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ARTICLE

Surveys of soil and water reveal a goldmine of *Phytophthora* diversity in South African natural ecosystems

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Abstract: *Phytophthora* species are well-known as destructive plant pathogens, especially in natural ecosystems. It is ironic, therefore, how little is known regarding the *Phytophthora* diversity in South African natural woody ecosystems. In this study, *Phytophthora* species were isolated using standard baiting techniques from 182 soil and water samples and these were identified based on ITS and *cox*I sequence data. The 171 resulting *Phytophthora* isolates resided in 14 taxa including six known species (*P. multivora, P. capensis, P. cryptogea, P. frigida, P. cinnamomi, P. cinnamomi* var. *parvispora*), the known but as yet unnamed *Phytophthora* sp. PgChlamydo, *P.* sp. emzansi, and *P.* sp. Kununurra and five novel taxa referred to as *P.* sp. stellaris, *P.* sp. Umtamvuna *P.* sp. canthium, *P.* sp. xWS, *P.* sp. xHennops. Four of the new taxa were found exclusively in water and two of these are hybrids. The most commonly isolated species from soil was *P. multivora*, a species recently described from Western Australia. *Phytophthora frigida* was isolated for the first time from stream water. With the exception of *P. cinnamomi,* very little is known regarding the biology, epidemiology or origin of *Phytophthora* in South Africa.

Key words:
Oomycetes
Phytophthora
ITS
nrDNA
*cox*I
Phylogeny
Taxonomy

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INTRODUCTION

Less than 2 % of the land surface of South Africa is covered with indigenous forests. The larger part of the country is grassland and dry savanna woodland such as semi-dessert with small shrubs and Acacia trees (Grundy & Wynberg 2001). Savanna woodlands cover 35–40 %, while plantation forests cover 1.5 % of the land area. Before 1940, small privately-owned plantations of *Acacia* or *Eucalyptus* species in Western Cape were associated with agriculture to protect crops from wind erosion and subsequent sand drift. After 1945, the Department of Forestry was established to protect indigenous forest by establishing a plantation industry based on non-native species such as *Eucalyptus* spp. from Australia and *Pinus* spp. from north and central America (Burgess & Wingfield 2001, Grundy & Wynberg 2001). South Africa is also home to three of the world's 25 Biodiversity host-spots (http://www.biodiversityhotspots.org) and many studies have been conducted to document and conserve animal and plant biodiversity. In contrast, there has been relatively little work on fungal biodiversity (Crous *et al.* 2006, Marincowitz *et al.* 2008) and almost nothing is known regarding the endemic *Oomycetes*, which are broadly treated with the fungi.

Several common *Phytophthora* species have been recovered from agricultural landscapes in South Africa, notably *P. cinnamomi, P. cactorum, P. citrophthora, P. citricola, P. megasperma, P. cryptogea, P. drechsleri, P. infestans, P. nicotianae, P. syringae,* and *P. porri* (Crous *et al.* 2000). Eight *Phytophthora* spp. have been recovered from plantations of non-native species, mainly located in KwaZulu-Natal, Eastern and Western Cape, and Mpumalanga. These include *P. boehmeriae, P. cinnamomi*, *P. cryptogea*, *P. nicotianae*, *P. meadii, P. frigida*, and *P. alticola* (Zeijlemaker 1971, Bumbieris 1976, Wingfield & Knox-Davies 1980, Linde *et al.* 1994, Roux & Wingfield 1997, Maseko *et al.* 2001, Maseko *et al*. 2007). All the *Phytophthora* species commonly found in agriculture and forestry are considered introductions to South Africa. *Phytophthora alticola* and *P. frigida* are the only species known exclusively from South Africa, and they could be endemic to the region.

The species most studied from natural ecosystems in South Africa is the devastating pathogen *P. cinnamomi* (von Broembsen 1984a, von Broembsen & Kruger 1985). It is also commonly recovered from dying *Proteaceae*, including commercially cultivated members such as *Protea* spp., *Leucodendron* spp., and *Leucospermum* spp., mostly in the

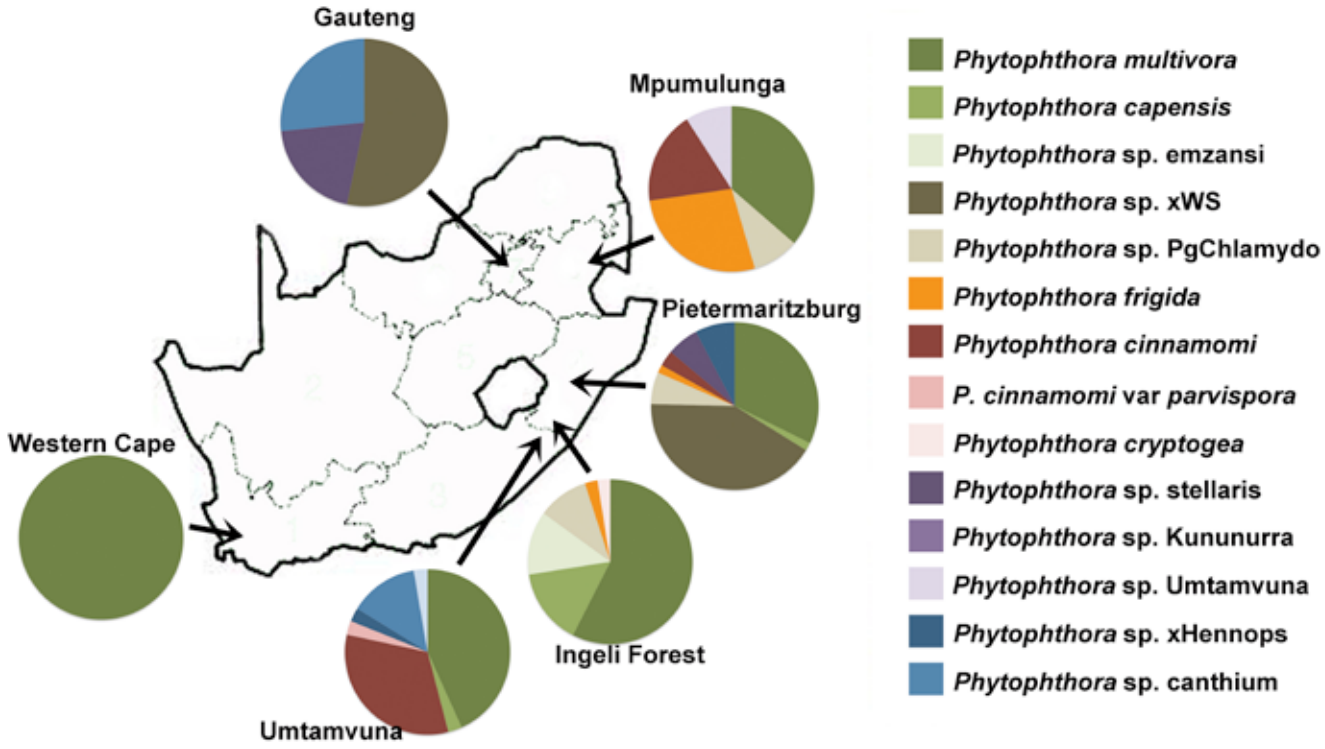

Fig. 1. Diversity and distribution of *Phytophthora* species from six sampling sites in South Africa.

Cape Province (Knox-Davies 1975, von Broembsen 1984b, von Broembsen & Kruger 1985).

In 2010, *P. capensis* and *P.* sp. emzansi[1] were identified from the cultivated endemic shrubs *Agathosma betulina*, *Olea capensis*, and *Curtisia dentate,* and also stream water, in Cape Province (Bezuidenhout *et al.* 2010). The original isolates of *P. capensis* had been reported as "*P. citricola* complex" (CIT4) in an earlier study (Oudemans *et al.* 1994). Additionally, *P. cinnamomi*, *P. cinnamomi* var. *parvispora*, *P. citricola*, *P. cryptogea*, *P. dreschsleri, P. multivora*, *P. nicotianae,* and *P. plurivora* were identified on diseased *Agathosma* spp. in commercial fields and nurseries (Bezuidenhout *et al.* 2010). In a recent study, *P.* sp. PgChlamydo and several hybrid species have recently been reported from a stream within a botanical garden in Gauteng (Nagel *et al*. 2013b). Other than the latter two studies and the considerable body of literature on the impact of *P. cinnamomi* in natural and managed ecosystems in South Africa, there have been no recent studies examining the diversity, biology or impact of *Phytophthora* species in natural ecosystems. The aim of the present study was thus to broaden our knowledge of the genus in the country by collections of the genus from such environments.

[1]Informal names have been used in the past for some species discussed here, and also for some that are newly reported. This practice is adopted pending fuller information being obtained, and formal names will be introduced where appropriate in a future publication

MATERIALS AND METHODS

Sampling and isolation

Sampling was conducted in five provinces of South Africa in different climatic zones (Table 1, Fig. 1): Mpumulunga (MP; sub-tropical climate), 26 soil samples and 3 water samples from natural vegetation (one soil sample was from a *Eucalyptus* plantation); Gauteng (GT; temperate climate, over 2000 m elevation), 4 water samples from a botanical garden; Western Cape (WC; Mediterranean climate), 6 soil samples and 2 water samples from natural vegetation; Eastern Cape, Umtamvuna Nature Reserve (UM; temperate climate), 21 soil samples and 6 water samples for filtering from natural vegetation; KwaZulu-Natal, Pietermaritzburg (PMB; temperate climate), 16 soil samples, 12 water samples for filtering and 13 water samples for baiting from a botanical garden; and KwaZulu-Natal, Ingeli Forest Reserve (ING; sub-tropical climate), 52 soil samples, 9 water samples for filtering and one water sample for baiting from natural forest.

For soil baiting, 2–300 g of soil was placed in a container (12 × 22 cm stainless steel, Sunnex, UK) containing 800 mL of non-sterile distilled water. Floating litter was removed, and two intact and edge-excised Rhododendron (*R. indioum* Claude Goyet) and pear leaves were floated on the surface of water for up to 7 d until lesions appeared. The margin of the necrotic regions was excised and cut into to small pieces (5 × 5 mm) and placed onto *Phytophthora* selective medium, NARPH (Hüberli *et al*. 2000). The plates were incubated at room temperature, of approximately 22 °C for 3–7 d, and mycelium on the plates was transferred to cornmeal agar (CMA).

Water samples (1 L) were baited in the laboratory using a technique modified from Jung *et al.* (1996), where stream

Table 1. Isolates of *Phytophthora* species collected from soil and water in this study.

Province	Location	Source	No. samples	No. +ve samples	No. isolates
Mpumulunga	Lydenburg	Forest soil	15	3	5
	Schagen	Stream water (baiting)	3	2	2
	Schagen	Soil near stream	9	2	3
	Schagen	Forest soil	1	1	1
	Jonkershoek	Forest soil	5	2	2
	Jonkershoek	Stream water (baiting)	1	0	0
Western Cape	Betty's Bay, Harold Potter NBG	Garden soil	2	2	3
	Betty's Bay, Harold Potter NBG	Stream water (baiting)	1	0	0
Gauteng	Roodepoort, Crocodile, River, Walter Sisulu NBG	Stream water (baiting)	2	2	10
	Centurion, Hennops River	Stream water (baiting)	2	2	5
Kwa Zulu Natal	Pietermaritzburg	Stream water (filtering)	13	8	25
	Pietermaritzburg	Stream water (baiting)	13	7	14
	Pietermaritzburg	Soil	16	13	25
Kwa Zulu Natal	Ingeli Forest	Stream water (filtering)	9	5	9
	Ingeli Forest	Stream water (baiting)	8	1	1
	Ingeli Forest	Soil	58	22	30
Eastern Cape	Umtamvuna	Stream water (filtering)	6	3	7
	Umtamvuna	Soil	23	15	29

water was placed in a 12 × 22 cm stainless steel container (Sunnex, UK), baited with *Rhododendron indioum* leaves and whole pear and apple fruits (previously washed and surface-sterilized with 95 % ethanol). The leaves were collected after 3–4 d when necrotic symptoms were visible, and the fruits after 7 d. The baits were rinsed with sterilized dH_2O and placed on paper towels to remove excess water, sterilized in 95 % ethanol for 10–20 s, rinsed in sterilized dH_2O and dried with paper towel. Sections containing lesions were then excised and plated onto NARPH medium and purified as described above. Other water samples were filtered shortly after collection, through a 47 mm circle filter paper with 0.45 µm pore size (Whatman™, Kent, UK) using a filtering funnel and glass flask connected to a vacuum pump. Filters were then placed topside down onto the surface of NARPH medium for 2 d, after which they were removed and individual colonies were transferred to new plates.

Single hyphal tips of all putative *Phytophthora* spp. from the isolations using the various techniques were transferred to V8 agar. After purification, they were stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria.

Identification of *Phytophthora* species

Mycelium of isolates grown in 10 % V8 broth was harvested, washed with sterile distilled water, removed of excess water with filter paper, placed in a 2 mL microfuge tubes, and lyophilised with VirTris Advantage BenchTop Tray Lyophilizer (SP Scientific, UK) overnight. The dried mycelium was then transferred to new microfuge tubes with two 3 mm metal beads. Extraction of total genomic DNA and amplification of target genes by polymerase chain reaction was carried out using a modification of the protocol described by Winton & Hansen (2001).

The Internal Transcribed Spacer regions of the rDNA (ITS1, 5.8S, and ITS2) were amplified using the primers ITS6 (Cooke & Duncan 1997) and ITS4 (White *et al*. 1990) and the cytochrome oxidase subunit I (*cox*I) was amplified using the primers FM84 and FM83 (Martin & Tooley 2003) with annealing temperatures of 55 ºC and 50 ºC respectively. Amplified DNA was purified with a high pure PCR product purification kit (Roche, RSA) and sequenced with the same primers. Cloning was carried out using pGEM-T® Easy vectors (Promega, USA) following manufacturer's instructions when the sequences of the isolates could not be read due to DNA polymorphism.

Sequences of the isolates were uploaded and aligned in Geneious v. R6 (Biomatters; available from http://www.geneious.com/). The most appropriate substitution model was determined using jModelTest (Posada 2008). The TIM3+G (ITS) and GTR+I+R (*cox*I) model were selected and used in the Bayesian analysis (Ronquist & Huelsenbeck 2003). All sequences for the isolates considered in this study were submitted to GenBank and given in Figs 2–3.

RESULTS

Isolation

Isolation success varied between sites and the methods used (Table 1); soil samples from the Western Cape, Mpumulunga,

Ingeli Forest Reserve, Umtamvuna and Pietermaritzburg yielded a success rate of 100, 28, 38, 65 and 81 % respectively. Isolation success from water-baited samples in the Western Cape, Mpumulunga, Gauteng, Ingeli Forest Reserve, and Pietermaritzburg gave a success rate of 0, 50, 100, 12.5 and 53.8 % respectively. Isolation by water filtering from Ingeli Forest Reserve, Umtamvuna and Pietermaritzburg gave success rates of 55 %, 50 % and 61 % respectively. In total, 171 *Phytophthora* isolates were recovered; 98 from soil, 32 from water baiting and 41 from water filtering (Table 1).

Identification of *Phytophthora* isolates

ITS sequence data were obtained for all isolates, and their identity confirmed by firstly conducting BLAST searched in GenBank *(*www.ncbi.nlm.nih.gov/genbank/*)* and secondly by alignment to sequences of type isolates from original publications or, if these were unavailable, to the representative isolates selected for the oomycete barcode paper of Robideau *et al.* (2011). A maximum of three sequences per taxa were selected for inclusion in the complete ITS phylogenetic analysis (TreeBASE 14082). There were three taxa for which the ITS could not be directly sequenced; one group residing in Clade 6 and two in Clade 9 of the phylogeny (Fig. 2). In each case, the ITS product of representative isolates from each group were cloned and 10 cloned fragments sequenced (11 isolates of *P.* sp. xWS, 8 isolates of *P.* sp. xHennops and 3 isolates of *P.* sp. stellaris). These were then aligned to known taxa and ITS alleles representing each of the taxa were selected for inclusion in the complete ITS phylogenetic analysis.

The aligned ITS dataset consisted of 945 characters of which 492 were parsimony informative. Analysis resulted in 24 trees of 1887 steps (CI = 0.54, RI = 0.91) (Fig. 2). Fourteen *Phytophthora* spp. were identified from amongst the 173 isolates (Table 1, Fig. 2). Of these, six were of known species (*P. multivora, P. capensis, P. frigida, P. cinnamomi, P. cinnamomi* var. *parvispora,* and *P. cryptogea*), three species matched previously designated taxa (*P.* sp. emzansi, *P.* sp. PgChlamydo, *P.* sp. Kununurra) and five taxa did not correspond to any known species and are designated here as *P.* sp. xWS, *P.* sp. stellaris, *P.* sp. xHennops, *P.* sp. Umtamvuna and *P.* sp. canthium.

For three of the unknown taxa, *P.* sp. xWS, *P.* sp. xHennops and some isolates of *P.* sp. stellaris, direct sequencing of the ITS region failed. After cloning of ITS amplicons, each of these species yielded at least ITS alleles corresponding in the phylogenetic analysis to other taxa. For *P.* sp. xWS, alleles corresponding to *P. thermophila* and *P. amnicola* in Clade 6 were obtained (12 SNPs). For *P.* sp. xHennops, alleles corresponding to *P. hydropathica* and an unknown species in Clade 9 were obtained (24 SNPs). For *P.* sp. stellaris, direct sequencing was not possible due to a 1bp indel in one of the ITS alleles, however the remaining variation (3 bp) between the ITS alleles was considered to be within the range of intraspecific variation. *P.* sp. stellaris is most similar to *P. insolata* but differs by 36 bp (4.4 %), *P.* sp Umtamvuna differs from *P. hydropathica* by 23 bp (2.55 %) and from *P.* sp. Kununurra by 19 bp (3.05 %) and *P.* sp. canthium differs from *P. kernoviae* by 42 bp (5.1 %). In the phylogenetics analysis Clade 9, separates into four sub-clades (Fig. 2).

*cox*I sequence data were obtained for most isolates and those of *P. multivora, P. capensis, P. cryptogea, P. frigida, P. cinnamomi, P. cinnamomi* var. *parvispora* and *P.* sp. emzansi returned 100 % matches to corresponding species in Blast searches on GenBank (data not shown). Phylogenic analyses was conducted including all related species in Clade 6 (Nagel *et al*. 2013), and those for which sequence data was available from Clade 9 (Fig. 3). All isolates designated as *P.* sp. stellaris had identical *cox*I alleles. Isolates designated as *P.* sp. xWS based on ITS sequence data had *cox*I alleles corresponding to either *P. thermophila* or *P. amnicola.* Isolates designated as *P.* sp. xHennops had *cox*I alleles corresponding to either *P.* sp. Kununurra or *P. hydropathica.* The latter two taxa, *P.* sp. xWS and *P.* sp. xHennops are considered to be hybrids and are designated as such by the use of the "x".

Distribution of *Phytophthora* isolates

With the exception of three isolates of *Phytophthora multivora* recovered from dying *Rapanea* collected in the Harlod Porter Botanical Garden in Western Cape Province, all other isolates were recovered from soil associated with asymptomatic plants or from water (Table 2, Fig. 1). *Phytophthora multivora* was the most frequently isolated species (40 % of all isolates) and was recovered from all locations except GT (Fig. 1). It was almost always recovered from the soil, except for three isolates recovered from filtered water (Table 2). *P. cinnamomi* (9.25 % of the isolates) was also recovered only from soil in UM, PMB and MP (Table 2, Fig. 1). Of the other known species, *P. capensis* (4.6 % of the isolates) was recovered from soil at PMB, UM and ING and once from filtered water in ING, *P. cryptogea* was recovered once from soil in ING and *P. cinnamomi* var *parvispora* was recovered once from filtered water in UMT (Table 2, Fig. 1).

Of the previously designated taxa, *P.* sp. emzansi (2.9 % of the isolates) was recovered only from Ingeli forest, where it was found in both soil and filtered water, *P.* sp. PgChlamydo (5.2 % of the isolates) was recovered from ING, PMB and MP, predominantly from water, but the isolate from MP was from soil on a riverbank (Table 2, Fig. 1). An isolate with a 100 % ITS sequence match to *P.* sp. Kununurra was recovered once from soil in MP.

The remaining isolations, with the exception of the isolate designated as *P.* sp. canthium from Umtamvuna, were of previously undescribed taxa recovered from water either through baiting or filtering (Table 2). *Phytophthora* sp. stellaris (4 % of the isolates recovered from MP and PMB) and *P.* sp. Umtamvuna (rarely recovered in UM), both represent novel species residing in ITS Clade 9. Hybrid taxon *P.* sp. xWS (20 % of the isolates) was recovered from PMB and GT and *P.* sp. xHennops was recovered from UMT, PMB and GT (Table 2, Fig. 1).

DISCUSSION

Fourteen *Phytophthora* taxa were isolated from soil and water associated with asymptomatic vegetation in natural ecosystems of South Africa. Six of the taxa were of the known species, *P. multivora, P. capensis, P. frigida, P. cinnamomi, P. cinnamomi* var. *parvispora,* and *P. cryptogea,* and three match

Table 2. Distribution of *Phytophthora* species across sampling substrates and locations.

Locality	Substrate	*Phytophthora multivora*	*Phytophthora capensis*	*Phytophthora* sp. emzansi	*Phytophthora* sp. xWS	*Phytophthora* sp. PgChlamydo	*Phytophthora frigida*	*Phytophthora cinnamomi*	*Phytophthora cinnamomi* var *parvispora*	*Phytophthora cryptogea*	*Phytophthora* sp. stellaris	*Phytophthora* sp. Kununurra	*Phytophthora* sp. Umtamvuna	*Phytophthora* sp. xHennops	*Phytophthora* sp. canthium
Ingeli Forest (ING)	Soil	21	5	2			1			1					
	Water bait					1									
	Water filter	2	1	3		3									
Umtamvuna (UTM)	Soil	15	1					12							1
	Water filter								1				1	5	
Pietermaritzburg (PMB)	Soil	20	1				1	2			1				
	Water bait				11	1					1			1	
	Water filter	1			15	3					2			4	
Western Cape (W C)	Soil	5													
Mpumulunga (MP)	Soil	4				1	1	2				1			
	Water bait						2								
Gauteng (GT)	Water bait				8						3			4	

previously informally designated taxa, *P.* sp. emzansi, *P.* sp. PgChlamydo, *P.* sp. Kununurra. The remaining five taxa did not correspond to any known species. Two of these taxa, found exclusively from water sampling, are thought to be hybrids.

Phytophthora multivora is a species recently described causing disease in natural ecosystems in Western Australia where it has a wide distribution and host range and is a pathogen of *Eucalyptus* spp., *Banksia s*pp., and *Agonis flexuosa* (Burgess *et al.* 2009, Scott *et al*. 2009). This species was previously misidentified as *P. citricola* (Burgess *et al.* 2009) and in Western Australia it has a wider distribution within natural ecosystems than *P. cinnamomi* (Burgess *et al.* 2009, Scott *et al.* 2009). It is also the dominant species in the urban environment on numerous hosts in *Myrtaceae* and *Proteaceae* (Barber *et al.* 2012). The variability within the *cox*I region led Scott *et al.* (2009) to hypothesize that *P. multivora* was endemic to Western Australia. However, similar variability was seen among isolates from South Africa in this study. Additionally, *P. multivora* was routinely isolated from the rhizosphere of non-symptomatic vegetation in South Africa, while in Australia it is associated with dead or dying vegetation. This is obviously an important species and further studies should be undertaken to determine its origin.

Phytophthora capensis and *P.* sp. emzansi have been recognized only recently from cultivated endemic plant species in the Western Cape (Bezuidenhout *et al.* 2010). The recovery of these species in other locations in South Africa shows they have a wider distribution within the region and additional isolates of *P.* sp. emanzsi will facilitate its formal description. Both species are related to *P. multivora,* and their presence in soil from natural forests where the vegetation was asymptomatic suggests they are probably endemic in South Africa.

Phytophthora frigida was first recovered from diseased roots or the rhizosphere of dying *Eucalyptus* in South Africa (Maseko *et al.* 2007). This species was not highly pathogenic to *Eucalyptus* when compared to *P. cinnamomi*, a known and serious root pathogen of *Eucalyptus* in South Africa (Linde *et al.* 1994). However, it may be a potential threat to other plants in the native vegetation, plantations or in agriculture due to the presence of inoculum in waterways. To date this species has not been recovered elsewhere in the world and it could be endemic to southern Africa. Interestingly, a recently described species from Western Australia, *P. elongata,* highly pathogenic to young *Eucalytus marginata,* is the closest relative of *P. frigida* (Rea *et al.* 2010).

Since the first report of *P. cinnamomi* in South Africa in 1933, this species has been the most widely studied *Phytophthora* species in the country. It is also the most destructive species in native vegetation of the Western Cape Province and in forestry plantations and fruit orchards widely distributed in South Africa (von Broembsen 1984a, 4b, Linde *et al.* 1994). Thus, the isolation of *P. cinnamomi* in this study was not surprising. *Phytophtora cryptogea*, although rarely encountered in this study has also been commonly isolated in

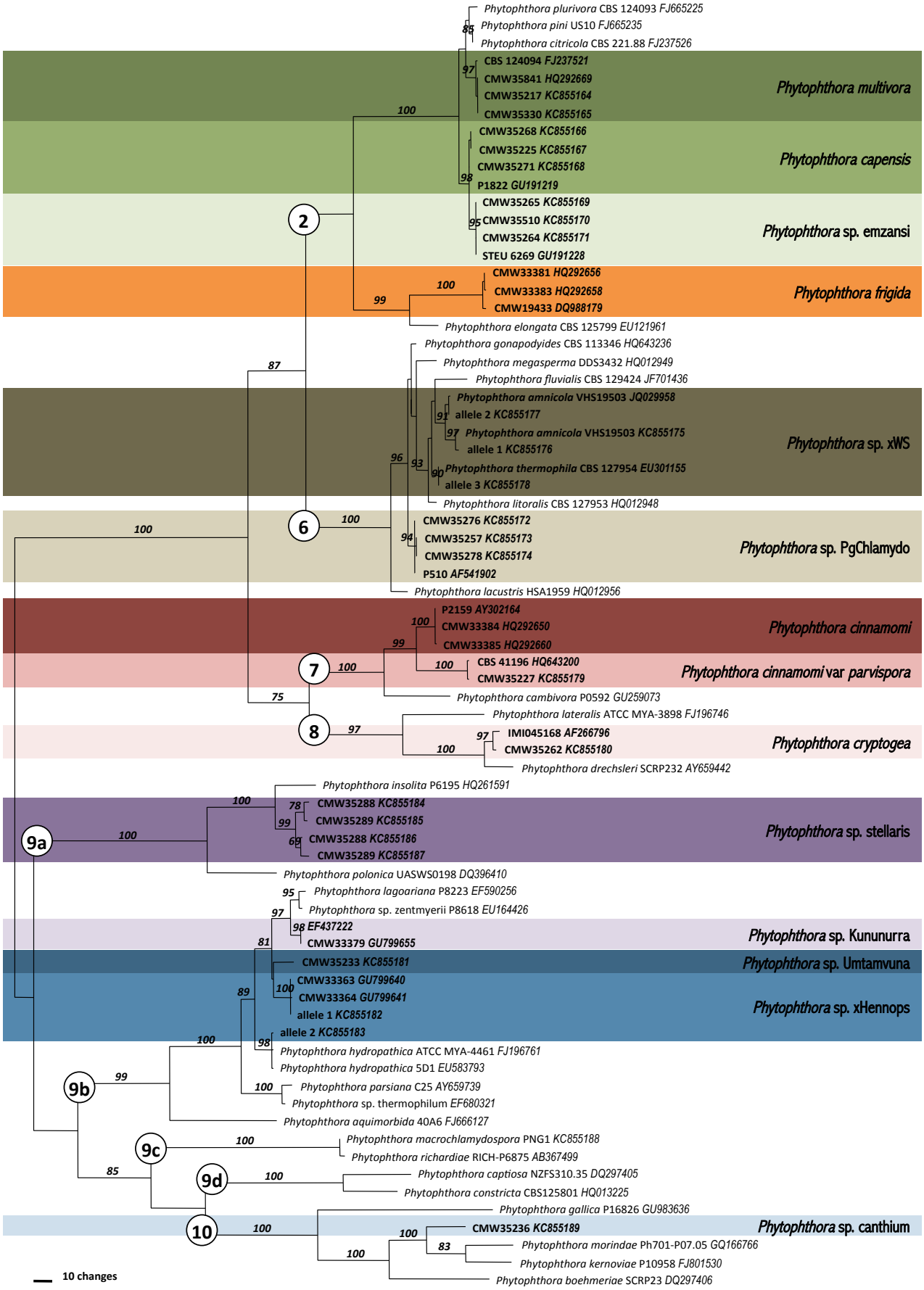

Fig. 2. A phylogram based on ITS sequence data indicating the placement of the 14 *Phytophthora* species recovered in this study in relation to closely related taxa. Numbers in circles represent the Clade as designated by Cooke *et al.* (2000). Numbers above the branch represent the bootstrap support based on parsimony analysis.

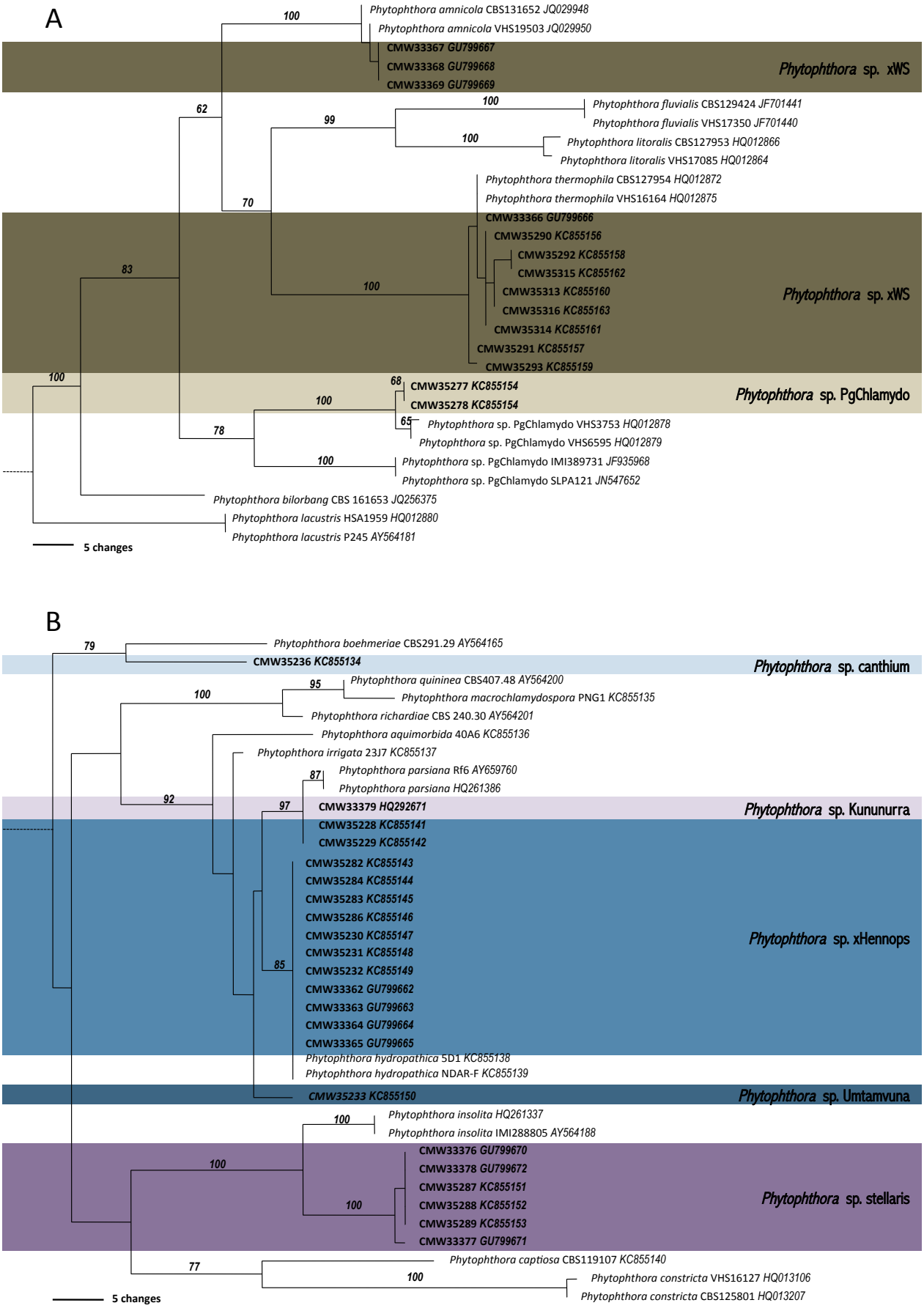

Fig. 3. Phylograms based on *cox*I sequence data indicating the placement of undescribed *Phytophthora* taxa recovered in this study in relation to closely related taxa in (A) *Phytophthora* Clade 6 and (B) *Phytophthora* Clade 9. Bootstrap support is given above the line. Numbers above the branch represent the bootstrap support based on parsimony analysis.

agricultural systems in South Africa (Crous *et al.* 2006, Nagel *et al.* 2013a).

Phytophthora sp. PgChlamydo has been recovered from stream water, soil, dying plants in many parts of the world including countries of Europe, North and South America, Australia and South Africa (Brasier *et al.* 2003, Greslebin *et al.* 2005, Burgess *et al.* 2009, Reeser *et al*. 2011, Nagel *et al*. 2013b, Hüberli *et al*. 2013). It is morphologically very similar to *P. gonapodyides* and differs only in its production of chlamydospores. Both species are frequently present in waterways and it is considered a weak pathogen and litter decomposer (Brasier *et al.* 2003, Jung *et al.* 2011).

Phytophthora sp. xWS differs from all known species or designated taxa in ITS Clade 6 (Jung *et al.* 2011). Polymorphism is observed in the ITS sequence data and two separate *cox*I alleles are obtained, one of which corresponds to *P. thermophila* and the other corresponds to *P. amnicola;* both these species have been described recently from Western Australia (Jung *et al*. 2011, Crous *et al.* 2012). *Phytophthora* sp. xWS appears to match a stable hybrid recently characterized from both Australia and South Africa (Hüberli *et al.* 2013, Nagel *et al.* 2013b).

One isolate designated as *P.* sp. canthium was recovered from soil in Umtamvuna Nature Reserve. This isolate is interesting because, based on ITS and cox1 sequence data, it resides in Clade 10. This is a sparsely populated Clade, basal to the *Phytophthora* phylogeny, presently comprising of only four species, *P. morindae, P. kernoviae, P. gallica,* and *P. boehmeriae. Phytophthora boehmeriae* is a species with a global distribution, including South Africa (Roux & Wingfield 1997). The other species in this Clade have a limited geographic distribution, with *P. kernoviae* being an invasive and damaging pathogen on ornamental and wild plant species in the UK (Brasier *et al.* 2005).

Four undescribed taxa found in this study reside in ITS Clade 9; one is an exact match for *P.* sp. Kunnunara, *P.* sp Umtamvuna is closely related to *P. hydropathica* and *P.* sp. stellaris resides in the sub-clade containing *P. insolata* and *P. polonica*. The remaining taxon, *P.* sp. xHennops, appears to be a hybrid; it has two ITS alleles, one of which is an exact match for the type isolate of *P. hydropathica.* There are also two alleles of the *cox*I among isolates of *P.* sp. xHennops (i.e. each isolate only has one *cox*I allele but was designated as *P.* sp. xHennops based on having two distinct ITS alleles), one is identical to *P. hydropathica*, the other to *P.* sp. Kununurra. Several species belonging to ITS Clade 9 have recently been described from irrigation water (Hong *et al*. 2008, Hong *et al*. 2010), however, the pathogenicity of those species is unknown and, like many Clade 6 Phytophthoras, they may be saprophytes of green litter (Brasier *et al.* 2003, Jung *et al*. 2011).

Phytophthora sp. stellaris has a single *cox*I allele, but two ITS alleles. Similarly, *P. amnicola* returned single alleles for five gene regions, while two ITS alleles, differing by a single indel of 5bp, were obtained (Crous *et al*. 2012). For *P*. sp. stellaris, an indel in the ITS allele of some isolates of *P.* sp. stellaris precluded direct sequencing, however the alleles differed by only by 3–5b p and we consider this to represent intraspecific variation.

This study has contributed considerably to the knowledge of *Phytophthora* species associated with natural vegetation in South Africa. Given that the relatively few locations considered resulted in a large number of undescribed taxa, additional surveys will undoubtedly reveal an even more substantial *Phytophthora* biodiversity in South Africa.

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