405 | *P. cinnamomi* | VI | 7a | *E. dunnii* | Piet Retif, Mpumalanga | DQ988172 | DQ988211 |
| CMW19404 | *P. cinnamomi* | VI | 7a | *E. smithii* | Hodgsons, KwaZulu-Natal | DQ988173 | DQ988212 |
| CMW19403 | *P. cinnamomi* | VI | 7a | *E. elata* | Piet Retif, Mpumalanga | | |

a Groups according to Waterhouse (1963).
b Clades according to Cooke *et al.* (2000); Kroon *et al.* (2004).

excluding template was also included. Amplicons were visualised on 1 % agarose gel stained with ethidium bromide and visualised under UV light. Resulting band size estimates we'e achieved using GeneRuler™ 100 bp DNA ladder (Fermentas). The PCR products were purified using a PCR products purification kit (Roche Molecular Biochemicals, Almeda, CA). They were then sequenced using the forward and reverse primers used in the amplification of the ITS and β-tubulin gene regions. Reactions were performed using the ABI PRISM™ Big dye terminator sequencing reaction kit according to the manufacture's instructions (Perkin-Elmer Applied BioSystems, Foster City, CA). Sequencing was done using an ABI 3100™ automated DNA sequencer.

### *Phylogenetic analysis of the sequence data*

In order to compare the new *Phytophthora* spp. from this study with other closely related species, additional sequences of representative species from Cooke *et al.* (2000) were obtained from GenBank. Phylogenetic analyses were done using MP

methods in PAUP software version 4.0b10 (Swofford 2003) and Bayesian analysis (Ronquist & Heuelsenbeck 2003).

The initial analysis was performed on an ITS dataset alone and subsequent analyses were performed on a combined dataset of ITS and β-tubulin sequence, after a partition homogeneity test (PHT) had been performed in PAUP to determine whether sequence data from the two separate gene regions were statistically congruent (Farris *et al.* 1994; Huelsenbeck *et al.* 1996). The most parsimonious trees were obtained using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option, off. Max-trees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis & Huelsenbeck 1992). Characters were unweighted and unordered, branch and branch node supports were determined using 1 K BS replicates (Felsenstein 1985).

Bayesian analysis was conducted on the same aligned combined dataset. First Mr Modeltest v2.2 (Nylander 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes 3.1 applying a general time reversible (GTR) substitution model with a gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. The MCMC analysis of four chains started from random tree topology and lasted 2 M generations. Trees were saved, each resulting in 1 K trees. Burn-in was set at 200 K generations after which the likelihood values were stationary, leaving 950 trees from which the consensus trees and PPs were calculated. PAUP 4.0b10 was used to construct the consensus tree and maximum PPs assigned to branches after 50 % majority rule consensus tree was constructed from the 950 sampled trees.

### *Morphological, cultural, and physiological characteristics*

Starter cultures (five per taxon examined) were grown on V8A incubated at room temperature for 5 d. A 4 mm cork borer was used to cut agar discs from the colony edges and these were placed at the centre of clarified V8 juice agar (Erwin & Riberio 1996), carrot agar (CA) (Erwin & Riberio 1996), CMA, potato dextrose agar (Difco; 24 $gl^{-1}$), and malt extract agar (MEA; 20 $gl^{-1}$ Biolab, Johannesburg). Two lines intersecting at right angles at the centre of a 90 mm Petri dish were drawn on the outside of Petri dishes as reference growth points for each isolate. Five isolates of each taxon were transferred to the different media in triplicate and incubated at temperatures ranging from 5–35 °C, at 5 °C intervals. The colony diameters were measured daily with electronic digital callipers until the colonies reached the edges of the Petri dishes.

Sporulation on solid media was induced by adding Petri's mineral salt solution to cultures growing on V8A or CMA (Ribeiro 1978). Sporangial production and release of zoospores into liquid media was achieved using a modified mycelial matt method (Chen & Zentmyer 1970). Agar blocks bearing mycelium were cut from the edges of colonies and transferred into 20 ml clarified V8 juice broth and incubated at room temperature for 4 d. Mycelial mats were harvested, rinsed twice with sterile distilled water and Petri's mineral salt solution was added. Sporangia were produced after 2–3 d incubation in the dark and zoospore release was achieved by chilling the Petri dishes at 10 °C for 30 min and then returning them to 25 °C.

Sexual structures of *Phytophthora* sp. B were induced by growing ten test isolates on 10 % V8 agar and MEA at room temperature for 10–15 d. Single zoospore isolates produced oospores independently without crossing and were thus considered homothallic. Isolates of *Phytophthora* sp. A did not produce oospores independently, and thus, were tested for their ability to cross using the method outlined in Erwin & Riberio (1996). Matings were performed by pairing all ten *Phytophthora* sp. A isolates with known A1 and A2 (CMW21989, CMW21993) strains of *P. nicotianae* on 65 mm Petri dishes containing CA. In order to determine which isolates produced the oogonia in compatible pairings, a sterile polycarbonate membrane (47 mm diam, 0.2 μm pore size; Millipore) was used as described by Ko (1978).

A light microscope was used to examine the reproductive structures and to compare the morphology of isolates with the aid of the revised tabular key of Stamps *et al.* (1990). For detailed microscopic examinations and measurements, sporulating mycelium was mounted on glass slides in lactophenol. Measurements of 50 randomly selected sporangia, oogonia, antheridia, chlamydospores, and hyphae were made for a single representative isolate of each of the two unknown *Phytophthora* spp. In addition, 20 of the above-mentioned structures were measured for each of the remaining nine isolates of each species.

The mean ranges and confidence limits for all taxonomically relevant structures were recorded and are presented as (min–) (0.95 lower conf limit– 0.95 upper conf limit) (–max). Photographs captured with a HRc Axiocam (Carl Zeiss, München) digital camera and complementary Axiovision® 3.1 software were used to measure all morphological characters.

Petri dishes containing CMA and amended with different concentrations of hymexazol were prepared to give final concentrations between 10 and 50 $\mu gml^{-1}$ at 10 $\mu gml^{-1}$ increments. A similar set of Petri dishes was amended with malachite green (125 $\mu gml^{-1}$). Small (5 mm diam) agar discs bearing mycelium of each of the ten test isolates of each unknown species were placed on the surface of the amended agar and incubated at 20 °C in the dark for 5 d (Shepherd 1976; Kennedy & Duncan 1995). Three replicate Petri dishes for each of the ten isolates were used and the sensitivity of the isolates to the test compounds was expressed as percentage growth rate *versus* that of isolates on control Petri dishes that were free of the test compounds.

Ten isolates of each of the unknown *Phytophthora* spp. were tested for their ability to utilise nitrate as a sole nitrogen source. Agar discs (5 mm diam) were placed on the surface of agar growth media containing L-asparagine (P3) and nitrate (P4) as described by Hohl (1975). After incubation for 5 d at 20 °C, the colony growth was measured and expressed as increase in colony diameter in millimetres per day (mm $d^{-1}$). Pigment production of the test isolates was assessed on casein hydrolysate tyrosine (CHT) agar (Shepherd 1976). Petri dishes were incubated at 20 °C the dark for 15–20 d. The resulting extent of pigmentation was compared with control cultures grown on casein hydrolysate agar (casein hydrolysate broth 29.33 $gl^{-1}$, Sigma-Aldrich, St. Louis, Biolab, JHB agar 15 $gl^{-1}$).

Petri dishes were examined on a light box and scored as having no (0), slight (1), moderate (2) or abundant (3) pigment (Shepherd 1976).

### *Pathogenicity tests*

Ten isolates of each of the unknown *Phytophthora* spp. and five isolates of *P. cinnamomi* were used to inoculate one-year-old *Eucalyptus dunnii* trees in the field (Table 1). The pathogenicity trial was located in a commercial stand of trees at Sutton plantation, near Ixopo in the KwaZulu-Natal Province, South Africa. *E. dunnii* trees were inoculated using a 9 mm diam cork borer to remove the bark from each tree at breast height in March and November 2002. An agar plug removed from a one-week-old PDA culture of each of the test isolates, was inserted into the wound and sealed with masking tape to reduce desiccation. Controls were included by inoculating trees with sterile PDA plugs. Ten trees were inoculated for each of the 25 test isolates and five trees were used as controls. A completely randomised block design was used for the inoculations and the entire trial was repeated in November during the summer season. Lesion lengths on the inner bark of trees were measured six weeks after inoculation. Lesion lengths were compared and analysed using one way analysis of variance (ANOVA), and the inoculations tests were also compared with each other using ANOVA and the STATISTICA (version 6), data analysis software.

## Results

### *DNA amplification and sequence data analysis*

The PCR product of the ITS-rDNA regions yielded a single band of approximately 900 bp for all the undescribed *Phytophthora* isolates used in this study. The aligned dataset consisted of 885 characters of which 381 were parsimony informative. These data contained significant phylogenetic signal ($P < 0.01$; $gl = -0.627$) to allow for meaningful analysis. Initial heuristic searches of unweighted characters in PAUP resulted in two most parsimonious trees of 900 steps ($CI = 0.627$, $RI = 0.90$). The two new *Phytophthora* species formed well-supported terminal clades (Fig 1, TreeBASE = SN3042). *Phytophthora* sp. A grouped together with an undescribed *Phytophthora* sp. associated with *Rosaceae* hosts (GenBank AF408625) of Abad *et al.* (2001) (Fig 1). *Phytophthora* sp. B isolates grouped together with *P. arecae*, *Peronophythora litchii*, *Phytophthora megakarya*, and *Peronospora sparsa* (Fig 1). However, *Phytophthora* sp. B isolates were closest to an isolate of another undescribed species from the US oak forests namely, *Phytophthora* sp. MD92, GenBank DQ313223).

PCR amplification of the β tubulin regions yielded a single band (*ca* 900 bp) for *Phytophthora* isolates examined in this study. The aligned dataset for the combined ITS and β -tubulin sequences consisted of 1689 characters, of which 318 were parsimony informative and were included in analysis. The partition homogeneity test showed no significant difference ($P = 0.91$) between the data from the different gene regions (sum of lengths of original partition was 778, range for 1000 randomisations was 771–779).

*Phytophthora* sp. A and *Phytophthora* sp. B also formed highly supported terminal clades in the combined ITS and β-tubulin tree (not shown; Tree BASE = SN3042). However, they grouped in an unresolved clade, but remained within their respective sub-groups with closely related species as the ITS tree. Based on sequence data comparisons for the combined ITS-rDNA and β-tubulin gene regions, isolates of *Phytophthora* sp. A and *Phytophthora* sp. B from cold-tolerant *Eucalyptus* spp. represent undescribed taxa. Descriptions for these new species are given below.

## Taxonomy

**Phytophthora frigida** Maseko, Coutinho & M.J Wingf., **sp. nov** (Figs 2–4).

MycoBank no.: MB511178

*Etym.*: 'frigida' refers to the fact that this species is cold tolerant.

*Phytophthora frigida* sp. nov. crescit stellater vel rosaceiter in mediis plurimis, et potest in frigidis crescere (ita nomen), heterothalla, sporangiis ovoideo-obpyriformibus papillatis. Inter Phytophthoris aeriis typica est, sporangiis caducis et chlamydosporis permultis.

*Typus*: **Republic of South Africa**: *Natal Province*: Sutton plantation, Ixopo, *Eucalyptus smithii*, Feb. 2001, *B.O.Z Maseko*, (PREM 59222 — **holotypus**. dried culture with asexual structures on CMA with a corresponding microscope slide; ex-type culture CMW20311; **paratypes** PREM, (59218, 59218, 59220, 59221), dried cultures with asexual structures on CMA with matching microscope slides; ex-paratype cultures CMW 19426 CMW 19428, CMW 19433, CMW 19435.)

*Primary hyphae* coralloid, irregular, and sympodially branched, fairly uniform in width measuring up to 5 μm, (3.5–) 4–5 μm (mean 4.5 μm) (Fig 2A–B). *Hyphal swellings* globose and intercalary (Fig 2C–D). *Chlamydospores* terminal, globose, (20–) 24–26 (–35) diam, (mean 25 μm), thin or thick-walled and brown (Figs 2E–F, 3D–F). *Sporangiophores* thin branches, arising near or directly from hyphal swellings. Sporangia terminal or sometimes intercalary, readily produced in solid or liquid media, conspicuously papillate, exit pore (3–) 5–6 (7–) μm, (mean 5 μm), ovoid, obpyriform or irregular shaped, ($l \times b$) (24–) 31–34 (–40) × (20–) 26–28 (–33) (mean 33 × 27 μm), caducous with short pedicels (Fig 4A–L). *Oogonia* produced only in dual cultures, terminal, spherical with smooth walls, often thicker and golden brown with age and mostly (25–) 31–34 (−42) (mean 33 μm) diam (Fig 3A–C). *Oospores* aplerotic (19–) 26–30 (−38) μm diam (mean 28 μm), wall 1.5–2 (−3) μm thick, often light yellow or colourless. *Antheridia* amphigynous (95 %), elongated, cylindrical or spherical to ellipsoidal (Fig 3B).

*Cultural characterisitics: Phytophthora frigida* produces stellate to rosaceous colony types (5 d at 20 °C in darkness) on V8A, CA, MEA, CMA, and PDA (10 d at 20 °C in darkness). Cottony colonies with irregular growth patterns are produced on V8A, CA, MEA, and PDA. Submerged colonies with only sparse aerial mycelium were produced on CMA (Fig 5). Primary hyphae corraloid measuring 5 μm in width (3.5–) 4–5 μm (mean 4.5; Fig 2). Hyphal swellings intercalary and globose in solid and water media. Sporangiophores branched in sympodia arising near or directly from hyphal swellings. The cardinal temperatures for *P. frigida* isolates examined were 10 °C

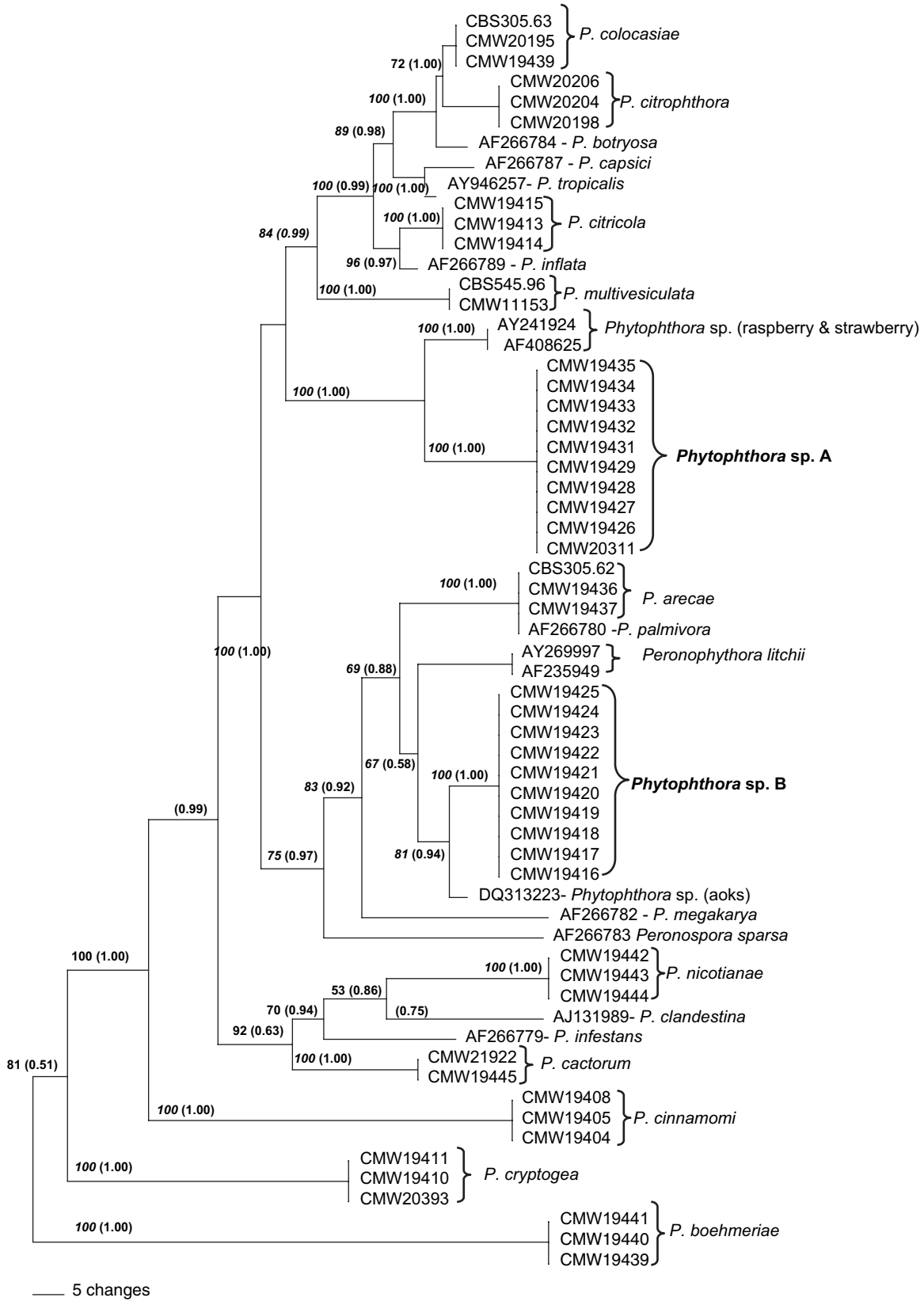


**Fig 1 – One of two most parsimonious phylogenetic trees of 900 steps obtained from analysis of ITS sequence data. Branch support (BS value) is given above the branches and PP from the Bayesian analysis in brackets.**

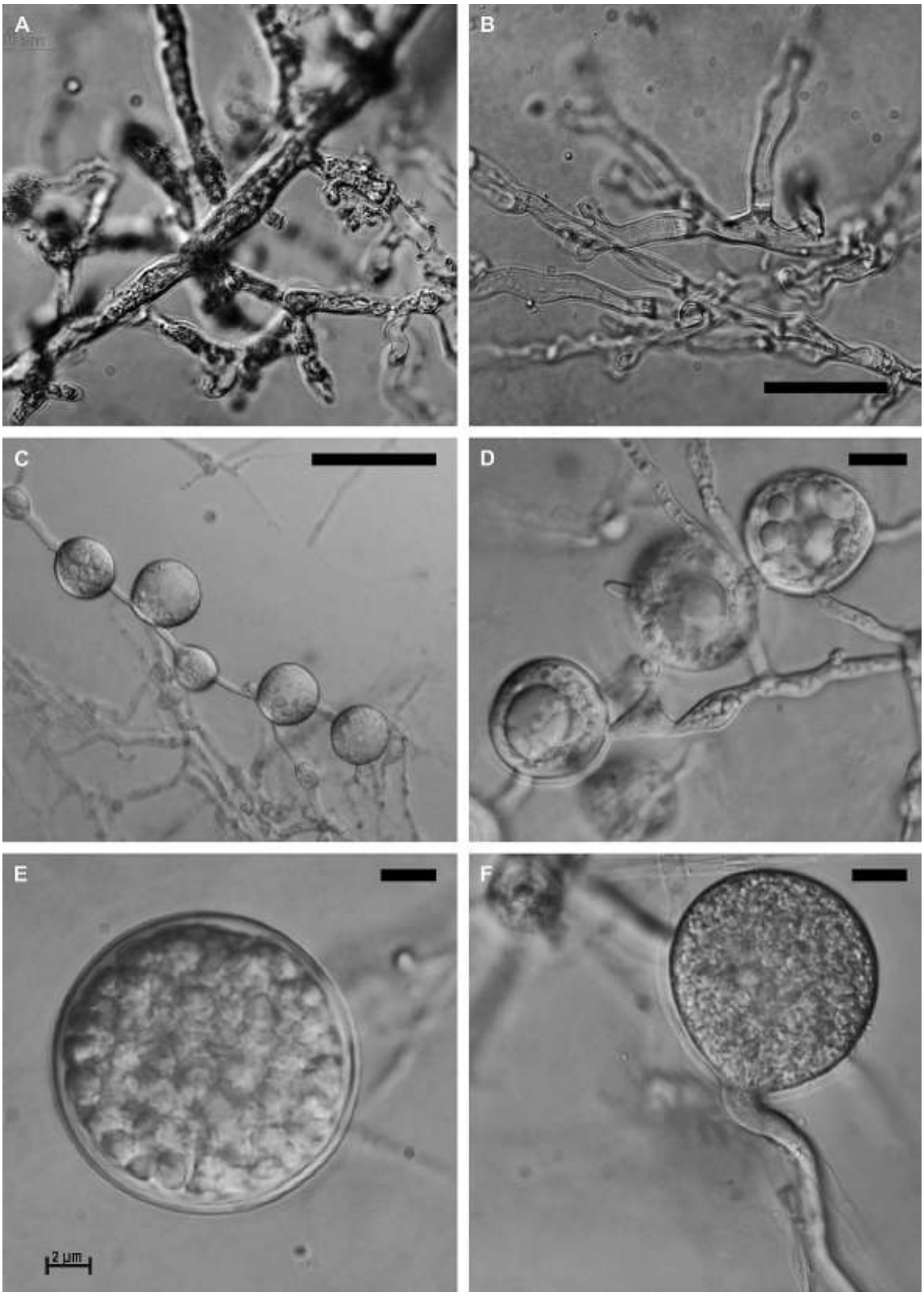


**Fig 2 – Primary hyphae and chlamydospores of *Phytophthora frigida*: (A–B) Coralloid mycelia with sympodial branching on solid media. (C–D) Intercalary globose hyphal swellings. (E) Thin-walled chlamydospores. (F) Germinating sporangium. Bars = 10 µm, except (E) = 2 µm.**

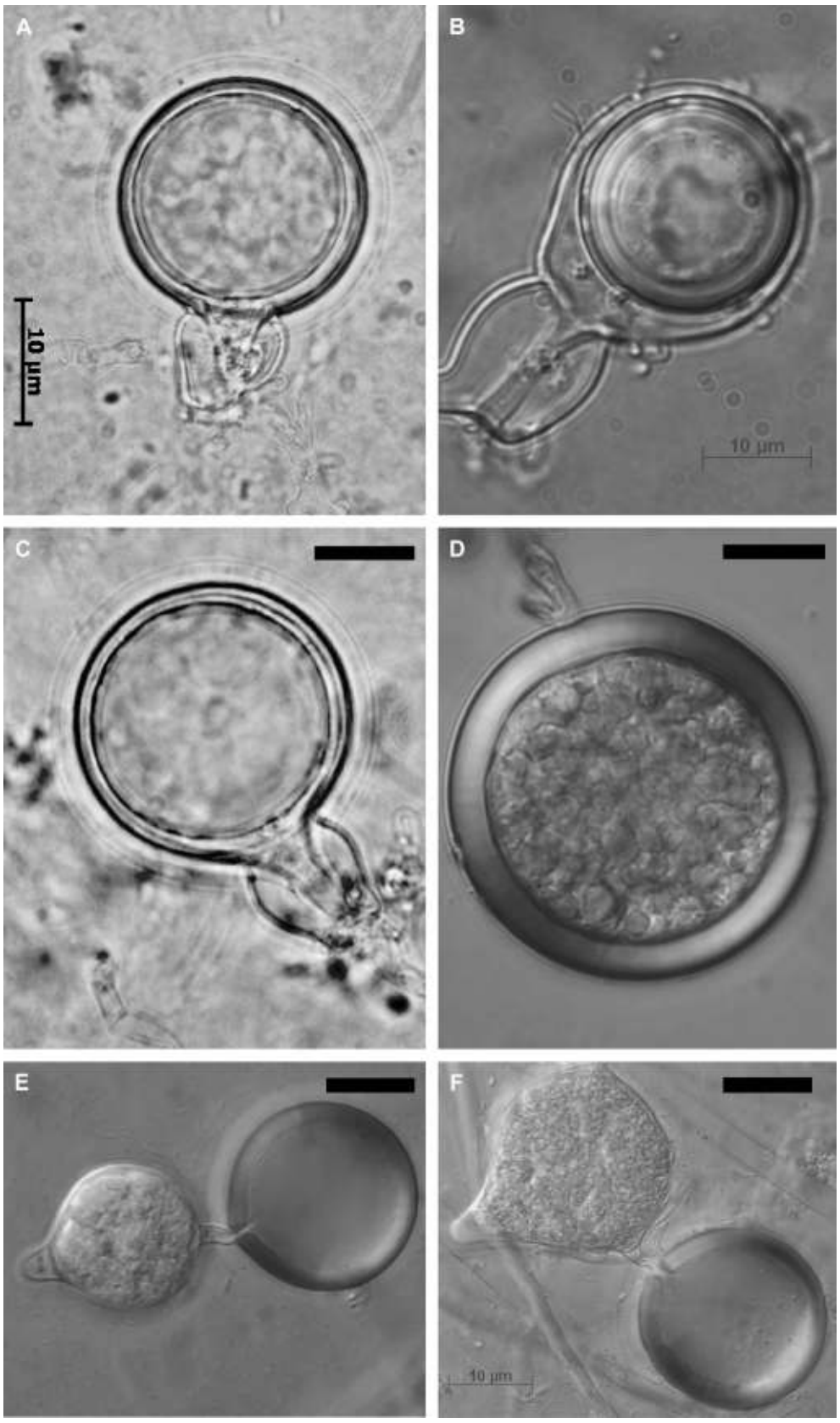


**Fig 3 – (A–C) Oogonia and oospores of *Phytophthora frigida* showing amphigynous antheridial attachment. (C–D) Thick-walled chlamydospores produced abundantly on liquid MEA. (E–F) Sporangium release from chlamydospore (rarely) observed. Bars = 10 µm.**

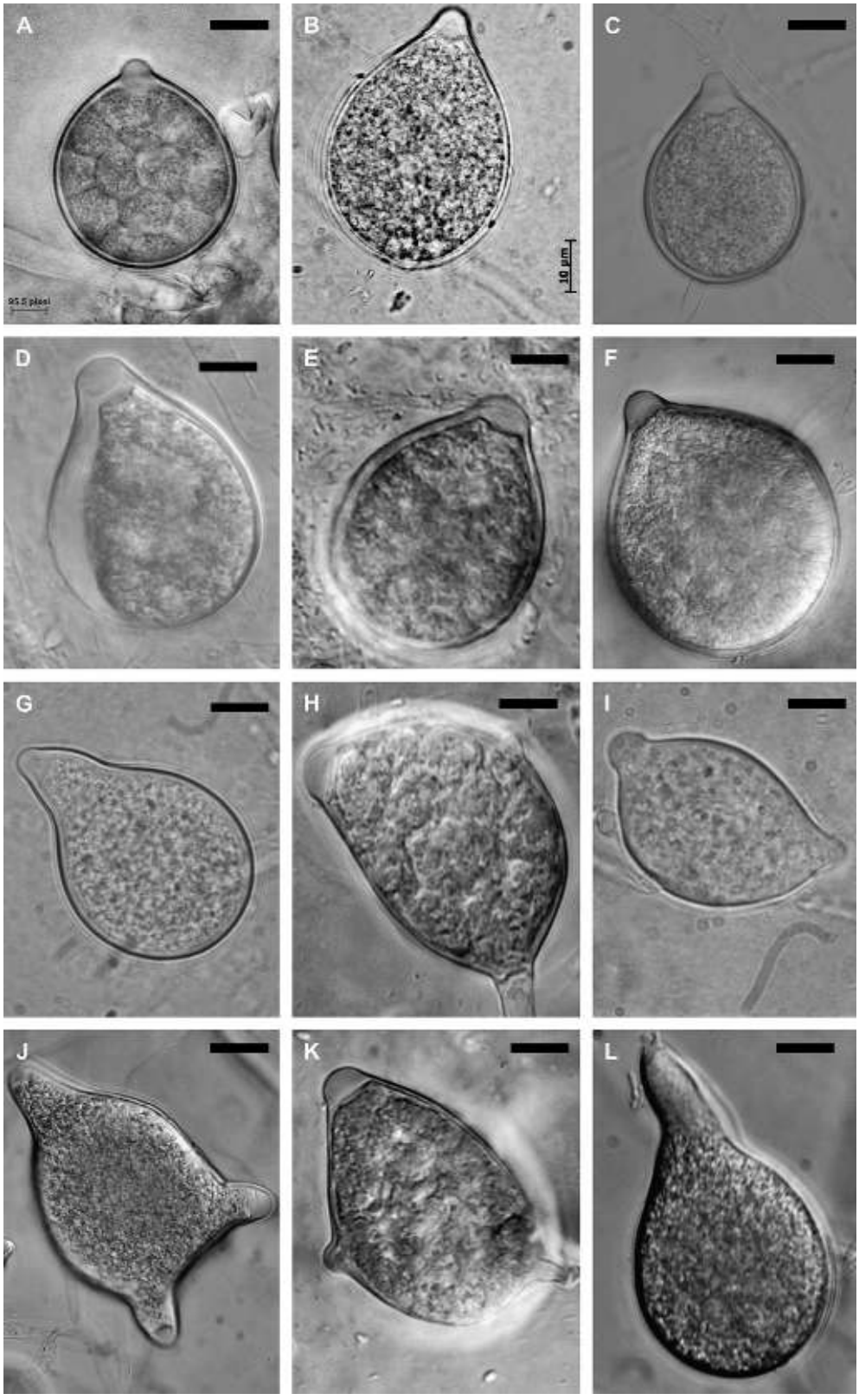


**Fig 4 – Sporangia of *Phytophthora frigida*. (A–F) Papillate and semi-papillate, ovoid-obpyriform, lateral attached and caducous sporangia. (G–L) Various distorted shapes observed in liquid media. (H) Papillate sporangium with conspicuous basal plug. (J, L) sporangia with elongated necks sometimes with three apices. Bars = 10 µm.**

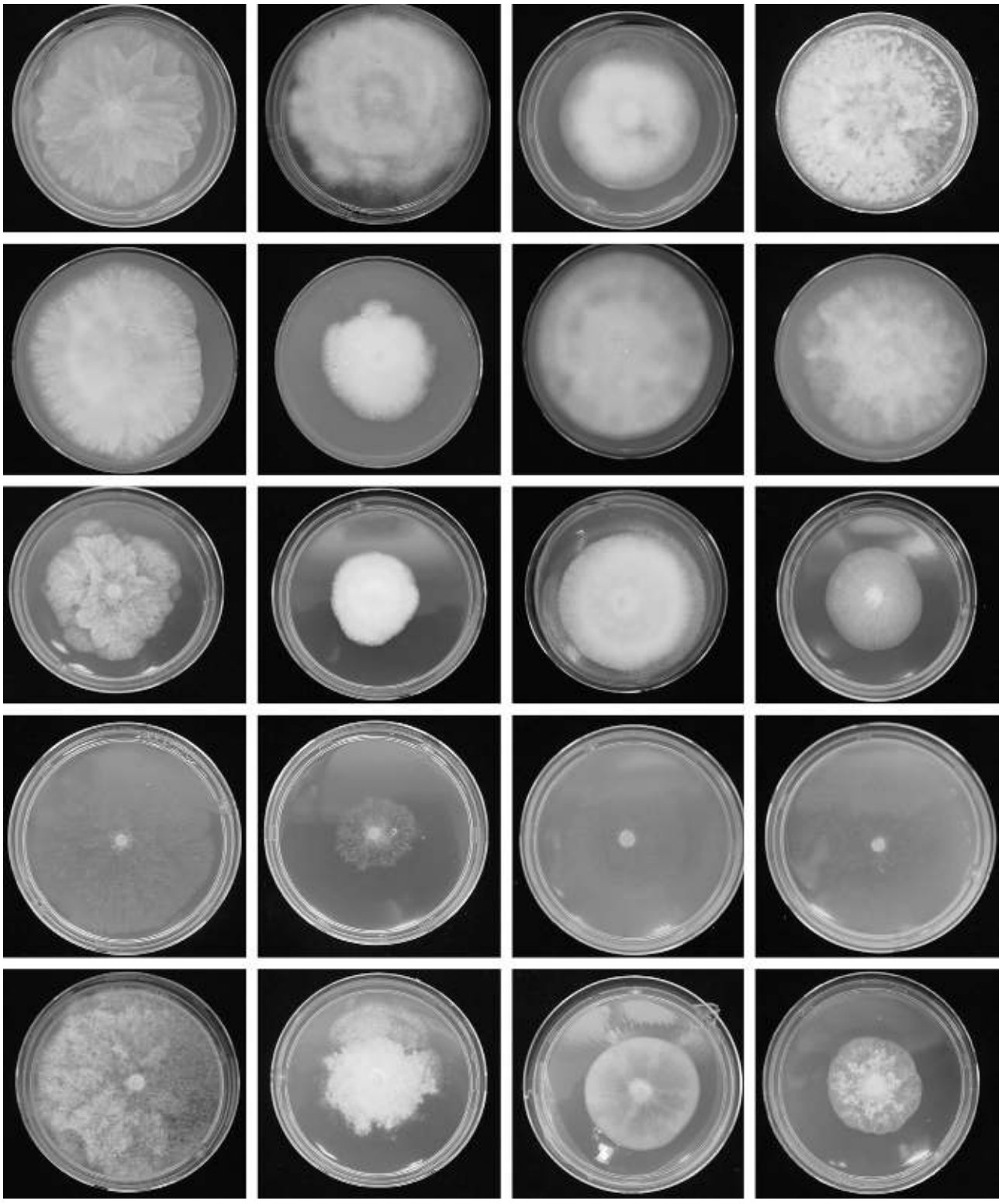

**Fig 5 – Colony types of *Phytophthora* species grown on V8A, CA, MEA, CMA after 5 d at 20 °C and PDA after 10 d. *P. frigida* (column 1; left, top to bottom), *P. multivesiculata* (column 2), *P. alticola* (column 3), *P. arecae* (column 4).**

(mean 2 mm $d^{-1}$) and 30 °C (mean 6 mm $d^{-1}$). None of the *P. frigida* isolates grew at the lowest (5 °C) or highest (35 °C) temperatures. The mean growth rates on five test media at 20 °C for all ten *P. frigida* isolates are presented in Table 2. The growth temperature relationships of *P. frigida* on V8 and CA are illustrated in Fig 6. All *P. frigida* isolates examined in this study were able to utilise nitrate as sole nitrogen source. Isolates produced a black pigment on CHT agar within two

**Table 2 – A comparison of morphological characteristics of *Phytophthora* isolates**

| Character | *Phytophthora* ***frigida*** **sp. nov.** | *Phytophthora* ***multivesiculata*** | *Phytophthora* ***alticola*** **sp. nov.** | *Phytophthora* ***arecae*** |
|---|---|---|---|---|
| No. of isolates | 10 | 2 | 10 | 3 |
| **Main hyphae** | Coralloid | Coiled | Smooth | Smooth |
| Hyphal swellings | Spherical | Catenulate | Irregular | Absent |
| Mean width (μm) | 4.5 | 6 | 5 | 4 |
| **Sporangia** | Papillate | Semi-papillate | Papillate | Papillate |
| Range (μm) | 24–40 × 20–33 | 30–60 × 20–41 | (30–45) × (20–35) | 35–60 × 25–35 |
| Length:breadth ratio (mean) | <1.6 | <1.6 | <1.6 | <1.6 |
| Shapes observed | Ovoid-obpyriform | Ovoid, obpyriform | Ovoid, ellipsoidal obturbinate | Ellipsoidal to obturbinate |
| Distorted shapes | + | + | | + |
| Caducity | Caducous | Caducous | Caduceus | Caducous |
| Pedicel (μm) | Short (<5 μm) | Short (<5 μm) | Short (<5 μm) | Short (<5 μm) |
| Sporangiophores | Simple sympodium | Simple and twisted sympodium | Simple or branched sympodium | Simple or branched sympodium |
| Exit pore (μm) | Broad (5–10) | Broad (8–12) | Narrow (<7 μm) | Narrow (<7 μm) |
| **Chlamydospores** | Abundant | In some isolates only | In some isolates only | Absent |
| Mean diam (μm) | <35 μm | | <35 μm | |
| **Oogonia** | | | | |
| Mean diam (μm) | 38 μm | 41 μm | 28 | 30 |
| Range diam (μm) | 24–48 | 28–50 | 20–35 | 23–44 |
| **Oospores** | | | | |
| Mean diam (μm) | 33 | 40 | 30 | 30 |
| Range (μm) | 25–42 | 28–46 | 24–36 | 25–40 |
| **Antheridia** | Amphigynous | Amphigynous | Amphigynous | Amphigynous |
| Sex | Heterothallic | Homothallic | Homothallic | Heterothallic |
| Growth media | Colony and cultural characteristics | | | |
| V8A (a) | Stellate-petaloid | Smooth | Smooth | Smooth |
| (b) | Moderately fluffy | Cottony | Fluffy | Fluffy |
| CA (a) | Stellate-petaloid | Smooth | Smooth | Smooth |
| (b) | Moderately fluffy | Cottony | Fluffy | Fluffy |
| MEA (a) | Stellate-Petaloid | Smooth | Smooth | Smooth |
| (b) | Moderately fluffy | Cottony | Fluffy | Fluffy |
| CMA (a) | Slight stellate | Smooth | Smooth | Smooth |
| (b) | Appressed | Appressed | Appressed | Appressed |
| PDA (a) | Stellate-petaloid | Smooth | Smooth | Smooth |
| (b) | Moderately fluffy | Cottony | Fluffy | Fluffy |
| | Mean growth rate $mmd^{-1}$ at 20 °C | | | |
| V8A | 5 | 5.8 | 3 | 6.2 |
| CA | 4.8 | 5 | 3.8 | 5.3 |
| CMA | 4.6 | 5.2 | 2.4 | 5.5 |
| MEA | 4 | 4.5 | 2 | 5 |
| PDA | 3 | 2.6 | 1.5 | 4.2 |
| P3 (asparagine) | 1.5 | 2.5 | 1 | 3 |
| P4 (nitrate) | 0.6 | 1.5 | 0.2 | 2 |
| Sensitivity malachite green | No growth | No growth | No growth | No growth |
| Percentage growth rate on CMA amended with different concentrations of hymexazol, isolate ranges in brackets | | | | |
| 10 $\mu g ml^{-1}$ | 92 (90–100) | 95 (90–100) | 80 (90–100) | 85 (90–100) |
| 50 $\mu g ml^{-1}$ | 70 (60–90) | 75 (45–96) | 55 (51–65) | 60 (40–65) |
| CHT Agar | Abundant (3) | Moderate (2) | None (0) | slight (1) |

V8A, V8 juice agar; CA, carrot agar; CMA, corn meal agar; MEA malt-extract agar; PDA, potato dextrose agar; P3, agar growth media containing L-asparagine (P3); P4, agar growth media containing nitrate (P4); CHT agar, casein hydrolysate tyrosine.

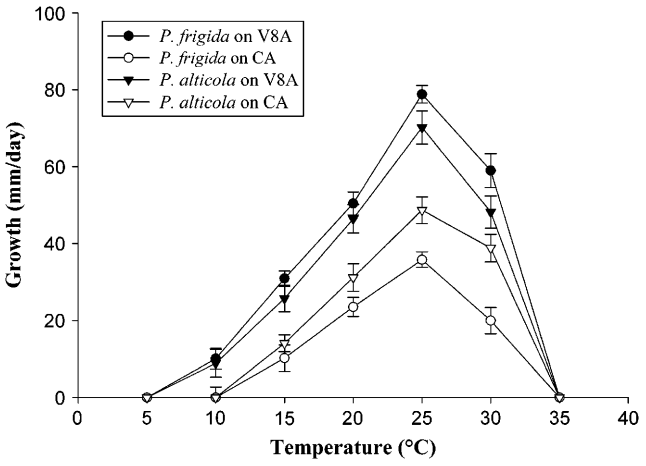


**Fig 6 – Growth–temperature graph of *Phytophthora frigda* and *P. alticola* on V8A and CA at temperatures ranging from 5–35 °C for 10 d.**

to three weeks. None of *P. frigida* isolates grew on a medium containing malachite green. All isolates examined in this study were tolerant to hymexazol (Table 2).

*Asexual structures:* Sporangia readily produced in solid and liquid media, caducous with short pedicels, terminal, and intercalary sporangia present. Sporangiophores irregular branched and with lax sympodia. Sporangia papillate often with various distorted shapes including bipapillate, elongated necks with three apices (observed in some isolates), conspicuous basal plugs, distinctly curved apices and lateral displacement of the papilla (Fig 4). Sporangia primarily ovoid-obpyriform, however, irregular shaped sporangia with variable shapes and sizes observed in liquid media. The size range of sporangia ($l \times b$) (24–)31–34(–40)×(20–)26–28(–33) (mean 33 × 27 μm). The exit pores range between (3–)5–6(7–) μm (mean 5 μm).

*Chlamydospores:* Numerous thin-walled chlamydospores are produced in liquid and on solid media. Round thin-walled chlamydospores are produced terminally with diameters ranging between (20–)24–26(–35) mean 25 μm. Characteristic thick-walled chlamydospores, producing sporangia were observed on solid media. These thick-walled chlamydospores could easily be confused with oogonia (Fig 3D–F). However, they did not have antheridia. Occasionally, direct sporangial germination from thick-walled chlamydospores observed (Fig 3E–F). The *P. multivesiculata* isolates examined did not produce chlamydospores as readily as *P. frigida,* although some isolates produced chlamydospores after long storage.

*Sexual structures:* Oogonia were produced only through pairing of opposite mating isolates, suggesting that the species is heterothallic. Isolates produced terminal oogonia, with spherical and smooth walls with diameters ranging between (25–)31–34(–42) (mean 33 μm). Oospores had thick inner walls and were aplerotic with diameters ranging between (19–)26–30(–38) μm, with mean of 28 μm. Antheridia were elongated, cylindrical and amphigynous (95 %) and spherical to ellipsoidal in shape (Fig 3A–C).

There are many key features that distinguish *P. frigida* from *P. multivesiculata* that is most closely related to it. In terms of mating behaviour, *P. frigida* is heterothallic whereas *P. multivesiculata* is homothallic. *P. frigida* has papillate sporangia rather than the semi-papillate sporangia and *P. frigida* has corraloid hyphae rather than coiled hyphae and large spherical hyphal swellings rather than catenulate hyphal swellings found in *P. multivesiculata*.

***Phytophthora alticola*** Maseko, Coutinho & M.J Wingf., **sp nov.** (Figs 8–9)
MycoBank no.: MB511177

*Etym*: Latin. The name refers to the fact that this fungus was first reported from high altitude sites.

*Phytophthora alticola* sp. nov. crescit lente sine ordinatione proprio incrementi; phylogenetice *P. arecae* persimilis sed homothalla, sporangiis ovoideis papillatis vel bipapillatis, saepe forma distorta, differt.

*Typus*: **Republic of South Africa**: *Natal Province:* Mid-illovo provenance/progeny trials, Richmond *Eucalyptus badjensis*, Mar. 2002, B.O.Z Maseko, (PREM 59215 — holotypus; dried culture with asexual and sexual structures on CMA with a corresponding microscope slide; ex-type culture CMW 19417; paratypes PREM 59214, PREM 59216, PREM 59217), dried cultures with asexual structures on CMA with matching microscopes slides, ex-paratype cultures CMW 19416, CMW 19424, CMW 19425.)

Primary hyphae (5–)4–6 μm (mean 5 μm) wide. Sporangia papillate, occasionally bipapillate, variable size and shape. Other sporangial shapes include ovoid, globose, obturbinate, limoniform and various distorted shapes. Terminal sporangia, caducous, short pedicel, conspicuous basal plugs. Sporangia (30–)33–36(45–)×(20–)26–29(35–) (mean 36 × 28 μm), length: breadth ratio range, 1:2 and 1:4 (mean 1.4). Exit pores (4–) 5–7 (8–) μm (mean 6 μm) diam. Chlamydospores, rarely produced, terminal and spherical, shape, between 20 and 35 μm (mean 28 μm; Fig 7). Oospores produced in single cultures, with thick inner walls, markedly aplerotic, diameters between 24–36 μm, with a mean of 28.3 × 30.5 μm. Antheridia mainly amphigynous, paragynous antheridia also present.

*Cultural characteristics: Phytophthora alticola* has smooth colonies with no distinctive growth pattern on V8A, CA, MEA, CMA, or PDA after 5 d incubation at 20 °C. Mycelium dome-shaped and fluffy with scant to moderate aerial mycelium on V8A, CMA, and MEA. However, colonies tend to be appressed with thinly spread aerial mycelium on CMA (Fig 5). The optimum growth temperature on V8 agar for the ten *P. alticola* isolates examined was 25–30 °C. The cardinal temperatures for *P. alticola* isolates examined were 15 °C (mean 1 mm $d^{-1}$) and 30 °C (mean 4 mm $d^{-1}$). None of the isolates examined grew at low temperatures (below 10 °C) or at high temperature (above 30 °C). *P. alticola* isolates examined grew slowly on all growth media tested at 20 °C. The mean growth rates for the isolates are listed in Table 2 and the growth temperature relationship on V8A and CA in Fig 6. All of the isolates examined were able to utilise nitrate as sole nitrogen source and did not produce pigment on CHT agar. None of the isolates were able to grow on malachite green media. All *P. alticola* isolates were sensitive hymexazol (Table 2).

*Primary hyphae* in *P. alticola* were smooth, with irregular hyphal swellings, in liquid media (5–)4–6 μm (mean 5 μm) wide. Sympodially branched hyphae and irregular hyphal swellings

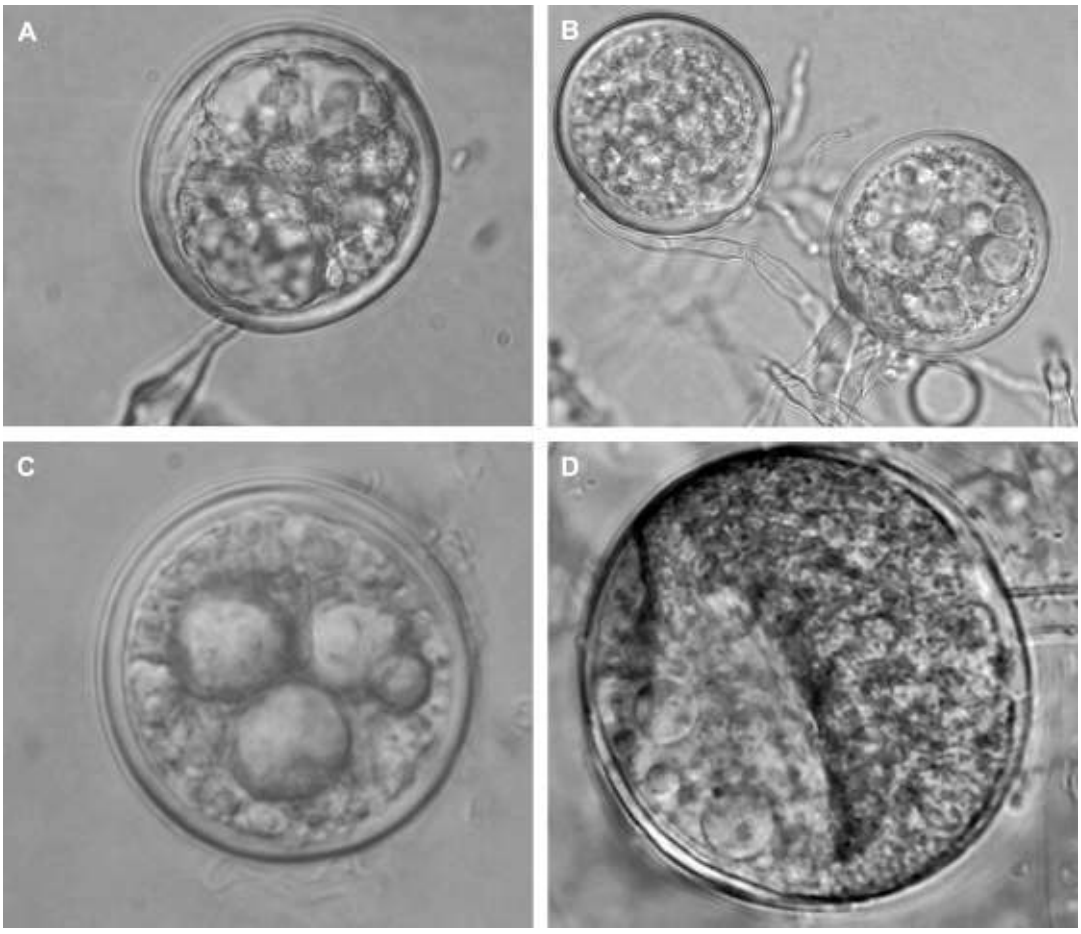


**Fig 7 – Chlamydospores of *Phytophthora alticola* on V8 Agar. (A–D) Terminal chylamydospores, large and spherical. Bars = 10 μm.**

were present. *P. arecae*, which is closely related to *P. alticola*, did not produce distinctive growth patterns but in that species, colonies were cottony, slightly radial and with abundant aerial mycelia on V8A, CA, MEA, PDA, and appressed colonies on CMA. *P. arecae* hyphae were smooth with no hyphal swelling and measured (3–)3.5–4.5 μm (mean 4 μm). There was a marked variation between isolates examined and the two species could not be readily distinguished from each other based on growth patterns in culture.

*Asexual structures:* All isolates of *P. alticola* examined produced sporangia on agar as well as in liquid media. Sporangia were conspicuously papillate and occasionally bipapillate (Fig 8), with variable sizes and shapes including ovoid, obpyriform, and various distorted shapes. Sporangia were terminal, caducous with short pedicels (Fig 8E). Sporangia were (30–)33–36 (−45) × (20–) 26–29 (−35) (mean 36 × 28 μm) in size and had a length:breadth ratio ranging between 1:2 and 1:4 (mean 1.4). The mean zoospores exit pore width was 6 μm. Spherical and terminal chlamydospores were produced in some isolates and their diameter range between (22–) 25–45 (mean 35 μm). The shapes of the sporangia of the closely related *P. arecae* vary from ovoid, obturbinate, elongated and ellipsoidal, and measured (35–) 40–44 (−60) × (25–) 28–30 (−35) (mean 42 × 30 μm) with mean length:breadth ratio (1:3–) 1.4 (−1.6). Round and terminal chlamydospores produced in older cultures and their diameter measuring (14–) 19–40 mean (30 μm) for *P. arecae*.

*Sexual structures:* All isolates of *P. alticola* examined in this study were homothallic. Oogonia formed readily formed in solid and liquid media. Oogonia were terminal with tapered stalks and were smooth-walled with diameters ranging between (24–) 26–28 (−31) (mean 26 μm). Oospores had thick inner walls with diameters ranging between (14–) 20–22 (mean 22 μm). Antheridia were predominantly amphigynous but paragynous antheridia were also observed in some isolates. Antheridia had a tendency to detach from the oogonia as illustrated in Fig 9. The obvious distinguishing feature between *P. alticola* and *P. arecae* is that the latter species is heterothallic.

### *Distribution and ecology*

*Phytophthora frigida* was first isolated from diseased plant material and rhizosphere soil samples taken around declining *Eucalyptus smithii* trees at Sutton during the spring of 1999. Since then, *P. frigida* has been associated with root and collar rot disease of *E. dunnii*, *E. smithii*, *Acacia mearnsii* and *A. decurrens* in several forest plantations. Although, *P. frigida*, is well adapted for wind or splash dispersal, it has not been

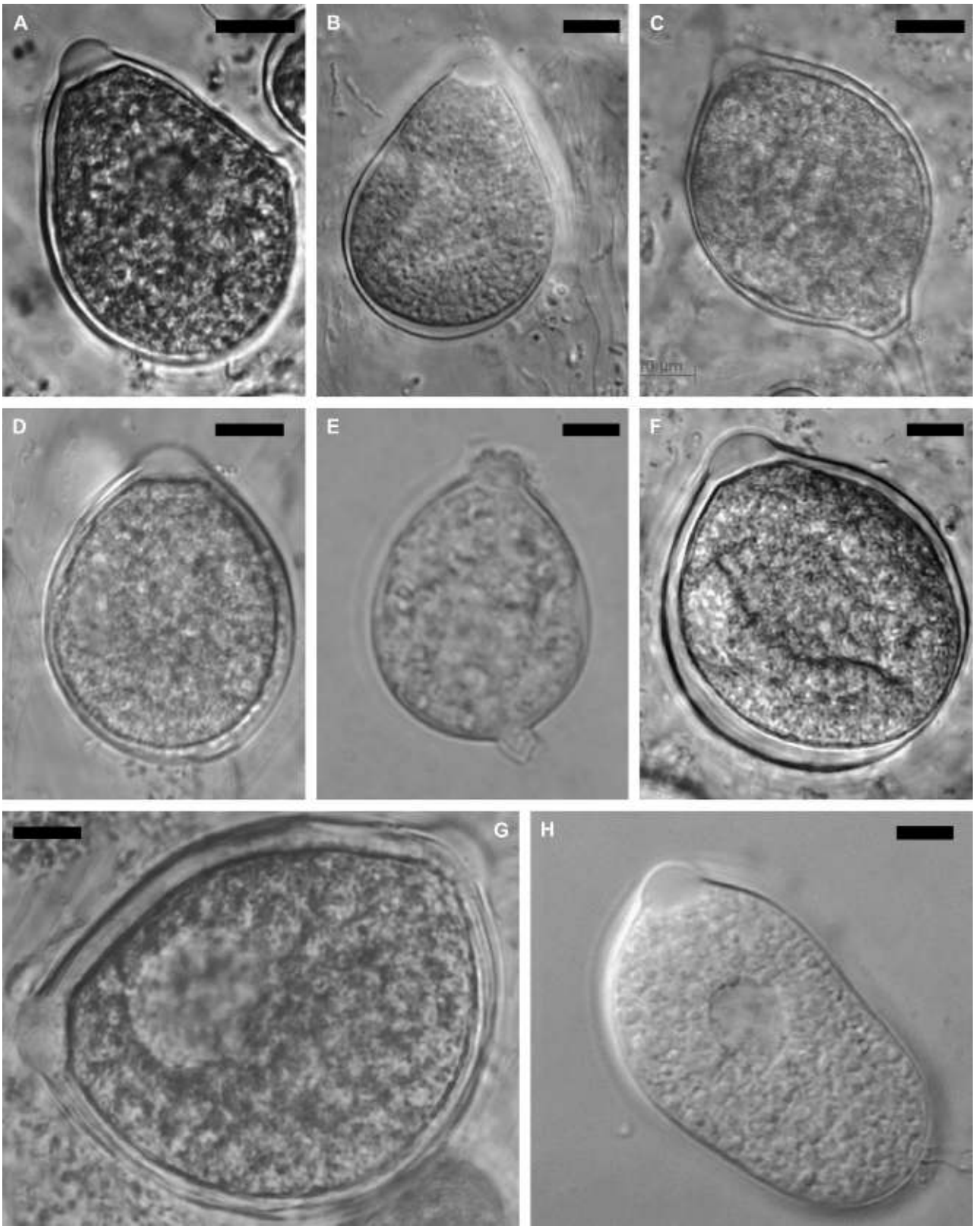


**Fig 8 – Sporangia of *Phytophthora alticola*. (A–C) Papillate, ovoidobpyriform, terminal attached and caducous sporangia. (D–F) Ovoid and papillate sporangia. Distorted shapes. (G) Bipapillate sporangium. (H) Peanut-shaped sporangium. Bars = 10 μm.**

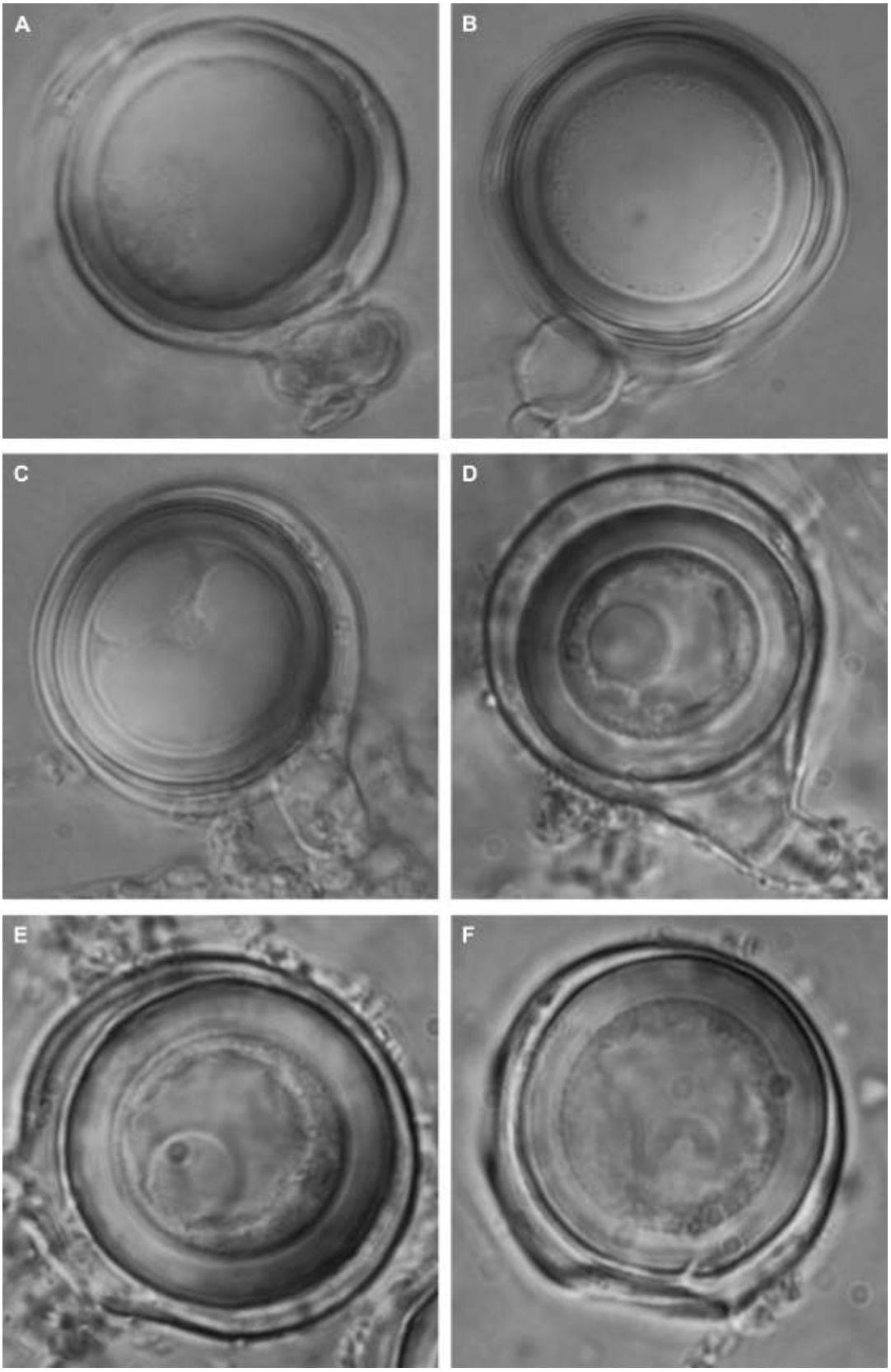


**Fig 9 – Oogonia, antheridial and oospores characteristics of *Phytophthora alticola*. (A) Spherical thick-walled oogonium with tapered base. (B–F) markedly aplerotic oospores, amphigynous antheridia often breaking-off from oogonia or attached to tapered oogonial stalk. Bars = 10 μm.**

associated with shoot dieback of the above forest tree species. *P. frigida* is occasionally recovered from baited soil samples of cold-tolerant *Eucalyptus* species during routine disease monitoring. In past surveys conducted during 1999–2003 on several *E. smithii* stands, *P. frigida* was found to have a wide distribution in the Mpumalanga and KwaZulu-Natal provinces of South Africa. To date, *P. frigida* is predominantly associated with root and collar rot disease of *E. smithii* and is less prevalent on other forest tree species.

*P. alticola* was first recovered in 2004 from dying *E. bajensis* in a mixed provenance/progeny trial at Mid-illovo and Paulpietersburg in the KwaZulu-Natal province. Subsequently, it was isolated from soil and diseased *E. dunnii* samples, established in a previous provenance/progeny trial with a history of site dieback in Paulpietersburg. In 2005, *P. alticola* was isolated from a stem canker of dying *E. macarthurii* in a plantation forest in a neighbouring country, Swaziland. The distribution of *P. alticola* is limited to provenance/progeny trials and single outlying plantations stands in the Mpumalanga and KwaZulu-Natal.

### *Pathogenicity tests*

All isolates inoculated on one-year-old *Eucalyptus dunnii* trees in the field were pathogenic and were consistently re-isolated from the resulting lesions. Discoloured lesions extending from the point of inoculation were produced in all inoculated trees. *P. cinnamomi* isolates were more aggressive than either *P. frigida* or *P. alticola* isolates (Fig 10). The mean lesion length produced by *P. cinnamomi* isolates was 12.7 cm compared with 7.7 and 3.8 cm produced by *P. frigida* and *P. alticola*, respectively. Control inoculations did not produce lesions. Mean lesion lengths for the different *Phytophthora* spp. compared were significant ($P > 001$) and different to each other and to those of the controls.

## Discussion

Two previously unknown *Phytophthora* spp. consistently associated with collar and root disease outbreaks on non-native cold-tolerant eucalypts in South Africa were identified in this study. Phylogenetic analyses of the DNA sequence data for the ITS regions of rRNA and β–tubulin region showed that these two taxa are distinct from all known species of *Phytophthora*. A number of unique morphological characteristics in these two species also support this view and we have thus described them as *P. frigida* and *P. alticola*.

The ITS phylogeny produced in this study showed that *P. frigida* is related to species within the ITS clade 2 of Cooke *et al.* (2000), and that *P. multivesiculata* was one of the species most closely related to *P. frigida*. However, *P. frigida* shares 95 % homology with an undescribed *Phytophthora* sp., which was isolated from raspberry, rose, and strawberry in 2001. At the time of the current study this undescribed *Phytophthora* sp. was not available for morphological comparison. Both *P. frigida* and the undescribed *Phytophthora* sp. of Abad *et al.* (2001) belong to a separate sub-group within the ITS clade 2 of Cooke *et al.* (2000), but the significant genetic distance

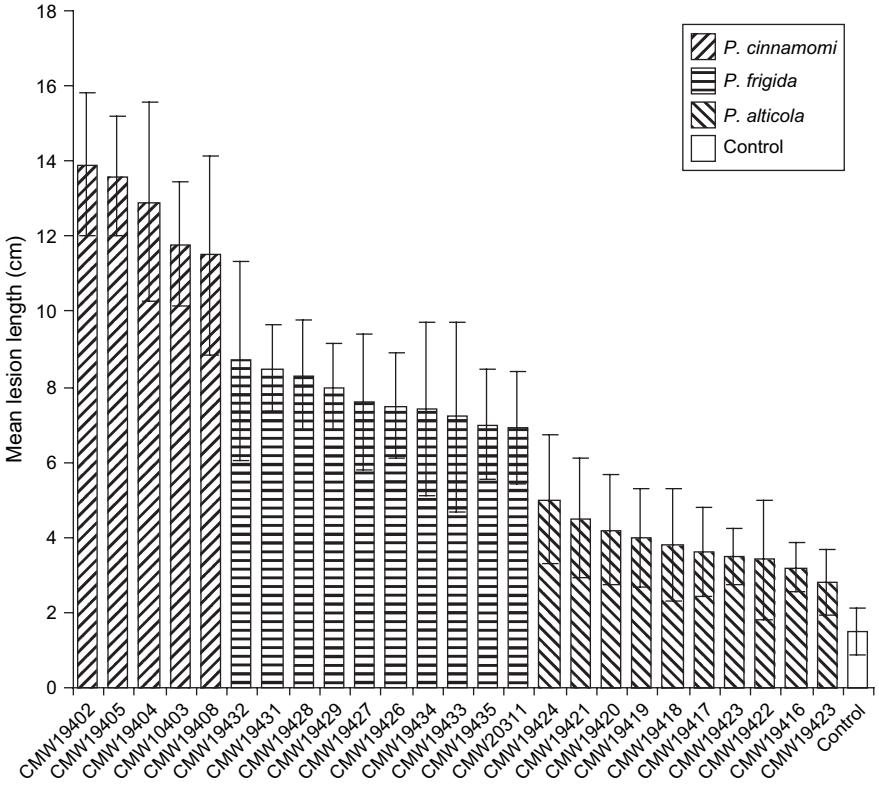


**Fig 10 – Mean lesion length of selected isolates of *Phytophthora alticola*, *P. frigida* and *P. cinnamomi*, 36 d after under-bark inoculation of 12- m-old *Eucalyptus dunnii* in the field.**

between the taxa provide good evidence that they are different species. The results of this study also show that the ITS clade 2 may include a greater number of sub-groups than previously reported by Cooke *et al.* (2000).

The distinctive morphological features of *P. frigida*, which include papillate and caducous sporangia, indicate that it is adapted for wind or splash dispersal. *P. frigida* is homothallic in culture and thus likely to be an inbreeding species. *P. frigida* has predominantly been found on *E. smithii*, planted in areas with an altitude above 1150 m in South Africa. However, its host range could possibly include *Acacia decurrens*, because a few isolates of *P. frigida* were recovered from soil collected from around diseased *A. decurrens* trees. The ability to grow at temperatures lower than 15 °C indicates adaptation to a cool temperate climate. Distinctive morphological characteristics include a stellate to petalloid growth pattern on all five media tested, and the ability to utilise L-asparagine better than nitrate as sole nitrogen source. Our observations for *P. multivesiculata*, the species most closely related to *P. frigida*, are generally consistent with those reported by Ilieva *et al.* (1998). However, these authors reported a maximum growth temperature for *P. multivesiculata*, which is higher than those emerging from the present study.

The ITS sequence data presented in this study have shown that *P. alticola* clusters with taxa in ITS clade 4 of Cooke *et al.* (2000). *P. arecae*, which is conspecific with *P. palmivora* (Mchau & Coffey 1994) and *Peronophythora litchii* (Riethmüller *et al.* 2002) are the species most closely related to *P. alticola*. A single undescribed species listed in GenBank as *Phytophthora* sp. MD 92 (GenBank DQ313223) and reported as coming from eastern US oak forests is phylogenetically closely related to *P. alticola* and could represent another host and location for this species.

*P. alticola* is a heterothallic species with ovoid-obpyriform conspicuously papillate sporangia. Consequently, it is in group II of the taxonomic scheme of Waterhouse (1963). In terms of DNA sequence data for the ITS region, it is phylogenetically placed in clade 4 of Cooke *et al.* (2000) and is related to *P. megakarya* and *P. arecae*. Superficially, *P. alticola* shares a number of morphological features with *P. arecae* and the two species could be confused. However, *P. alticola* isolates produce smooth, dome-shaped cultures with moderate aerial mycelium on V8A and MEA, with faint stellate growth patterns on CA, PDA and submerged colonies on CMA. In contrast, *P. arecae* produces smooth colonies with fluffy aerial mycelium with faint stellate growths on V8A, CA, MEA, PDA, and submerged, thin mycelial growth with no obvious patterns on CMA. The most obvious differences distinguishing *P. alticola* from *P. arecae* include significantly slower growth rates in culture, irregular rather than absent hyphal swellings, ovoid-obpyiform rather than ellipsoid to obturbanate sporangia; large terminal chlamydospores in the former and no chlamydospores in the latter and oogonia produced abundantly rather than rarely in the latter.

*P. frigida* and *P. alticola* were consistently isolated from diseased plant material and from rhizosphere soil associated with dying trees. Inoculation experiments conducted on one-year-old *E. dunnii* in the field confirmed that both species are pathogenic. We thus believe that they are agents of the dieback and early death of the affected cold-tolerant *Eucalyptus* spp. in South Africa. These two new species have thus far been recovered from *A. decurrens* and non-native cold-tolerant eucalypts planted in high altitude areas. This is in contrast with previous reports that only three *Phytophthora* spp. are associated with die-back of cold-tolerant eucalypts in South Africa (Linde *et al.* 1994c). However, *P. frigida* and *P. alticola* were substantially less pathogenic than *P. cinnamomi*, and their relative importance as tree pathogens will need to be determined.

Although various studies on *Phytophthora* spp. have been conducted, there has never been a detailed survey of these pathogens in South Africa. As many *Phytophthora* species are a threat to agricultural crops, forest trees species and native vegetation, such surveys would be valuable and should be encouraged. The discovery of two new pathogenic *Phytophthora* spp. in this study provides a strong indication that other new species of *Phytophthora* await discovery in South Africa. Examples include the recently discovered, *P. captiosa* and *P. fallax* (Dick *et al.* 2006) from exotic *Eucalyptus* species in New Zealand.

## Supplementary material

Supplementary (Fig_D06_00234) associated with this article can be found at doi: 10.1016/j.mycres.2007.08.011.

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