FUSE
Fungal Systematics and Evolution
VOLUME 13
JUNE 2024
PAGES 111–122

doi.org/10.3114/fuse.2024.13.05

A diverse range of *Phytophthora* species from botanical gardens in South Africa, including the novel Clade 5 species, *Phytophthora mammiformis sp. nov*.

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Key words:
detection
new taxon
oomycete
pathogens
sentinel plants

Abstract: The genus *Phytophthora* contains many destructive and globally important plant pathogens. In the last decade, targeted sampling efforts have resulted in a dramatic increase in the number of known species, as well as a better understanding of their global distribution. Routine activities undertaken in botanical gardens, combined with great numbers of local and international visitors, place botanical gardens at risk to the accidental introduction and establishment of pathogens such as *Phytophthora* spp. In this study, the occurrence of *Phytophthora* was investigated in two botanical gardens in the KwaZulu-Natal Province of South Africa. Symptomatic collar and stem tissues were collected, and root and rhizosphere soil samples were taken from trees exhibiting symptoms of decline. Standard baiting techniques and direct plating of symptomatic tissues revealed the presence of seven species of *Phytophthora* residing in four phylogenetic clades. Five of these species, *P. cinnamomi, P. citrophthora, P. multivora, P. parvispora* and the informally designated taxon *Phytophthora* sp. stellaris were known to be present in South Africa and *P. aquimorbida* was recorded for the first time. Of these, *P. citrophthora* represented a novel host-pathogen association causing bleeding cankers on indigenous *Celtis africana*. A multilocus phylogenetic analysis based on ITS, *βtub, cox1* and *hsp90* sequences showed the presence of an undescribed species belonging to the *Phytophthora* ITS Clade 5. This species is described here as *Phytophthora mammiformis sp. nov*. This study highlights the importance of monitoring botanical gardens for the detection and discovery of pathogens and emphasises their value as sites for the discovery of novel host-pathogen associations.

Citation: Paap T, Balocchi F, Burgess TI, Bose T, Wingfield MJ (2024). A diverse range of *Phytophthora* species from botanical gardens in South Africa, including the novel Clade 5 species, *Phytophthora mammiformis sp. nov*. *Fungal Systematics and Evolution* **13**: 111–122. doi: 10.3114/fuse.2024.13.05
Received: 27 February 2024; **Accepted:** 24 April 2024; **Effectively published online:** 17 May 2024
Corresponding editor: C.M. Visagie

INTRODUCTION

The accidental movement of invasive plant pathogens, most commonly introduced to new areas through the global trade of plant material, continues to threaten plant health (Brasier 2008, Santini *et al.* 2018). Plants in urban environments are frequently the first point of contact for newly arrived alien species; this due to their proximity to hubs of international trade (Paap *et al.* 2017). Botanical gardens may be particularly vulnerable to the arrival and establishment of alien pathogens (Hulbert *et al.* 2019). They are frequently located close to high-risk sites (*e.g.*, ports, airports), and with a high diversity of plant species maintained in these collections, there is increased potential for recently arrived alien pathogens to locate suitable hosts (Barham *et al.* 2016, Paap *et al.* 2017). This risk is further increased as a result of routine activities conducted in botanical gardens (including the collection, cultivation, sharing and sale of plant materials), as well as the mobility of staff and visits by millions of people each year (Wondafrash *et al.* 2021).

There are several challenges to understanding microbial plant pathogen invasions and ultimately, invasion events involving pathogens are difficult to predict (Paap *et al.* 2022). In addressing these challenges, the sentinel plant concept is emerging as a powerful tool for early warning and early detection of pathogen threats (Eschen *et al.* 2019, Migliorini *et al.* 2023a, Raffa *et al.* 2023), with botanical gardens proving to be valuable resources for sentinel research (Britton *et al.* 2010, Barham *et al.* 2016, Wondafrash *et al.* 2021).

The genus *Phytophthora* contains many globally important plant pathogens responsible for economically and environmentally devastating disease outbreaks (Erwin & Ribeiro 1996, Cahill *et al.* 2008, Scott *et al.* 2019, Bose *et al.* 2023). In the last decade, targeted sampling efforts have resulted in a dramatic increase in the number of known species, as well as a better understanding of the global distribution of these important pathogens (Scott *et al.* 2019, Burgess *et al.* 2021). Previous studies have highlighted the value of sampling highly managed and publicly accessible areas, including urban parks

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and botanical gardens, for identifying *Phytophthora* first reports and new host-pathogen associations (Barber *et al.* 2013, Hulbert *et al.* 2019, Green *et al.* 2020, La Spada *et al.* 2022). The aim of this study was to determine the range of *Phytophthora* species associated with trees exhibiting symptoms typical of *Phytophthora* infection, in two botanical gardens in the KwaZulu-Natal (KZN) Province of South Africa.

MATERIALS AND METHODS

Study area

Two gardens in the KZN Province of South Africa were surveyed: the KZN National Botanical Garden (KZN NBG) in Pietermaritzburg, and the Durban Botanic Gardens (DBG) in Durban. The KZN NBG is maintained by the South African National Biodiversity Institute (SANBI), while the DBG is managed by the Parks Department of the eThekwini Municipality. Both were established in the mid-19th Century. DBG served as a trial centre for planting coffee, tea and other agricultural crops, to assist in the introduction of economically valuable plants. It remains as Africa's oldest surviving botanical garden (McCracken 1996). One of the original objectives of the KZN NBG was to grow plants, primarily trees, for distribution in the then colony of Natal (Willis & Nene 2010). The establishment of both these gardens coincided with the development of a vast global network of colonial botanical gardens, coordinated by the Royal Botanic Gardens (Kew, Britain), and motivated by the quest to collect and document exotic and previously unknown plant specimens (Fry 2013). Consequently, DBG and KZN NBG both have an early history of receiving containerised live plants, in soil, from other countries that were linked to this historical network.

Sample collection

Targeted sampling, limited to trees exhibiting symptoms typical of *Phytophthora* infection *i.e.*, crown decline or bleeding cankers, was conducted in the DBG and KZN NBG between January and September of 2017, with additional samples collected in April 2021 (Table 1). These surveys were undertaken as part of the South African National Biodiversity Institute (SANBI) funded Sentinel Plant Project (https://www.fabinet.up.ac.za/index.php/sentinel-plant-network), which uses plant collections in botanical gardens to identify new and emerging pest risks.

Rhizosphere soils were collected from around the base of trees exhibiting symptoms of premature crown decline (Fig. 1A, D). Care was taken to collect fine roots, especially where these were lesioned or rotting (Fig. 1C). A total of 14 rhizosphere soil samples were collected (eight from the DBG and six from the KZN NBG). An additional sample was collected from a bleeding canker on the trunk of a *Celtis africana* in the KZN NBG (Fig. 1B).

Isolation of *Phytophthora*

Rhizosphere soil samples were baited following the best practice methodology proposed by Burgess *et al.* (2020). Tissue samples collected from canker margins were washed in distilled water, blotted dry and plated onto the *Phytophthora* selective media, NARP (50 mg nystatin, 200 mg ampicillin, 10 mg rifampicin and 25 mg pentachloronitrobenzene per 1 L distilled water and 17 g cornmeal agar). Plates were kept in the dark at 22 °C and examined daily. Single hyphal tip isolations of all putative *Phytophthora* isolates were prepared using a stereomicroscope and transferred onto fresh half-strength potato dextrose agar (1/2 PDA; 19.5 g PDA powder, Merck, South Africa, 7 g Difco™ agar, 1 L distilled water). The resulting cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Lunnon Road, Pretoria, South Africa. The holotype of the novel Clade 5 *Phytophthora* species was deposited in the H.G.W.J. Schweickerdt Herbarium (PRU) and the ex-holotype culture in the culture collection of Innovation Africa @UP (CMW-IA), housed at FABI (University of Pretoria, Pretoria, South Africa).

DNA extraction, amplification, sequencing

Genomic DNA was extracted from mycelia harvested from 10-d-old *Phytophthora* cultures using a ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, USA). For all isolates, the internal transcribed spacer (ITS) region of the rDNA (ITS1-5.8S-ITS2) was amplified using the primers ITS6 (Cooke *et al.* 2000) and ITS4 (White *et al.* 1990). ITS sequences were used to confirm the identification at species level.

For three isolates representing a novel Clade 5 species, three additional regions, *β*-tubulin (*βtub*), cytochrome c oxidase subunit I (*cox1*) and heat shock protein 90 (*hsp90*), were amplified using primers Btub_F1A/BTub_R1(Kroon *et al.* 2004, Blair *et al.* 2008), OomCoxI-Levup/Fm85mod (Robideau *et al.* 2011) and HSP90_F1/ HSP90_R2 (Blair *et al.* 2008).

Amplification reaction mixtures included 2.5 µL of 10× KAPA Taq Buffer A (Kapa Biosystems, Cape Town, South Africa), 0.5 µL of 0.1 mM dNTPs (Promega, MI), 1 µL each of forward and reverse primers, 0.2 µL of KAPA *Taq* DNA Polymerase (Kapa Biosystems, Cape Town, South Africa), 18.8 µL PCR grade water and 1 µL of DNA template (total volume 25 µL). The PCR amplifications consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s; annealing temperatures of 58 °C for 30 s (ITS), 60 °C for 30 s (*βtub*), 55 °C for 60 s (*cox1*), and 62 °C for 30 s (*hsp90*); 72 °C for 1 min; and final elongation at 72 °C for 7 min.

Resulting PCR products were cleaned using Sephadex® G-50 columns (Sigma-Aldrich, South Africa). The forward and reverse sequences were sequenced separately using the BigDye™ Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA), following the manufacturer's protocol. The obtained products were cleaned, and sequencing of the products was carried out using an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems, USA) at the Sequencing Facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria, South Africa. The forward and reverse sequences obtained for each isolate were visualised and assembled into consensus sequences with CLC Main Workbench v. 23.0.4 (Qiagen, Hilden, Germany). All sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (Table 2 and Suppl. Table S1).

Phylogenetic analyses

ITS consensus sequences were aligned to the NCBI GenBank database (NCBI, http://www.ncbi.nlm.nih.gov) using the BLAST utility to obtain a preliminary identification for all isolates. Based on the similarity output results, sequences were aligned to the ex-type sequences of the closest match for each species, as described by Abad *et al.* (2023b). To conduct the phylogenetic

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Table 1. Geographic location of the 14 rhizosphere soil and one tissue sampling sites, tree species sampled and *Phytophthora* taxa isolated by baiting or direct plating.

Sampling area[1]	Coordinates	Sampled tree species[2]	Sample type	Sample date	*Phytophthora* taxa[3]
DBG	-29.845978, 31.00746	*Triadica sebifera*	rhizosphere soil	May 2017	MUL, STE
	-29.847208, 31.008236	*Ficus cotinifolia*	rhizosphere soil	Sep. 2017	AQU, MUL
	-29.844749, 31.006725	*Galpinia transvaalica*	rhizosphere soil	Sep. 2017	—
	-29.846167, 31.007277	*Hymenaea verrucosa*	rhizosphere soil	Sep. 2017	CL5
	-29.847366, 31.005584	*Persea americana*	rhizosphere soil	Sep. 2017	MUL
	-29.847937, 31.007120	*Taxodium distichum*	rhizosphere soil	Sep. 2017	—
	-29.846167, 31.007277	*H. verrucosa*	rhizosphere soil	Apr. 2021	CL5
	-29.846072, 31.007186	*H. verrucosa*	rhizosphere soil	Apr. 2021	CL5
KZN NBG	-29.606701, 30.346100	*Araucaria columnaris*	rhizosphere soil	Jan. 2017	—
	-29.605127, 30.346551	*Betula nigra*	rhizosphere soil	Jan. 2017	PAR
	-29.607853, 30.458500	*Cedrus deodara*	rhizosphere soil	Jan. 2017	—
	-29.605832, 30.346424	*Cupressus sempervirens*	rhizosphere soil	Jan. 2017	MUL
	-29.606536, 30.345536	*Quercus suber*	rhizosphere soil	Jan. 2017	CIN
	-29.605034, 30.347700	*Platanus* x *hispanica* (syn. = *P.* x *acerifolia*)	rhizosphere soil	May 2017	MUL
	-29.605245, 30.346806	*Celtis africana*	bleeding canker	Apr. 2021	CIT

[1] DBG = Durban Botanic Garden; KZN NBG = KwaZulu-Natal National Botanical Garden.

[2] Tree species names as accepted by Plants of the World Online (https://powo.science.kew.org/).

[3] AQU = *P. aquimorbida*; CIN = *P. cinnamomi*; CIT = *P. citrophthora*; MUL = *P. multivora*; PAR = *P. parvispora*; STE = *P.* sp. stellaris; CL5 = *P. mammiformis sp. nov*.

Table 2. GenBank accession numbers for ITS Clade 5 *Phytophthora* species used for phylogenetic analyses. Accession numbers in bold font are from the present study. Sequences for *P. agathidicida* NZFS 3772 and *P. podocarpi* NZFS 3642 were extracted from the whole genome sequences available for these species.

Species	Strain[1,2]	GenBank accessions			
		ITS	***ßtub***	***cox1***	***hsp90***
P. agathidicida	CPHST BL 154[T]	MG602692	MH493902	MK493471	MK020269
	NZFS 3772	LGTR00000000	LGTR00000000	LGTR00000000	LGTR00000000
P. castaneae	CPHST BL 47G[T]	MG865470	MH493918	MH136866	EU080806
	P 3389	HQ261600	EU079815	HQ261347	EU079818
P. cocois	CPHST BL 157[T]	MG865478	MH493925	MH136874	MK020290
	ICMP 16949	KP295305	—	KP295221	KP295275
P. heveae	CPHST BL 67[T]	MG865505	MH493947	MH136899	MK020314
	ICMP 16914	KP295303	EU080697	KP295218	KP295272
P. mammiformis	**CMW 51734[T]**	**OR978479**	**OR972571**	**OR972568**	**OR972574**
	CMW 57832	**OR978480**	**OR972572**	**OR972569**	**OR972575**
	CMW 57837	**OR978481**	**OR972573**	**OR972570**	**OR972576**
P. podocarpi	NZFS 3642[T]	LGSN00000000	LGSN00000000	LGSN00000000	LGSN00000000

[1] Ex-types ([T]).

[2] Isolate abbreviations: CMW = Forestry and Agricultural Biotechnology Institute, Pretoria, South Africa; CPHST BL = Centre for Plant Health Science and Technology Beltsville Laboratory; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; NZFS = Forest Health Reference Laboratory, Scion, Rotorua, New Zealand; P = isolate codes from World Phytophthora Collection, University of California, Riverside.

identification for three isolates identified as a novel species belonging to the *Phytophthora* ITS Clade 5, we retrieved ITS, *ßtub, cox1* and *hsp90* sequences for the ex-type isolates and additional isolates representing well described species within ITS Clade 5, from the NCBI GenBank database (Table 2). *Phytophthora podocarpi* (NZFS 3642) was used as the outgroup. The sequences corresponding to all four gene regions of *P. podocarpi* were retrieved from the supplementary data files of Abad *et al.* (2023a).

Individual datasets were aligned using MUSCLE v. 3.8.31 (Edgar 2004) and leading and trailing gaps were trimmed using Mesquite v. 3.61 (Maddison & Maddison 2019). A concatenated dataset was then generated using FASconCAT (Kück & Meusemann 2010). The alignments are available through Mendeley Data (doi: 10.17632/d4g92wvrhp.1).

Maximum likelihood (ML) analysis and model testing were performed for both single gene and concatenated (partitioned data) datasets using IQ-TREE v. 2.2.2.6 (Nguyen *et al.* 2015) with

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Fig. 1. Examples of symptoms on specimens in the Durban Botanic Garden and KwaZulu-Natal National Botanical Garden from which *Phytophthora* was isolated **A.** Crown thinning of *Persea americana*. **B.** Basal bleeding canker on *Celtis africana*. **C.** Necrotic fine roots of *Platanus* × *hispanica*. **D.** Crown thinning of *Cupressus sempervirens.*

1 000 bootstrap replicates. Bayesian analyses (BI) of the sequence datasets were conducted using Mr. Bayes v. 3.2.7a (Huelsenbeck & Ronquist 2001). Four Markov Chain Monte Carlo (MCMC) chains were initiated from a random starting tree and run for 5 M generations. The stop value was set at 0.01, the temperature at 0.2, and trees were sampled after every 100 generations. A quarter of the sampled trees were discarded as burn-in, and the remaining trees were used to generate majority rule consensus trees. Finally, the phylogenetic trees were visualised and rooted using FigTree v. 1.4.4 (Rambaut 2010).

Colony morphology, growth rates and cardinal temperatures

The colony morphology of three isolates of the novel Clade 5 species was described following the suggested system of Erwin & Ribeiro (1996). Inoculum plugs (5 mm diam) were taken from the margins of 8-d-old cultures grown on 1/2 PDA at 20 °C in the dark and transferred to four culture media: 10 % carrot agar (CA; 100 mL fresh carrot juice; 15 g Difco agar, Becton, Dickinson and Company, Sparks, USA; 900 mL of distilled water), 2 % malt extract agar (MEA; 20 g malt extract, Merck, South Africa; 15 g Difco agar and 1 L of distilled water), 10 % clarified V8 agar (V8A; 100 mL clarified V8 juice, Campbell Soup Company USA; 15 g Difco agar; 900 mL of distilled water), and 1/2 PDA. The colony morphologies were evaluated and described after incubation at 20 °C for 7 d in the dark. For temperature growth studies, all isolates were sub-cultured onto V8A plates and incubated for 24 h at 20 °C to stimulate growth. The plates were then moved to incubators fixed at 4, 6, 10, 15, 20, 25, 30 and 35 °C. Radial growth rate was measured 3–7 d after the onset of linear growth, along two lines crossing the middle of the inoculum plug at right angles, and the mean growth rates (mm/d) were assessed. Three replicate plates were used for each isolate at each temperature. Plates with no colony growth were returned to 20 °C for 7 d to check the isolate viability.

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Morphology of asexual and sexual structures

Morphological features of the three isolates of the novel *Phytophthora* species were examined. To produce sporangia, 15 × 15 mm square agar discs were cut from the growing edge of 5–8-d-old colonies on V8A and grown in 90-mm-diam Petri dishes with 10 % V8 broth. Petri dishes were incubated at room temperature in natural daylight. After 48 h the V8 broth was decanted, followed by two rinses with distilled water and non-sterile soil extract added. Shape, type of apex, caducity and special features of sporangia and the formation of hyphal swellings and aggregations were recorded after 48–72 h. For each isolate, dimensions of 50 sporangia were measured using a Zeiss AXIO Imager.A2 compound microscope equipped with a Zeiss AxioCaM 512 colour camera driven by Zen Blue v. 3.2 software (Carl Zeiss CMP, Göttingen, Germany).

The formation of chlamydospores, gametangia (oogonia and antheridia) and their characteristic features were examined on V8A after 4–14 d growth at 20 °C in the dark. For each isolate, 50 oogonia, oospores and antheridia were chosen at random and measured under a compound microscope as described before. The oospore wall index was calculated following the approach of Dick (1990).

Designation of status

The status of each *Phytophthora* species detected during the current study was specified as native or introduced in South Africa. For species lacking evidence for a centre of origin, we followed Burgess *et al.* (2017). Species were designated as introduced if they are known globally from agriculture and horticulture, or native if currently only known from South Africa (Suppl. Table S1).

RESULTS

Phytophthora species associated with symptomatic trees

Phytophthora species were isolated from ten of the 14 rhizosphere samples processed by baiting (six from the DBG and four from the KZN NBG). An additional *Phytophthora* isolate was recovered by direct plating tissue from a bleeding canker of *Celtis africana.* Multiple species were recovered from two rhizosphere samples, resulting in a total of 13 unique *Phytophthora* isolates.

Based on morphology and ITS sequence analyses, seven species were identified. These included five formally described species, one known but not yet formally described species and one putative novel Clade 5 species (Table 1, Suppl. Table S1). Four species were recovered from the DBG (*P. aquimorbida, P. multivora, Phytophthora* sp. stellaris and the novel Clade 5 species), with four species also recovered from the KZN NBG (*P. cinnamomi, P. citrophthora, P. multivora* and *P. parvispora*). *Phytophthora multivora* was the most frequently isolated species (from five samples), and the only species isolated from both botanical gardens (Table 1).

The three isolates of the novel Clade 5 species were collected from two adjacent *Hymenaea verrucosa* trees, a species native to East Africa. The first of these isolates was collected from a single tree sampled in September 2017, with the two remaining isolates recovered during a subsequent sampling of both trees in April 2021 (Table 1).

The remaining five species were each only recovered from a single sample, with *P. aquimorbida* isolated from rhizosphere soil of *Ficus cotinifolia* (together with *P. multivora*), *P. cinnamomi* from *Quercus suber* rhizosphere soil, *P. citrophthora* from a bleeding canker on *C. africana, Phytophthora* sp. stellaris from *Triadica sebifera* rhizosphere soil (together with *P. multivora*) and *P. parvispora* from *Betula nigra* rhizosphere soil (Table 1).

Phylogenetic analyses

In ML and BI analyses of ITS Clade 5 species, using both concatenated and single gene datasets, all three isolates of the novel *Phytophthora* species obtained in this study formed a distinct highly supported clade (Fig. 2 and Suppl. Fig. S1). Analyses of the ITS, *hsp90* (Suppl. Fig. S1) and the concatenated (Fig. 2) datasets placed the novel species nearest to *P. heveae*, although this relationship received weak branch support. Phylogeny of the *cox1* gene region resolved the clade of the novel *Phytophthora* species as sister to *P. castanea*e, while in the *βtub* tree it emerged as a sister to another clade containing *P. agathidicida*, *P. castanea*e and *P. cocois* (Suppl. Fig. S1). Although most gene regions resolved each species of the clade in highly supported subclades, none of the analyses resolved the arrangement of the species within the clade with significant support.

Taxonomy

Phytophthora mammiformis T. Paap, T. Bose, Balocchi, ***sp. nov.*** MycoBank MB 853463. Fig. 3.

Etymology: Referring to the characteristic mammiform shape of the sporangia.

Description: *Sporangia* only observed in non-sterile soil extract after 4–7 d; borne terminally on thin, simple (unbranched)

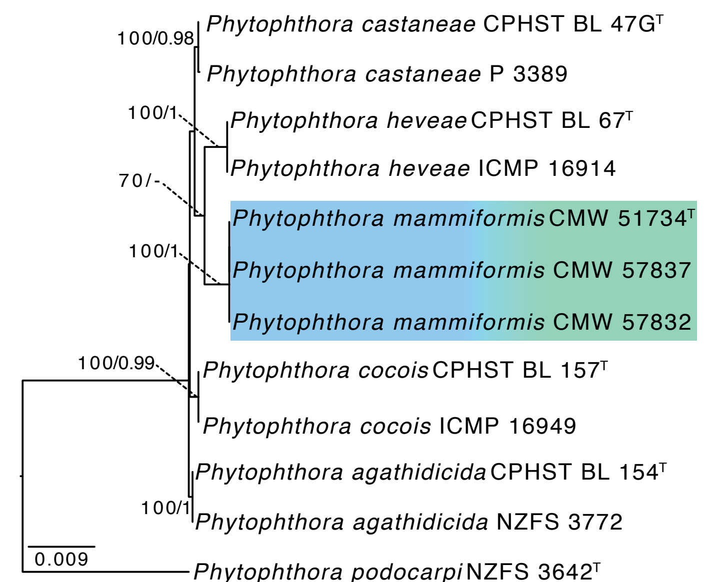

Fig. 2. Maximum likelihood phylogeny obtained using a concatenated dataset (ITS, *βtub, cox1* and *hsp90*) for ITS Clade 5 *Phytophthora* species. The clade of *Phytophthora mammiformis* is highlighted with a coloured block. *Phytophthora podocarpi* NZFS 3642 was used as an outgroup. Numbers on the branches are bootstrap support values (≥ 70 %)/posterior probabilities (≥ 0.8). Superscript T denotes material with a type status.

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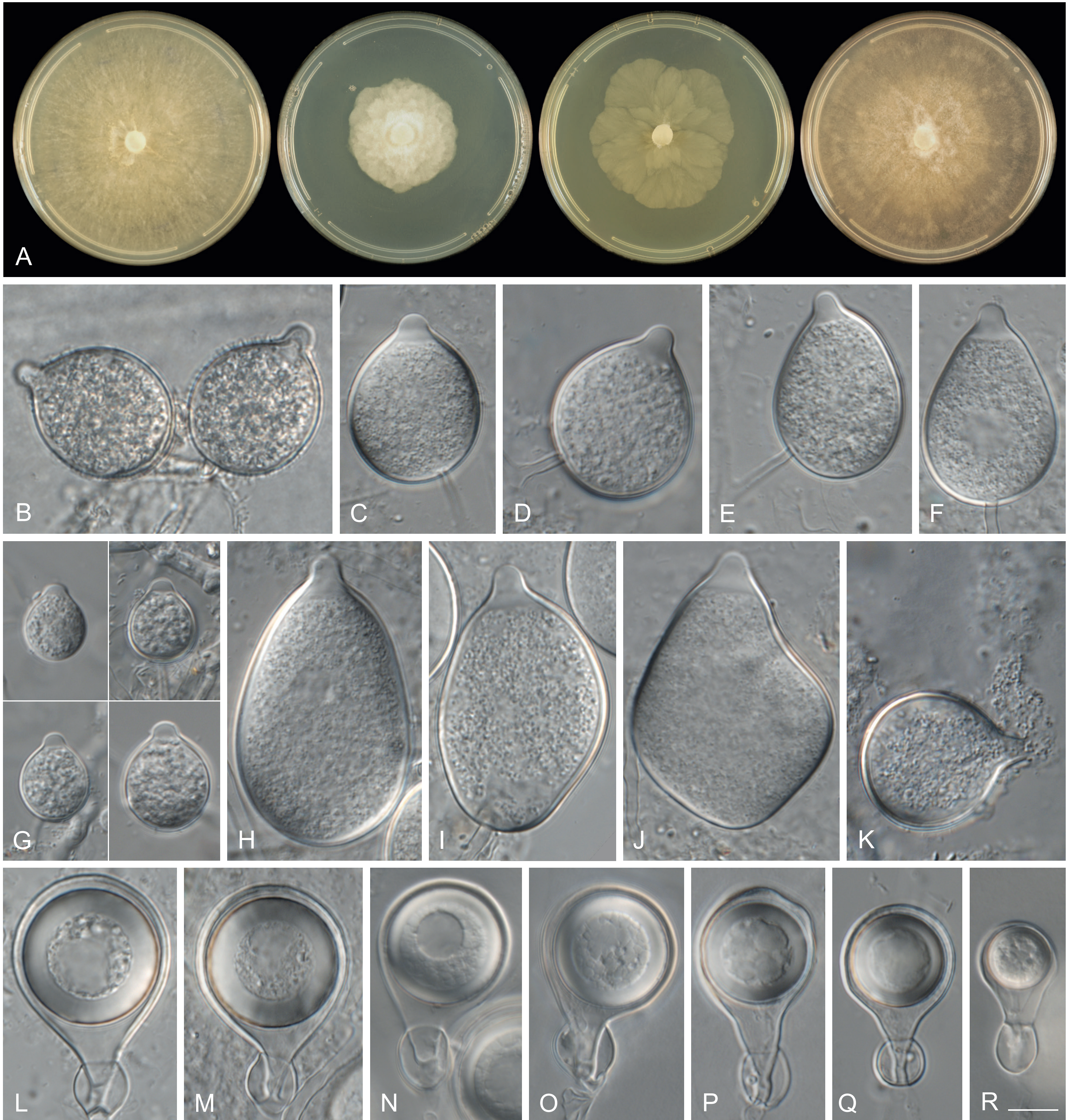

Fig. 3. Photoplate of *Phytophthora mammiformis*. **A.** Colony morphology of *Phytophthora mammiformis* (CMW 51734) after 7 d growth at 20 °C on V8 agar, half strength potato dextrose agar, malt extract agar and carrot agar (left to right). **B–R.** Morphological structures of *Phytophthora mammiformis*. **B–K.** Sporangia; (B–F) papillate globose to ovoid sporangia with basal (F) and lateral (C–E) attachments; (G) smaller globose sporangia; (H) large elongated ovoid sporangium; (I–J) large and distorted sporangia, (K) globose sporangia releasing zoospores. **L–R.** Oogonia; (L, M, Q) globose oogonia with funnel-shaped base; (N–O) comma-shaped oogonia; (P) slightly eccentric and elongated oogonium; (R) smaller globose but extended oogonium. Scale bar = 10 µm (applies to B–R).

sporangiophores or laterally (Fig. 3E), rarely having a bulbous base, rarely intercalary (< 1 %), persistent, non-proliferating, papillate, very rarely semi-papillate or bi-papillate. Sporangia predominantly subglobose (49 %; Fig. 3C), globose (18 %; Fig. 3D) or ovoid (27 %; Fig. 3F), rarely (< 1 %) elongated ovoid (Fig. 3H) or distorted (Fig. 3I, J); sometimes asymmetrical (9.5 %; Fig. 3E), commonly attached laterally (Fig. 3C–E). Sporangia dimensions ranging 13.5–71 × 12–40 µm (av. 29.1 ± 8.7 × 25.3 ± 6.2 µm) with frequent small sporangia (~15 µm; Fig. 3G), L/B ratio 1.10–1.19 (av. 1.15 ± 0.14), exit pore 3–8 µm (av. 5.1 ± 1 µm). Slight *hyphal swellings* occasionally observed. *Chlamydospores* absent. *Homothallic*, oogonia developed abundantly in V8A after 4 d. Abortion rate was low (< 5 %). *Oogonia* globose (63 %; Fig. 3L, M), comma-shaped (10 %; Fig. 3N, O), slightly eccentric (24 %; Fig. 3P) or elongated (3 %), with smooth walls; 14–30 µm (av. 24.8 ± 2.6 µm) and with a tapering often funnel-like base. *Oospores*

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globose, uncoloured, slightly aplerotic (53 %) or aplerotic (47 %), diameter range 12–26 µm (av. 21.1 ± 2.2 µm), thick-walled (2.6 ± 0.56 µm), oospore wall index 0.57 ± 0.08 (0.38–0.75). *Antheridia* amphigynous (Fig. 3L–R), varying from globose to cylindrical or ellipsoid, 6–15.5 × 6–13 µm (av. 10.3 ± 1.7 × 9.8 ± 1.3 µm).

Cultures: Cultures on V8A and CA with no distinct pattern, limited aerial mycelium, velvety to slightly woolly. On 1/2 PDA cottony with a less defined petaloid pattern. On MEA with a less defined stellate pattern and very limited aerial mycelium (Fig. 3A). The optimum growth temperature on V8A was 25 °C (7.38 ± 0.92 mm/d; Fig. 4), the minimum was between 4 °C (no growth, non-lethal) and 10 °C (1.57 ± 0.43 mm/d); and the maximum between 30 °C (4.63 ± 1.41 mm/d) and 35 °C (no growth, lethal).

Typus: **South Africa**, KwaZulu-Natal Province, Durban Botanic Garden, from rhizosphere soil of declining *Hymenaea verrucosa* (-29.846167, 31.007277), Sep. 2017, *T. Paap* [**holotype** PRU(M) 4587, preserved as dried culture in metabolically inactive state; culture ex-type CMW-IA 6962 = CMW 51734]. DNA barcodes: OR978479 (ITS), OR972571 (*βtub*), OR972568 (*cox1*), OR972574 (*hsp90*).

Additional material examined: **South Africa**, KwaZulu-Natal Province, Durban Botanic Garden, from rhizosphere soil of declining *Hymenaea verrucosa* (-29.846167, 31.007277), Apr. 2021, *T. Paap*, culture CMW 57832 [DNA barcodes: OR978480 (ITS), OR972572 (*βtub*), OR972569 (*cox1*), OR972575 (*hsp90*)]; (-29.846072, 31.007186), and culture CMW 57837 [DNA barcodes: OR978481(ITS), OR972573 (*βtub*), OR972570 (*cox1*), OR972576 (*hsp90*)].

Notes: There are now five accepted species in Clade 5 and a comprehensive comparison of their morphological features is provided in Table 3. Species within this clade are morphologically similar; all are homothallic and produce oogonia with a tapering base and amphigynous antheridia containing thick-walled oospores. All produce globose to ovoid, persistent, papillate sporangia on unbranched sporangiophores or simple sympodia. While many morphological features are similar, *P. mammiformis* differs from the most closely related species, *P. heveae,* in producing smaller, more globose and strongly papillate (mammiform) sporangia on unbranched sporangiophores and lacking the globose to subglobose hyphal swellings found in *P. heveae.*

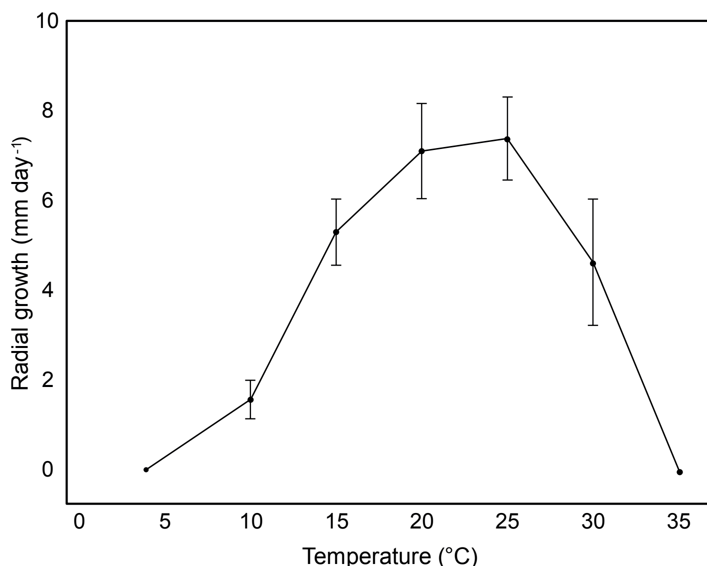

Fig. 4. Mean radial growth rate (mm/d ± SD) of *Phytophthora mammiformis* (three isolates) on V8 agar at different temperatures.

DISCUSSION

Seven *Phytophthora* taxa were isolated from rhizosphere soil and cambial tissue samples associated with symptomatic trees in two botanical gardens of the KwaZulu-Natal Province of South Africa. Five of the taxa represent species known to be present in South Africa, including *P. cinnamomi, P. citrophthora, P. multivora* and *P. parvispora,* as well as the informally designated taxon *Phytophthora* sp. stellaris. *Phytophthora aquimorbida* was detected for the first time in South Africa. In addition, three isolates described here as *P. mammiformis sp. nov*. were isolated and characterised.

Phytophthora multivora (Sub-clade 2c) was the most frequently isolated species in this study and the only taxon isolated from both botanical gardens. Multiple studies in South Africa have frequently recovered *P. multivora* (Bezuidenhout *et al.* 2010, Hulbert *et al.* 2019, Migliorini *et al.* 2023b), with Oh *et al.* (2013) routinely isolating this species from asymptomatic natural vegetation. A global population study by Tsykun *et al.* (2022) considered South Africa to be the native range of the species, which is further supported by its lack of association with severe disease outbreaks on native plants. *Phytophthora multivora* has an extensive global distribution and wide host range (Migliorini *et al.* 2019, Tsykun *et al.* 2022). It has emerged as a significant pathogen outside of its putative natural range, where it can be found causing disease in nurseries, the urban environment and natural ecosystems (Scott *et al.* 2009, Barber *et al.* 2013, Jung *et al.* 2016).

We recovered *P. multivora* in the DBG from rhizosphere samples of declining *Ficus cotinifolia*, *Persea americana* and *Triadica sebifera*, and in the KZN NBG from declining *Cupressus sempervirens* and *Platanus* × *hispanica.* While our study did not examine pathogenicity towards these hosts, Migliorini *et al.* (2019) demonstrated high susceptibility of *T. sebifera* to *P. multivora.* Rodriguez-Padron *et al.* (2018) found *P. multivora* infecting *Pe. americana* in the Canary Islands, with pathogenicity trials inducing moderate to non-significant root necrosis, depending on the isolate and experiment. Pathogenicity towards *C. sempervirens, Pl.* × *hispanica* and *F. cotinifolia,* to the best of our knowledge, has not been demonstrated in previous studies. Our observation of necrotic fine roots of *Pl.* × *hispanica* suggests this may be a host of *P. multivora,* however, Koch's postulates are required to confirm this.

Apart from *P. mammiformis*, the remaining *Phytophthora* species were each recovered only once. *Phytophthora aquimorbida* (Sub-clade 9a) was isolated from a rhizosphere sample of *F. cotinifolia* in the DBG. Described from irrigation runoff water in Virginia, USA, this species has high optimum and maximum temperatures (Hong *et al.* 2012). Our detection of *P. aquimorbida* in the DBG is the first report of this species in South Africa, also to the best of our knowledge, this is the first time *P. aquimorbida* is reported outside of the USA. Hong *et al.* (2012) found *P. aquimorbida* to be pathogenic to *Rhododendron*, however, pathogenicity to other plant species has not been determined.

Phytophthora cinnamomi (Clade 7c) was recovered from a rhizosphere sample of declining *Quercus suber* in the NBG. This

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Table 3. Comparison of morphological features, dimensions (mean ± SD) and culture characteristics of *Phytophthora mammiformis* and previously described *Phytophthora* Clade 5 species. All measurements except growth rate are in µm.

Species /Trait	*P. mammiformis*	*P. agathidicida*[1,3]	*P. castanae*[1,2,3]	*P. cocois*[1,3]	*P. heveae*[1,2,3]
Hyphal swellings	slight swellings	slight swellings	absent	absent	globose to subglobose
Sporangia	persistent	persistent	persistent	persistent	persistent or caducous*
Characteristics	papillate, very rarely semi-papillate	papillate	papillate	papillate	papillate
Shapes observed	subglobose, globose, ovoid, rarely elongated ovoid or broad ellipsoid	globose to ovoid-ellipsoid	limoniform, ovoid, obpyriform, obturbinate	globose to ovoid	ovoid to obpyriform, ellipsoid, irregular
Distorted shapes	infrequently asymmetrical, distorted or bi-papillate	—	infrequently asymmetrical or bi-papillate	—	frequently asymmetrical
Sporangiophores	unbranched, rarely with bulbous base	long thin, unbranched	unbranched	unbranched	simple sympodial
Exit pores ± SD	5.1 ± 1 (3–8)	—	—	—	—
Length × breadth mean	29.1 ± 8.7 × 25.3 ± 6.2	39.6 × 28.4	27.5 × 22.5	38.4 × 25.4	45 × 29.6
Length × breadth range	13.5–53 (71) × 12–40	14.9–75 × 12.4–50	10–42.5 × 10–37.5	18.6–50 × 12.4–35	27–66 × 20–49
Range of isolates means	27.6–31.5 × 22.8–27.6	—	—	—	—
Length: breadth ratio (isolate means)	1.15 ± 0.14 (1.10–1.19)	1.39	1.22	1.51	1.5 (1.1–2.9)
Proliferation	absent	present (internal)	absent	absent	absent
Chlamydospores	absent	absent	present (spherical)	absent	absent
Range diam.			12–19.2		
Sexual system	homothallic	homothallic	homothallic	homothallic	homothallic
Oogonia	globose with smooth walls	globose with ornamented walls (mildly stipulate)	globose with ornamented walls (warty protuberances)	globose with mostly smooth walls	globose with smooth walls
Mean diam.	24.8 ± 2.6 (14.2–30.1)	31.9 (22.2–45)	27 × 25 (19–31)	26.2 (22.3–35)	22.3 (17–32)
Range of isolate means	23.7–25.4	—	—	—	—
Tapering base	tapering base	tapering base	tapering base	tapering base	tapering base
Oospores	slightly aplerotic or aplerotic	plerotic	both	plerotic	aplerotic
Mean diam.	21.1 ± 2.2 (12.2–26.4)	27.7 (19.8–35)	20 (17–27.5)	24.2 (19.8– 29.7)	21.5 (15–26.8)
Range of isolate means	20.4–21.5	—	—	—	—
Wall diameter	2.62 ± 0.56 (1.5–4.5)	4.0	2.8	3.7	2.6
Oospore wall index	0.57 ± 0.08 (0.38–0.75)	0.64	0.67	0.66	0.53

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Table 3. (Continued).

Species /Trait	*P. mammiformis*	*P. agathidicida*[1,3]	*P. castanae*[1,2,3]	*P. cocois*[1,3]	*P. heveae*[1,2,3]
Antheridia	amphigynous	amphigynous	amphigynous	amphigynous	amphigynous
Length × breadth mean	10.3 ± 1.7 × 9.8 ± 1.3	12 × 11.1	—	—	—
Length × breadth range	6–15.5 × 6–13	7.4–17.5 × 7.4–15	—	—	—
Growth characteristics					
Max. temperature (°C)	>30–<35	25	30	30	32.5
Opt. temperature (°C)	25	21.5	22.5	22	22.5
Min. temperature (°C)	>4–<10	6	6	10	10
Lethal temperature (°C)	35	—	—	—	—
Growth rate on V8A at optimum (mm/day)	7.38 ± 0.92	5.7	5.8	5.8	5.8
Colony morphology (V8A)	no distinct pattern	weakly stellate radial	no distinct pattern	no distinct pattern	no distinct pattern

[1] Weir *et al.* (2015), [2] Erwin and Ribeiro (1996), [3] Abad *et al.* (2023). *: There is ambiguity on whether *P. heveae* produces caducous sporangia (see Weir *et al.* 2015).

species is listed among 100 of the world's worst invasive alien species and is well established in South Africa (Engelbrecht *et al.* 2022). It is a destructive pathogen of native vegetation of South Africa's Cape Floristic Region, as well as in forestry plantations and fruit orchards across the country (Von Broembsen 1984, Linde *et al.* 1994, Paap *et al.* 2023). Its association with declining *Q. suber* was not unexpected, as this pathogen has been implicated in *Q. suber* decline and mortality in its natural range of the Iberian Peninsula and Italy (Brasier 1996, Seddaiu *et al.* 2020).

Phytophthora citrophthora (Sub-clade 2a) was reported in South Africa from citrus foot and root rot in the early 1900s, and is an important pathogen of citrus globally (Meitz-Hopkins *et al.* 2014). This species also affects numerous other woody plants of ornamental and horticultural importance (Abad *et al.* 2023a). *Celtis australis* has been reported as a host in Argentina (Palmucci & Wolcan 2020), however, our detection of *P. citrophthora* causing bleeding cankers of *C. africana* is the first report of this specific host-pathogen association. This also appears to be the first report of *P. citrophthora* causing disease on native South African flora, although Koch's postulates should be completed to confirm this observation.

Phytophthora parvispora (Sub-clade 7c) was considered to be a variety of *P. cinnamomi* prior to Scanu *et al.* (2014) redesignating it as a unique taxon, based on a multigene phylogeny and examination of morphological and physiological properties. Scanu *et al.* (2014) suggested that almost all findings of *P. parvispora* are linked to the trade in plants-for-planting, with current known outbreaks confined to plants in cultivated settings. This includes the report of Bezuidenhout *et al.* (2010), who found *P. parvispora* during surveys of diseased *Agathosma* in nurseries and fields of cultivated *Agathosma* in the Western Cape Province of South Africa. It remains to be determined whether *P. parvispora* has as wide a host range and potential to cause disease in natural ecosystems as is true for *P. cinnamomi*. However, our detection of *P. parvispora* from *Betula nigra* adds a seventh family (*Betulaceae*) to the already diverse range of host families, including monocotyledonous, dicotyledonous and coniferous plant families, recorded for this species.

Oh *et al.* (2013) conducted sampling in the KZN NBG, with rhizosphere samples yielding five species of *Phytophthora* including *P. multivora*, *P. capensis, P. frigida, P. cinnamomi* and a novel species which they designated as *Phytophthora*. sp. stellaris. This species was also recovered from stream bait and filter samples taken in the same garden. This Sub-clade 9b species is most closely related to *P. insolita* and currently little is known regarding the biology, epidemiology or origin of this species. However, in recent years extensive surveys have been undertaken globally and there is no similar sequence submitted to GenBank, thus it is likely that *Phytophthora* sp. stellaris is native to South Africa.

Our discovery of a novel Clade 5 species and description of *P. mammiformis* adds a fifth accepted species to the clade, with this still being a relatively small number compared to other clades. Species in this clade appear to have a pan-tropical origin (Abad *et al.* 2023a), and it has been suggested that additional species may be found by conducting surveys in natural ecosystems in these regions (Weir *et al.* 2015, Abad *et al.* 2023a). It is plausible that *P. mammiformis* also has a pan-tropical origin and may have been unintentionally introduced to the DBG during plant exchange conducted between countries linked to the historical botanical gardens network.

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The pathogenicity and host range of *P. mammiformis* remains to be determined, however, other species in this clade are all important pathogens. These include *P. agathidicida,* causal agent of kauri (*Agathis australis*) dieback in New Zealand; *P. castaneae,* causing trunk and fruit rot in chestnuts and fruit and heart rot in coconuts; *P. cocois*, also causing coconut fruit and heart rot; and *P. heveae*, which causes several tropical tree crop diseases including black stripe of rubber, and bud rot and nut fall of coconut (Abad *et al.* 2023a).

Of the species detected in our study, *P. cinnamomi* is the only one currently listed as an invasive (1b, invasive species that must be controlled) in the South African National Environmental Management: Biodiversity Act (NEM:BA, Act 10 of 2004) Alien and Invasive Species Regulations (NEM:BA A&IS Regulations; Department of Environment, Forestry and Fisheries 2020a, b). Although not regulated, *P. citrophthora* and *P. parvispora* have been known to be present in South Africa for many years. While there is global evidence for the importance of these species as pathogens in cultivated settings, further studies are required to determine their capacity to invade natural ecosystems of South Africa. Estimating the risk associated with newly detected novel and/or putatively alien *Phytophthora* species remains challenging. However, Burgess *et al.* (2021) suggest that extrapolating information from well-known *Phytophthora* species could assist in predicting the potential behaviour of newly described species [see also Scott *et al.* (2019), Barwell *et al.* (2021) and Marcot *et al.* (2023)]. Based on this approach, we recommend further studies, including host range pathogenicity trials, be undertaken to investigate the potential of *P. aquimorbida* and *P. mammiformis* to cause disease on native South African flora. This knowledge will further inform risk analyses, and ultimately determine whether sufficient threat is posed to support listing under the NEM:BA A&IS Regulations.

The results of this study highlight the extensive *Phytophthora* species diversity that can be found within botanical gardens, even with limited sampling. Similar to previous studies (Hulbert *et al.* 2019, Green *et al.* 2020, La Spada *et al.* 2022), the value of monitoring botanical gardens to identify *Phytophthora* first reports and new host-pathogen associations has again been demonstrated. These findings also support the value of investing in plant health monitoring and biosecurity best practice capacity development among garden staff. Doing so could assist in expanding the role of botanical gardens from sentinel sites to becoming models for best practices in plant health, thereby supporting global efforts to limit the spread and impact of plant pathogens.

ACKNOWLEDGEMENTS

The South African Department of Forestry, Fisheries and the Environment (DFFE) are thanked for funding, noting that this publication does not necessarily represent the views or opinions of DFFE or its employees. We also acknowledge additional funding from the University of Pretoria, members of the Tree Protection Cooperative Programme (TPCP) and the DSI/NRF Centre of Excellence in Plant Health Biotechnology (CPHB). The staff of the Durban Botanic Gardens and the KwaZulu-Natal National Botanical Garden in South Africa deserve special thanks for their support.

Conflict of interest: The authors declare that there is no conflict of interest.

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Fig. S1: Maximum likelihood phylogeny using the single gene (ITS, *βtub, cox1* and *hsp90*) datasets for ITS Clade 5 *Phytophthora* species. The clades of *Phytophthora mammiformis* are highlighted with a coloured block. *Phytophthora podocarpi* NZFS 3642 was used as an outgroup. Numbers on the branches are bootstrap support values values (n = 1 000, only ≥ 70 % displayed)/posterior probabilities (only ≥ 0.8). Superscript T denotes material with a type status.

Table S1. GenBank accession numbers of *Phytophthora* isolates obtained from two botanical gardens in the KwaZulu-Natal Province of South Africa and status in South Africa.

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