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Multiple *Phytophthora* species associated with a single riparian ecosystem in South Africa

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Abstract: The diversity of *Phytophthora* spp. in rivers and riparian ecosystems has received considerable international attention, although little such research has been conducted in South Africa. This study determined the diversity of *Phytophthora* spp. within a single river in Gauteng province of South Africa. Samples were collected over 1 y including biweekly river baiting with *Rhododendron indicum* leaves. *Phytophthora* isolates were identified with phylogenetic analyses of sequences for the internal transcribed spacer (ITS) region of the ribosomal DNA and the mitochondrial cytochrome oxidase c subunit I (*cox*I) gene. Eight *Phytophthora* spp. were identified, including a new taxon, *P.* taxon Sisulu-river, and two hybrid species from Cooke's ITS clade 6. Of these, species from Clade 6 were the most abundant, including *P. chlamydospora* and *P. lacustris*. Species residing in Clade 2 also were encountered, including *P. multivora*, *P. plurivora* and *P. citrophthora*. The detection of eight species in this investigation of *Phytophthora* diversity in a single riparian river ecosystem in northern South Africa adds to the known diversity of this genus in South Africa and globally.

Key words: Clade 6, *Phytophthora chlamydospora, P. citrophthora, P. lacustris, P. multivora, P. plurivora, P. taxon* Sisulu-river, South Africa

INTRODUCTION

Rivers and streams in riparian ecosystems play an important role in the dissemination of species in the oomycete genus *Phytophthora* and the diseases caused by these organisms. For example, decline of alder (*Alnus* spp.) in Europe and the United Kingdom, caused by *P. alni*, is much more common in trees that are within 1 m of a river (Gibbs et al. 1999). Riparian alder stands are more likely to become diseased if they share catchment areas with diseased stands (Jung and Blaschke 2004). When *P. lateralis,* which causes a lethal disease of Port Orford cedar (*Chamaecyparis lawsoniana*) in Oregon and California, is present in a stream *C. lawsoniana* trees with roots exposed to the river die within a few years (Hansen et al. 2000). Likewise *Phytophthora* diseases in agricultural and horticultural nurseries are closely linked to the presence of *Phytophthora* spp. present in irrigation water (Oudemans 1999, Yamak et al. 2002, Gevens et al. 2007, Werres et al. 2007, Ghimire et al. 2009, Orlikowski et al. 2009). Although the incidence of sudden oak death caused by *P. ramorum* appears unlinked to rivers and streams (Davidson and Shaw 2003), riparian systems play an important role in early disease detection because zoospores make their way into nearby streams when *P. ramorum* is present in an area (Sutton et al. 2009).

Multiple *Phytophthora* spp. often occur simultaneously in rivers, and high diversity of Clade 6 *Phytophthora* spp. often is present (Brasier et al. 2003, Jung et al. 2011, Hansen et al. 2012). For example, in North Carolina stream monitoring for *P. ramorum* revealed numerous *Phytophthora* species, including *P. cambivora*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. gonapodyides*, *P. heveae* and *P. pseudosyringae* (Hwang et al. 2008, 2011). In a similar study conducted in Oregon and Alaska, 18 *Phytophthora* spp. were recovered from streams of which *P. gonapodyides* and *P. chlamydospora* were the most frequently encountered (Reeser et al. 2011). Nine *Phytophthora* spp. were retrieved from rivers and streams in Western Australia and included only two described species (*P. cinnamomi* var. *parvispora* and *P. inundata*) (Hüberli et al. 2013). Likewise a survey of rivers in Argentinean *Austrocedrus chilensis* stands revealed the presence of five *Phytophthora* spp., namely *P. syringae*, *P. gonapodyides*, *P. cambivora*, *P. chlamydospora* and *P.* taxon raspberry (Greslebin et al. 2005). Eight *Phytophthora* spp. were retrieved from water and soil from oak forests in France (Hansen and Delatour 1999), and *P. gonapodyides* was notable for its ubiquity in the water sampled. Likewise eight *Phytophthora* spp. were identified from stream and soil baiting in China (Huai et al. 2013), where *P. chlamydospora* was the most frequently encountered species.

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The most common technique used to retrieve *Phytophthora* spp. from water is by baiting samples (Cooke et al. 2007, Gevens et al. 2007, Ghimire et al. 2009). Baiting involves placing living plant material in the water, enabling *Phytophthora* zoospores to infect this tissue, but not all *Phytophthora* spp. infect all bait equally well (Ferguson and Jeffers 1999, Cooke et al. 2007, O'Brien et al. 2009). When studying a single or relatively low number of known *Phytophthora* spp., the choice of appropriate bait is relatively easily. However, in natural ecosystems, numerous *Phytophthora* spp. are likely to be encountered (Balci and Halmschlager 2003, Brasier et al. 2003, Hüberli et al. 2010). In such cases bait selection is more complex and the possibility exists that the chosen baits could inhibit detection of one or more *Phytophthora* spp.

Phytophthora spp. are well known plant pathogens in South Africa (Nagel et al. 2013a) and include root rot of avocado (*Persea americana*) (Zentmyer 1979, Lonsdale et al. 1988) and grapevine (*Vitis* spp.) (van der Merwe et al. 1972, Marais 1979) caused by *P. cinnamomi*, and rot and wilt of several solanaceous and cucurbitaceous crops caused by *P. capsici* (Thompson et al. 1994; Labuschagne et al. 2000, 2003; Meitz et al. 2010). Likewise *Phytophthora* diseases are widespread in commercial forestry plantations, such as black butt disease of black wattle (*Acacia mearnsii*) caused by *P. nicotianae*, *P. boehmeriae* and *P. meadii* (Roux and Wingfield 1997), root and collar rot of *Pinus* spp. and *Eucalyptus* spp. caused by *P. cinnamomi* (Linde et al. 1994), and several other *Phytophthora* spp. such as *P. alticola*, *P. boehmeriae*, *P. fridiga* and *P. nicotianae* associated with diseases of *Eucalyptus* spp. (Linde et al. 1994; Maseko et al. 2001, 2007). Knowledge of *Phytophthora* spp. from native plant species is restricted to the Western Cape province, where *P. cinnamomi* infects numerous species of native Bruniaceae (Lamiales), Ericaceae (Ericales) and Proteaceae (Proteales) species making up the Fynbos vegetation (van Wyk 1973, von Broembsen 1984b, von Broembsen and Kruger 1985). In addition, several *Phytophthora* spp., including *P. cinnamomi*, *P. cryptogea*, *P. drechsleri*, *P. multivora*, *P. nicotianae* and *P.* taxon emzansi, are associated with diseases of native *Agathosma* spp. (Rutaceae, Sapindiales) used in traditional medicine (Bezuidenhout et al. 2010).

Similarly knowledge of *Phytophthora* diversity in rivers is restricted to the Western Cape province. In the late 1970s *P. cinnamomi* was present in all the major rivers of the province (von Broembsen 1984a). In the same areas *P. citricola*, *P. cryptogea* and *P. drechsleri* were present in rivers used for irrigation (von Broembsen 1989). *Phytophthora capensis* also was isolated once from a stream, although it initially was identified as *P. citricola* (Oudemans et al. 1994, Bezuidenhout et al. 2010). All these reports date to the 1980s, and *Phytophthora* species diversity in the rivers of the Western Cape province warrants reassessment. A recent investigation, conducted more or less at the same time as the present study, found a great diversity of *Phytophthora* spp. from soil and water samples from several woody ecosystems of South Africa (Oh et al. 2013).

It is important to augment our knowledge of *Phytophthora* spp. in South African river and riparian ecosystems to identify species of potential risk to agricultural, forest and natural ecosystems. This study focused on the diversity of *Phytophthora* spp. in a river associated with both a native and disturbed riparian zones in Gauteng province.

MATERIALS AND METHODS

Sampling and isolation.—Sampling was done from the headwaters of the Crocodile River (West) in Gauteng province, South Africa. Its headwaters are characterized by a relatively narrow (less than 5 m) stream that is mostly shallower than 1 m. The water is commonly higher with a more rapid flow rate in the summer months (November–February) because of higher rainfall. Crocodile River is one of the largest tributaries of the Limpopo River and has its source in the Witwatersrand mountain range. From there it flows through the Walter Sisulu National Botanical Garden (NBG), Roodepoort, Johannesburg, and residential areas and small holdings of the Muldersdrift area. Further downriver it unites with other tributaries and eventually flows into the Hartebeespoort dam. It continues to flow north, merging with the Marico river near the border of Botswana, to form the Limpopo river. The Walter Sisulu NBG is one of nine NBGs in South Africa and features the Rocky Highveld Grassland biome, characterized by a combination of grassland and savannah vegetation (Bredenkamp and van Rooyen 1996). In addition, as the Crocodile River runs through the garden, it is surrounded by a densely forested riparian zone. Outside the NBG, urbanization is developing rapidly and the river is typically surrounded by several agricultural and commercial enterprises and disturbed vegetation that includes invasive tree species, such as *Acacia mearnsii*, *Eucalyptus* spp. and *Salix* spp.

Samples were collected from three sites inside and two downstream sites outside the garden (FIG. 1). Sampling Site 1 had no riparian forest zone, but was surrounded by grassland. Site 1 was separated from the other sites by a waterfall. Sites 2 and 3 had dense riparian forests on either side of the river. Site 3 lacked the complete canopy spanning the river that characterized Site 2. The downstream sampling sites occurred approximately 10 km downstream of the garden, and the riparian zone consisted mostly of disturbed vegetation. The river at Site 4 was narrow and fast flowing, whereas at Site 5 it became broader with slower.

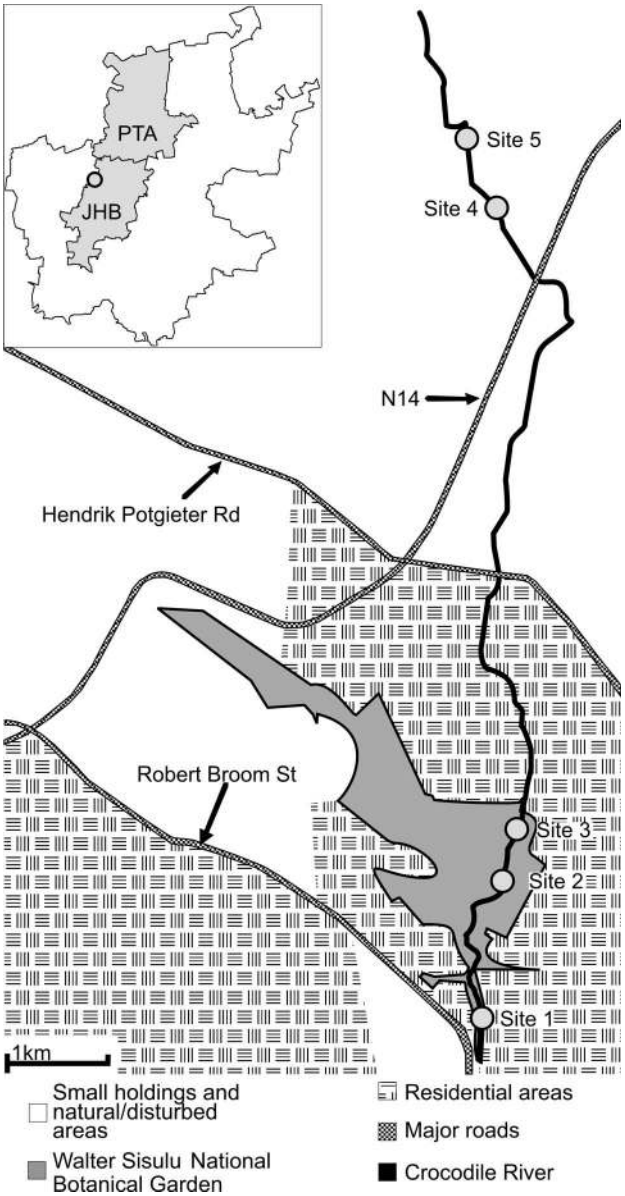

FIG. 1. Area where the stream baiting for *Phytophthora* spp. was conducted with the five sampling sites indicated. The insert is an outline of Gauteng province, and indicated in gray are the Johannesburg and Pretoria metropolitan areas. Circle indicates the location of the sampling area.

Samples were collected every 2 wk with on-site river baiting, where mesh bags containing *Rhododendron indicum* leaves were anchored in the river, as done in other studies (Hwang et al. 2008, Hüberli et al. 2013). One bag with four leaves was used per sampling site. Leaf baits were collected after 2 wk exposure and sampling was conducted over 1 y, 2009–2010, to reduce any effect of seasonal variation. Bait leaves were rinsed in distilled water and sections containing lesions were excised. These sections were surface disinfested in 70% ethanol for 10 s, rinsed in distilled water and plated onto NARPH media (Hüberli et al. 2000). NARPH plates were incubated 3–5 d at 22 C and all putative *Phytophthora* colonies were transferred to 10% V8 agar (V8A) (100 mL Campbell's V8 juice, 3 g $CaCO_3$, 16 g agar, 900 mL distilled water). Cultures of putative *Pythium* spp. isolated from leaf baits were discarded. Cultures were maintained on V8A and cornmeal agar (CMA, Sigma-Aldrich, Steinheim, Germany) at 25 C. Isolates included in the phylogenetic analyses are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA sequencing comparisons.—DNA was extracted from all isolates after they were grown 2 wk on 10% V8A at room temperature. Mycelium was harvested by scraping the surface of cultures with a sterile scalpel blade and transferring this to 1.5 mL Eppendorf tubes. DNA was extracted with the protocol described by Myburg et al. (1999).

Polymerase chain reaction (PCR) was used to amplify two gene regions. The mitochondrial cytochrome oxidase c subunit I gene (*cox*I) was amplified for all isolates with primers FM84 and FM83 (Martin and Tooley 2003) to screen for various groupings or species. In addition, the internal transcribed spacers (ITS1–5.8S–1TS2) region of the nuc rDNA (ITS) was amplified for representative isolates in each group, with the ITS6 and ITS4 primers (White et al. 1990, Cooke et al. 2000). PCR mixtures contained 1× PCR reaction buffer (Roche Diagnostics, Mannheim, Germany), 2 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 2.5 units FastStart *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany), 200 μM of each dNTP, 0.45 μM of each primer, 2 μL template DNA (20–50 ng) and sterile water to a final volume of 25 μL and were performed in a 2720 thermal-cycler (Applied Biosystems, Foster City, California). PCR conditions were the same as those used in previous studies for ITS (Cooke et al. 2000, Martin and Tooley 2003). All DNA and PCR samples were electrophoretically analyzed on a 1.5 % agarose gel using gel red (Biotium, Hayward, California) as fluorescent dye and viewed under UV illumination.

PCR amplicons were sequenced in both directions with the same primers used in PCR amplification. The BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) was used and 1/16th reactions were set up to a final volume of 10 μL. Sequencing reactions were run on a ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, Foster City, California). PCR and sequencing reactions were purified by sodium acetate and ethanol precipitation (Zeugin and Hartley 1985).

Forward and reverse sequence reads were combined in CLC Main Workbench 6.0 (CLC Bio, Aarhus, Denmark). Before phylogenetic analyses, sequences for both the *cox*I and ITS gene regions were used to verify the identities of the isolates as *Phytophthora* against data in GenBank (www.ncbi.nlm.nih.gov) using the basic local alignment search tool (BLAST). Additional sequences of closely related *Phytophthora* species (SUPPLEMENTARY TABLE I) were retrieved from GenBank and from previous studies (Jung and Burgess 2009, Scott et al. 2009, Jung et al. 2011) and were aligned with the sequences generated in this study

TABLE I. *Phytophthora* spp. information and GenBank accession numbers for isolates used in the phylogenetic analyses

Identity	Reference collection No.[a]	Date	GenBank accession No.	
			ITS	*cox*I
Phytophthora amnicola ×*Phytophthora* taxon PgChlamydo	CMW37727	2009		JQ890348
	CMW37728	2009		JQ890349
	CMW37729	2010		JQ890350
	CMW37730	2010		JQ890351
	CMW37942	2009		JX272329
	CMW37943	2009		JX272330
Phytophthora thermophila × *Phytophthora amnicola*	CMW37731	2009		JQ890352
	CMW37732	2009		JQ890353
	CMW37733	2010		JQ890354
	CMW37734	2010		JQ890355
	CMW37947	2010		JX272331
	CMW37946	2009		JX272332
	CMW37948	2009		JX272333
	CMW37944	2010		JX272334
	CMW37945	2009		JX272335
Phytophthora taxon Sisulu-river	CMW37889	2009	JX272355	JX272336
	CMW37937	2009		JX272337
	CMW37995	2009	JX272356	JX272338
	CMW37996	2009		JX272339
Phytophthora lacustris	CMW37939	2009	JX272357	JX272340
	CMW37998	2010	JX272363	JX272346
	CMW37902	2010	JX272358	JX272341
	CMW37896	2010	JX272359	JX272342
	CMW37999	2009	JX272364	JX272347
	CMW37897	2009	JX272360	JX272343
	CMW37898	2009	JX272361	JX272344
	CMW37941	2010	JX272362	JX272345
Phytophthora chlamydospora	CMW37892	2009	JX272365	JX272348
	CMW37893	2010	JX272366	JX272349
	CMW37894	2010	JX272367	JX272350
	CMW37997	2010	JX272368	JX272351
Phytophthora citrophthora	CMW37890	2010	JX272354	JX272327
Phytophthora multivora	CMW37891	2009	JX272353	JX272327
Phytophthora plurivora	CMW37938	2009	JX272352	JX272328

[a] CMW = culture collection of the Forestry and Agricultural Biotechnology Institute (FABI).

(TABLE I) with MAFFT (mafft.cbrc.jp/alignment/server/index.html) (Katoh et al. 2005).

Isolates obtained in this study grouped in clades 2 and 6 of the classification of Cooke (Cooke et al. 2000). The ITS and *cox*I data were not combined because of differences in the mode of inheritance between nuclear and mitochondrial genes. Furthermore, sequence data from clades 2 and 6 were compiled into two separate datasets and subjected to phylogenetic analyses. The shorter sequence lengths of many reference taxa in the Clade 2 *cox*I dataset would have truncated the Clade 6 dataset if the two clades had been combined. The outgroup for both the ITS and *cox*I Clade 6 phylogenies was an isolate of *P. multivesiculata*. Although this species does reside in Clade 2, it was chosen because it is basal to all other known Clade 2 species. The outgroup taxon for the Clade 6 ITS phylogeny was *P. cinnamomi* because of the proximity of Clade 7 to Clade 6 in some phylogenies (Cooke et al. 2000). *Phytophthora nicotianae* was chosen as outgroup for the Clade 6 *cox*I phylogeny because sequences of *P. cinnamomi* spanning the whole *cox*I region used in this study were unavailable.

Maximum parsimony (MP) analysis was performed with phylogenetic analysis using parsimony (PAUP*) 4.0b10 (Swofford 2002). The most parsimonious phylogenetic trees were generated through a heuristic search whereby the initial tree was generated randomly by 100 stepwise additions of taxa and subsequent trees were generated with the tree bisection reconnection branch swapping algorithm.

TABLE II. Sampling of *Phytophthora* spp. from five sites along the Crocodile River

	T-A[a]	A-PG[a]	*P. lacustris*	*P. chlamydospora*	*P.* taxon Sisulu-river	*P. citrophthora*	*P. multivora*	*P. plurivora*	Total
Site 1	2	2	0	2	0	0	0	0	6
Site 2	5	18	1	4	4	1	1	1	35
Site 3	13	6	1	1	1	0	0	0	22
Site 4	12	3	5	2	0	0	0	0	22
Site 5	9	1	7	0	0	0	0	0	17
Total	41	30	14	9	5	1	1	1	102

[a] Hybrid identity: T-A = *P. thermophila* × *P. amnicola;* A-PG = *P. amnicola* × *P.* taxon PgChlamydo.

All characters were unordered and of equal weight and gaps in the alignments were regarded as fifth characters. A thousand bootstrap replicates were performed to calculate branch and branch node support values (Felsenstein 1985).

Bayesian statistical inferences were used to generate phylogenetic trees and node support probability values through the metropolis-coupled Monte Carlo Markov chain (MC^3) algorithm (Geyer 1991) to support results obtained through MP. Each locus was subjected to hierarchical likelihood ratio tests (hLRT) using MrModeltest 2.2 (Nylander 2004) to determine the optimal evolutionary model. Bayesian analyses were done with MrBayes 3.1 (Ronquist and Huelsenbeck 2003), and each analysis was run for 3 000 000 generations. Tracer 1.4 (Rambaut and Drummond 2003) was used to determine burn-in values before parameter and tree summarization.

RESULTS

Sampling and isolation.—A total of 102 *Phytophthora* isolates were retrieved from the five sites across the 12 mo sampling period. The majority of the isolates were sampled during winter and spring months. The most isolates were retrieved from Site 2, followed by both sites 3 and 4 (TABLE II). The fewest isolates were retrieved from Site 1.

DNA sequencing comparisons.—Four separate phylogenies were created for clades 2 and 6 taxa (TreeBASE S14125). All datasets had significant phylogenetic signal ($P < 0.01$) compared to random trees (Hillis and Huelsenbeck 1992). Maximum parsimony analyses for the Clade 2 ITS dataset yielded 19 most parsimonious trees (MPTs) with a tree length of 133 steps (SUPPLEMENTARY FIG. 1) and that for the *cox*I dataset of Clade 2 resulted in 39 MPTs with tree length of 197 steps (SUPPLEMENTARY FIG. 2). Maximum parsimony analyses of the ITS dataset for Clade 6 isolates resulted in a single MPT with a length of 285 steps (SUPPLEMENTARY FIG. 3) and 20 MPTs with a length of 469 steps for the *cox*I dataset (FIG. 2). The trees obtained for each analysis differed only in the length of the branches and by differences between the relationships of isolates within a species. The phylogenies generated by Bayesian inference corresponded well to those of the maximum-parsimony analyses.

Species could be readily identified in the analyses. In both the ITS (SUPPLEMENTARY FIG. 1) and *cox*I (SUPPLEMENTARY FIG. 2) phylogenies of Clade 2, all currently recognized *Phytophthora* spp., formed clades well supported by bootstrap and posterior probability values. The ITS and *cox*I phylogenies differed in terms of the relationships between species, but both agreed on the identities of the Clade 2 isolates recovered in this study, namely *P. citrophthora* (CMW37890), *P. multivora* (CMW37891) and *P. plurivora* (CMW 37938). Similarly both the ITS (SUPPLEMENTARY FIG. 3) and *cox*I (FIG. 2) phylogenies of the Clade 6 taxa supported the known *Phytophthora* spp. with high bootstrap and posterior probability values, although the relationships between species again differed between the two gene regions.

The Clade 6 ITS phylogeny distributed our isolates into three groups, which corresponded to two known *Phytophthora* spp. (*P. chlamydospora*, *P. lacustris*) and one group that is closely related to *P. asparagi*. The *cox*I phylogeny of this clade grouped the isolates into five distinct clades corresponding to *P.* taxon *chlamydospora*, *P. lacustris* and *P. asparagi*-like. The additional groups from the *cox*I phylogeny were further identified as *P. amnicola* (CMW37727–37730, CMW37942, CMW37943) and *P. thermophila* (CMW37731–37734, CMW37946–37948, CMW37944, CMW37945). These isolates were not be included in the ITS analyses because they gave rise to unusable data, characterized by double peaks in the chromatograms at specific sites. These isolates had been characterized and shown to be interspecific hybrids (Nagel et al. 2013b). Isolates identified in the *cox*I phylogeny as *P. amnicola* were characterized as hybrids between *P. amnicola* and *P. chlamydospora*, previously known as *P.* taxon PgChlamydo (Hansen et al. 2015) and were named *Phytophthora amnicola* × *Phytophthora* taxon PgChlamydo (A-PG) (Nagel et al.

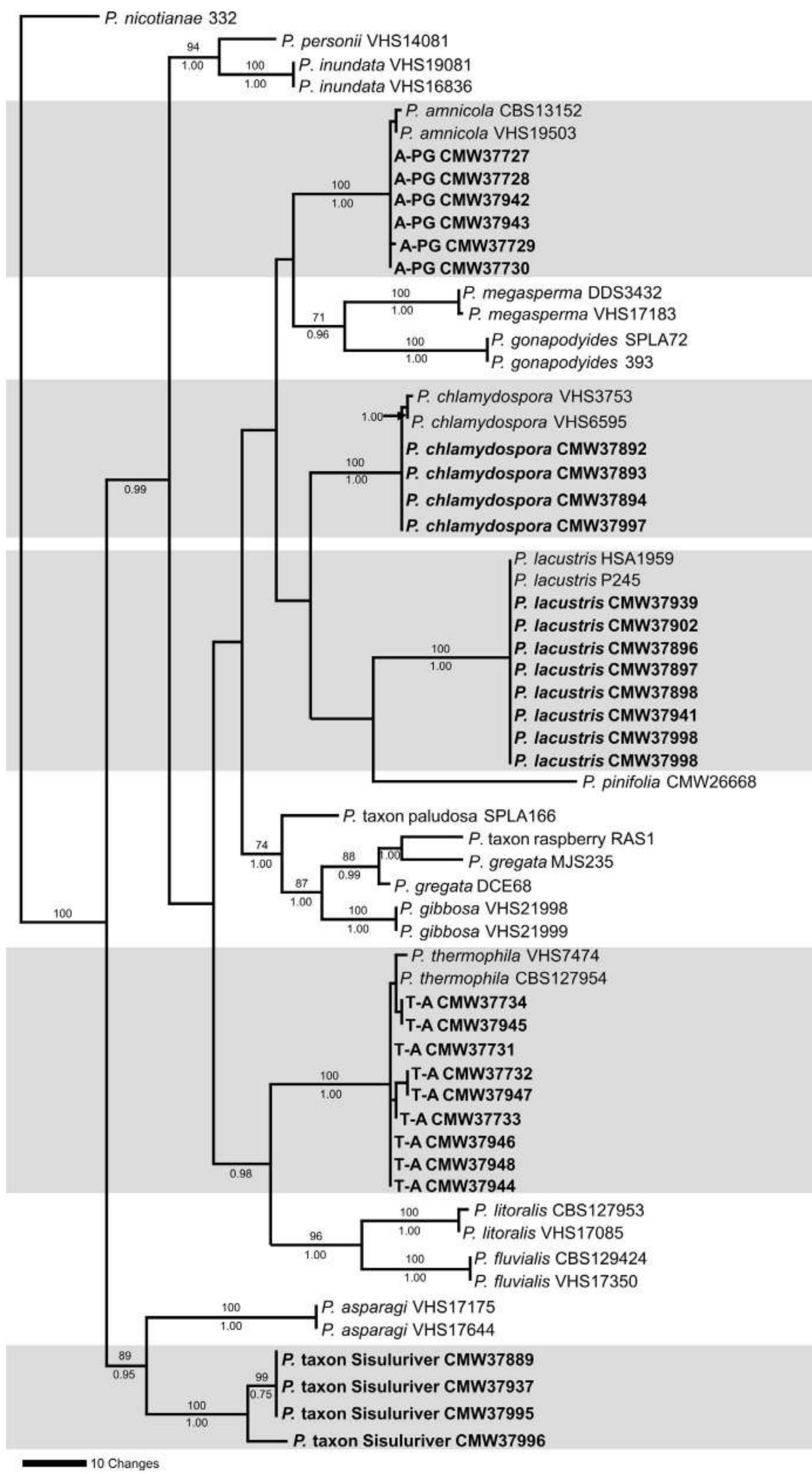

FIG. 2. Phylogenetic tree based on the *cox*I locus of the *Phytophthora* Clade 6 generated by a maximum parsimony heuristic search. Bootstrap support values appear above and posterior probabilities below branches. This tree is rooted with *P. nicotianae* as outgroup. Sequences generated in this study are indicated in boldface.

2013b). Likewise the isolates identified in the *cox*I phylogeny as *P. thermophila* were hybrids between *P. thermophila* and *P. amnicola* and following the same naming convention are identified as *Phytophthora thermophila* × *Phytophthora amnicola* (T-A). Although the identities of all other isolates were confirmed with phylogenetic analyses of the *cox*I locus, only a subset for each species was included in the ITS dataset (SUPPLEMENTARY FIG. 3).

A clade closely related to *P. asparagi* occurred in both the ITS and *cox*I phylogenies of Clade 6 (FIG. 2). These isolates consistently grouped closest to *P. asparagi* but always in a separate, well-supported clade. This group differed from *P. asparagi* by 52–54 steps in the *coxI* phylogeny and 22–24 steps in the ITS phylogeny. Given the large difference between this group and *P. asparagi*, it is regarded as a unique and previously unknown species, referred to here as *P.* taxon Sisulu-river.

Considerable variation was seen in the isolation frequencies between the taxa detected from the riparian system (TABLE II). The hybrid species described in Nagel et al (2013b), *P. thermophila* × *P. amnicola* (T-A) and *P. amnicola* × *P.* taxon PgChlamydo (A-PG) were the most frequently sampled. Isolates of A-PG were recovered from all sampling sites, although most frequently from Site 2, Isolates of T-A also were recovered from all five sampling sites, but were most prevalent at sites 3 and 4. The next most frequently encountered species was *P. lacustris*, followed by *P. chlamydospora* and *P.* taxon Sisulu-river. Isolates of *P. lacustris* were recovered from all but Site 1. Isolates of *P. chlamydospora* were recovered from all but Site 5. Isolates of *P.* taxon Sisulu-river were retrieved from sites 2 and 3. *P. citrophthora*, *P. multivora* and *P. plurivora* each were isolated only once. These three isolates were retrieved from Site 2.

DISCUSSION

Numerous *Phytophthora* spp. were collected by baiting with leaves of *Rhododendron indicum* in Crocodile River, Gauteng province, South Africa. Phylogenetic analyses revealed these to be mostly in Clade 6, but some Clade 2 species also were encountered. *Phytophthora* species in Clade 6 often are abundant in rivers and riparian ecosystems (Reeser et al. 2011, Hüberli et al. 2013). This is consistent with the view that Clade 6 species are adapted as saprotrophs on fallen leaves and other plant debris in rivers (Brasier et al. 2003, Jung et al. 2011). Eight *Phytophthora* spp. were identified including *P. citrophthora*, *P. multivora* and *P. plurivora* representing Clade 2 and two hybrid species, T-A and A-PG, *P. lacustris*, *P. chlamydospora* and *P.* taxon Sisulu-river from Clade 6. Other than for *P. citrophthora*, *P. multivora* and *P. chlamydospora*, these species have not been reported from South Africa. In addition, the novel species *Phytophthora* taxon Sisulu-river was discovered, which has a phylogenetic placement close to that of *P. asparagi*.

The five sampling sites differed in the number and identities of *Phytophthora* isolates recovered. The abundance of retrieved isolates at Site 2 could be explained by the presence of a complete foliar canopy, which decreases the direct solar radiation and increases diversity of native plants in the riparian zone. This might also account for the scarcity of isolates retrieved from Site 1 that had no canopy and where the riparian zone consisted mostly of grassland. In addition, the waterfall separating sites 1 and 2 probably restricts the movement and survival of *Phytophthora* spp. between Site 1 and the lower riparian zone.

Phytophthora citrophthora and *P. multivora*, but not *P. plurivora*, were reported previously from South Africa. *Phytophthora citrophthora* is found on all continents except Antarctica and has a wide host range (Erwin and Ribeiro 1996). It is best known as the causal agent of gummosis of *Citrus* trees, was first identified in South Africa during the 1920s and recently was implicated in a trunk disease of clementines (*Citrus reticulata*) in Western Cape province (Schutte and Botha 2008). *Phytophthora multivora* was implicated in the decline of *Eucalyptus* spp., *Banksia* spp. and *Agonis* spp. in Australia (Scott et al. 2009) and was isolated from diseased *Agathosma* spp. in Western Cape province (Bezuidenhout et al. 2010). *Phytophthora plurivora* is known from various European countries and the USA, where it occurs on a wide variety of hosts, including *Abies* spp., *Acer* spp. and *Quercus* spp. (Jung and Burgess 2009), but it was not previously reported from South Africa. Because *P. multivora* and *P. plurivora* often were identified previously as *P. citricola*, their global distribution is probably larger than is reported. For the same reason the distribution of these two species in South Africa also might be under estimated.

The identification of *P.* taxon Sisulu-river has important implications because it expands the known diversity of Clade 6, especially subclade III. *Phytophthora asparagi* and *P.* taxon sulawesiensis are the only other taxa in subclade III (Brasier et al. 2003, Jung et al. 2011). *Phytophthora* taxon Sisulu-river is more closely related to *P. asparagi* than to *P.* taxon sulawesiensis. *Phytophthora asparagi* is a well known species that causes spear and root rot of *Asparagus officinalis* in Australia, Europe, New Zealand and USA (Förster and Coffey 1993, Cunnington et al. 2005, Saude et al. 2008, Crous et al. 2012) and basal root rot of plants in the family Agavaceae in Australia (Cunnington et al. 2005). Little is known regarding

P. taxon sulawesiensis, except that it was isolated from a declining clove (*Syzygium aromaticum*) tree from Sulawesi, Indonesia. The host range of *P.* taxon Sisulu-river is unknown, but its inclusion in subclade III clarifies that taxa in this subclade are phylogenetically far removed from others in Clade 6, suggesting it represents an undersampled subclade. Thus studies of *Phytophthora* spp. diversity from unsampled environments and regions seem likely to result in the discovery of additional subclade III species.

This study is the first to report *P. lacustris* from Africa, and it represents only the second report of *P. chlamydospora* from South Africa. These species are found in Europe, North America and Australia (Brasier et al. 2003, Reeser et al. 2011, Stukely 2012). Both appear to be strongly associated with aquatic habitats where they are thought to exist as saprotrophs on plant debris (Brasier et al. 2003, Nechwatal and Mendgen 2006). However, they also infect living plants, such as *Salix* spp. and *Fraxinus* sp. in the case of *P. lacustris* (Brasier et al. 2003, Orlikowski et al. 2011) and *Prunus* sp., *Rhododendron* sp. and *Taxus* sp. in the case of *P. chlamydospora* (Brasier et al. 2003, Schwingle et al. 2007). *Phytophthora chlamydospora* recently was identified from soil and streams from the Mpumalanga and KwaZulu-Natal provinces of South Africa (Oh et al. 2013). It is not yet known what plants these species infect in South Africa, but *Salix* spp. were abundant in the disturbed areas sampled and could be the hosts.

The two hybrid species from South Africa that were characterized by Nagel et al. (2013b) were dominant in the sampled river. Isolates of T-A made up approximately 40% and those of A-PG 30% of the total isolates recovered in this study. Apart from the current study location, these hybrids have been found only in Australia (Nagel et al. 2013b). Nothing is known regarding their hosts, geographical distribution or pathogenicity. The parental species of these hybrids are thought to be *P. thermophila* and *P. amnicola* for T-A and *P. amnicola* and *P. chlamydospora* for A-PG (Nagel et al. 2013b). *Phytophthora thermophila* is found only in Australia, where it mostly is associated with environmental samples from rivers and soil and rarely from roots of *Eucalyptus marginata* (Jung et al. 2011). Likewise *P. amnicola* is known only from Australia where it was found in river and soil beneath a diseased *Patersonia* spp. plant (Crous et al. 2012). There is no evidence to suggest that *P. amnicola* and *P. thermophila* were introduced into South Africa.

The diversity of species retrieved from the stream in this study is comparable to that found in similar surveys. Studies of the diversity of *Phytophthora* species from streams retrieved between five (Greslebin et al. 2005) and eighteen (Reeser et al. 2011) species, but most isolate between seven and nine species (Hansen and Delatour 1999, Hwang et al. 2011, Huai et al. 2013, Hüberli et al. 2013). Several *Phytophthora* species are ubiquitous in riparian ecosystems, including *P. chlamydospora*, which frequently was found in riparian ecosystems in North America (Reeser et al. 2011), Argentina (Greslebin et al. 2005) and China (Huai et al. 2013).

Many Clade 6 *Phytophthora* spp. frequent in other countries are not encountered in South Africa. For example, *P. gonapodyides* was isolated frequently from water in North America (Reeser et al. 2011), UK (Brasier et al. 2003), France (Hansen and Delatour 1999) and Argentina (Greslebin et al. 2005). Likewise *P. bilorbang* (*Phytophthora* taxon OakSoil) is well known from France (Hansen and Delatour 1999, Brasier et al. 2003), North America (Reeser et al. 2011) and Australia (Aghighi et al. 2012). In Australia several other species, such as *P. amnicola*, *P. fluvialis*, *P. inundata* and *P. thermophile*, were isolated frequently from water (Jung et al. 2011, Crous et al. 2012, Hüberli et al. 2013). Given their wide distribution elsewhere, perhaps some of these species occur in South Africa but the limited geographic area sampled in the present study was insufficient to test this.

This is the first study to consider *Phytophthora* species diversity in rivers outside Western Cape province. None of the species reported from the Western Cape (von Broembsen 1989, Bezuidenhout et al. 2010) were recovered in our survey. It is difficult to contrast the species diversity found in these studies, because they were done more than 20 y ago in an ecosystem far removed from the location of our study, at a time when the taxonomy of *Phythophthora* was not yet informed by DNA sequence comparisons. Our study was conducted in a relatively small area at the headwaters of the Crocodile River. Even so an unexpectedly large *Phytophthora* diversity was found, including two interspecific hybrids, one new taxon and several *Phytophthora* species previously unknown in South Africa. Furthermore, the plant hosts of these species are unknown; it also clear is not whether any of these *Phytophthora* spp. are pathogenic to the plants species in this area. The species identified may represent threats to cultivated and natural plants in South Africa. Further effort will be required to ascertain their role as pathogens. Clearly expanded surveys and studies should be undertaken to address these questions.

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