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Phytophthora acaciae sp. nov., a new species causing gummosis of black wattle in Brazil

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ABSTRACT

A new *Phytophthora* species was found associated with gummosis in black wattle plantations in the subtropical, humid, south of Brazil. The new species *Phytophthora acaciae* is formally named herein based on phylogenetic and morphological analyses. This is the fourth *Phytophthora* species found from this pathogen complex in black wattle plantations causing gummosis in Brazil. The other three species are *P. nicotianae, P. boehmeriae,* and *P. frigida. Phytophthora acaciae* is heterothallic with amphigynous antheridia, noncaducous, papillate sporangia and is placed in the *Phytophthora* clade 2 based on nuc rDNA internal transcribed spacer (ITS1-5.8S-ITS2 = ITS) sequences. Maximum parsimony and maximum likelihood phylogenetic analyses of *P. acaciae* isolates based on multigene sequences, including partial DNA sequences of three nuclear proteincoding genes (β -tubulin, translation elongation factor-1 α , and ras-related protein), two mitochondrial protein-coding genes (cytochrome *c* oxidase subunits I and II), in addition to ITS sequence data, support the delimitation of this new species on *Acacia mearnsii* from the other previously described clade 2 *Phytophthora* species. Pathogenicity trial confirmed that the new species causes necrotic lesions on the plant stem, with either the presence or absence of gum.

INTRODUCTION

Black wattle (*Acacia mearnsii*), a tree species native to southeastern Australia, is planted in many parts of the world, including Asia (China, India, Indonesia, and Japan), South Africa, and Brazil, for tannin production, woodchips, cellulose, firewood, charcoal, and other wood products. In Brazil, this species was introduced in the 1920s for bark tannin production, and currently it is the third most important forest species planted in the country, occupying an area of approximately 170,000 ha (Chan at al. 2015).

Gummosis, caused by *Phytophthora* spp., is a major disease of black wattle in Brazil and the most common disease symptom is trunk rot. When the bark is removed, dark brown, irregular, necrotic lesions are observed underneath, which can be accompanied by gummosis or no gum exudation. The incidence of the disease can reach up to 43% in Brazilian plantations, and in severe cases the infection can lead to plant death (Santos et al. 2005).

The disease is also important in South Africa, where it has been associated with *Phytophthora nicotianae* var.

ARTICLE HISTORY

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parasitica (Dastur) G.M. Waterh. (Zeijlemker 1971), *P. meadii* McRae, and *P. boehmeriae* Sawada (Roux and Wingfield 1997). In Brazil, the disease has been recognized since the 1950s, but the first report of the association of symptoms with *Phytophthora* was not published until 1998, although the species was unidentified at that time (Santos et al. 1998). *Phytophthora nicotianae* was the first species identified as causal agent of gummosis of black wattle in Brazil (Santos et al. 2005), and later *P. boehmeriae* (Santos et al. 2006) and *P. frigida* Maseko, Cout. & M.J. Wingf. (Alves et al. 2016) were also found to be associated with the disease complex.

Disease occurrence and damage, including gummosis, is recurrent in black wattle commercial plantations in the subtropical, humid, south of Brazil. Field surveys revealed a population of *Phytophthora* isolates causing trunk rot in black wattle, but the species identification was inconclusive when based solely on morphological or molecular (internal transcribed spacer [ITS]) characteristics. This led us to examine the identity of the *Phytophthora* species by genealogical concordance phylogenetic species recognition (GCPSR; Taylor et al. 2000). This method was successful

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B Supplemental data for this article can be accessed on the publisher's Web site.

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for delimiting species boundaries in *Phytophthora* in previous studies (Blair et al. 2008; Gómez-Alpizar et al. 2008; Kroon et al. 2004; Martin and Tooley 2003; Martin et al. 2014) and is often used as the basis for recognizing new species (Maseko et al. 2007; Nelson and Abad 2010; Oliva et al. 2010; Abad et al. 2011).

The goals of our research were to identify unknown *Phytophthora* isolates associated with gummosis on black wattle and phylogenetically characterize them based on DNA sequence variation in seven loci, which included partial DNA sequences of three nuclear protein-coding genes (β -tubulin [β -*TUB*], translation elongation factor-1 α [*TEF1\alpha*], and ras-related protein [*RAS* and *RAS* intron 1]), two mitochondrial protein-coding genes (cytochrome oxidase subunit I [COXI] and subunit II [COXII]), and the nuc rDNA ITS1-5.8S-ITS2 (= ITS). A second goal was to characterize the isolates based on morphological characters, temperature-growth relationships, and pathogenicity on *A. mearnsii*.

MATERIALS AND METHODS

Sampling and isolation.—A dozen black wattle commercial plantations in southern Brazil were surveyed for gummosis, and parts of trunks with symptoms of disease were collected and taken to the laboratory for isolation of the pathogen (SUPPLEMENTARY TABLE 1). Infected bark tissue was washed thoroughly with tap water to remove impurities, and excess water was removed with filter paper. Bark segments were cut into small pieces and disinfected with 70% ethanol for 1 min followed by 0.5% sodium hypochlorite solution for 1 min and then rinsed three times with sterile distilled water. Bark segments were dried on sterilized filter paper and then placed on 2% water agar medium (20 g agar, 1 L distilled water) amended with ampicillin (50 ppm), benomyl (10 ppm), and chloramphenicol (20 ppm) (Santos et al. 1998). Petri dishes were kept at 25 C in the dark and examined after 4-6 d using a microscope. Colonies were transferred to potato dextrose agar (PDA) medium (200 g potato, 20 g dextrose, 18 g agar, 1 L distilled water). Twenty-five pure culture isolates from eight locations were obtained by the hyphal tip method and maintained on carrot agar (CA) medium (200 g carrot, 17 g agar, 1 L distilled water) under mineral oil and deposited in the fungal collection at Brazilian Agriculture Research Corporation (Embrapa Forestry, Colombo, Paraná, Brazil).

Morphophysiological and cultural characterization.

—All isolates of our new species of *Phytophthora* from black wattle were examined morphologically. Colony morphology was evaluated from 7-d-old cultures grown at 24 C on CA medium, following the methods of Erwin and Ribeiro (1996). Soil extract was prepared by adding 100 g of soil into 1 L of distilled water, shaking every 20 min for 2 h, settling overnight, and filtering. Sporangia were produced by transferring small mycelial plugs from the edge of active colonies growing on CA medium to Petri dishes containing nonsterile 10% soil extract and incubating under continuous fluorescent lights at room temperature (RT) for 96 h. Sporangial morphology was evaluated under a microscope after 72 h incubation in 10% soil water extract. For the determination of mating types A1 and A2 among our Phythophthora strains, mating type assays were performed with single-spore isolates by pairing 5-mm plugs of mycelia from all Phythophthora, sp. nov., isolates on CA medium 3 cm from a known A1 or A2 mating type tester strain of *P. frigida* then incubating in the dark at 25 ± 2 C for 5 d. Thereafter, the A1 mating type isolates AN02 and AN05 were paired with the A2 mating type isolates AN87 and AN73 to verify the formation of oospores by our new species of Phythophthora.

Characteristic morphological features and measurements of 50 randomly selected mature sporangia, chlamydospores, and gametangia were determined by making slides in lactophenol from the cultures. Measurements of sporangium length and width and chlamydospore, oogonia, and oospore diam and wall thickness were made using an ocular micrometer on a light microscope. Morphological comparisons were also made with the other *Phytophthora* species described from black wattle in Brazil, including *P. frigida*, *P. boehmeriae*, and *P. nicotianae*.

Cardinal temperatures were determined for isolates of *Phytophthora* sp. nov (n = 25), and *P. frigida* (n = 3) by cultivation on CA for 5 d at 24 ± 2 C. Mycelial plugs (6 mm diam) were transferred to Petri dishes (85 mm) containing CA medium, and isolates were incubated at temperatures of 6, 12, 18, 24, 30 and 36 \pm 2 C, in the dark, for 7 d. Triplicate Petri dishes were made for each isolate in this experiment. Radial colony growth was recorded after 3, 5, and 7 d along two perpendicular lines that intersected the center of the inoculum plug. Optimum, minimum, and maximum cardinal growth temperatures were determined. The morphophysiological characterization of our *Phytophthora* isolates were based on Waterhouse (1963, 1970), Newhook et al. (1978), Stamps et al. (1990), Erwin and Ribeiro (1996), and Ristaino (2012).

DNA extraction and sequencing.—The same isolates of *Phytophthora* sp. nov. (n = 25), were grown 7 d in

50 mL of carrot broth (200 g carrot, 1 L distilled water) at RT. The mycelium was filtered, harvested, and stored at -20 C. Genomic DNA was extracted from approximately 100 mg of frozen mycelium using the modified cetyltrimethylammonium bromide (CTAB) extraction protocol described by Doyle and Doyle (1991). DNA was dissolved in 50 µL of Milli-Q water and stored -20 C. Concentrations of total DNA were estimated by NanoDrop (ThermoFisher Scientific, Waltham, MA).

Six loci from nuclear and mitochondrial genes were sequenced using the following sets of paired oligonucleotide primers for each locus: ITS6/ITS4 (White et al. 1990) to amplify the ITS region; FM80RC/FM85 (COXI) (Mantin and Tooley 2003) and FM35/FMPhy-10 (COXII) (Martin 2000; Martin et al. 2004) to amplify two mitochondrial protein-coding genes; and TUBUF2/ TUBUR1 for β -TUB, ELONGF1/ELONGR1 for TEF1 α (Kroon et al. 2004), RASF/RASR for RAS, and IRF/IRR for RAS intron 1 (Gómez-Alpizar et al. 2008) to amplify three nuclear protein-coding genes (SUPPLEMENTARY TABLE 2). The polymerase chain reaction (PCR) conditions for each locus were (i) ITS rDNA: 94 C for 5 min, 35 cycles of denaturation at 94 C for 1 min, annealing at 48 C for 1 min, and extension at 72 C for 1 min, and final cycle at 72 C for 5 min; (ii) COXII: 94 C for 3 min, 35 cycles of denaturation at 94 C for 30 s, annealing at 58 C for 1 min and 30 s, and extension at 72 C for 1 min and 30 s, and final cycle at 72 C for 10 min; (iii) COXI, RAS, and RAS intron 1: 96 C for 2 min, 35 cycles of denaturation at 96 C for 1 min, annealing at 56 C for 1 min, and extension at 72 C for 2 min, and final cycle at 72 C for 10 min; and (iv) β -TUB and *TEF1* α , 94 C for 2 min, 35 cycles of denaturation at 94 C for 30s, annealing at 60 C for 30 s, and extension at 72 C for 1 min, and final cycle at 72 C for 10 min.

The amplified PCR products were visualized by electrophoresis on agarose gels (2%) in 1× TAE buffer (40 mM Tris, 20mM acetic acid, 1 mM EDTA, pH 8.0). Amplicons were purified with *ExoSAP*-IT reagent (Thermo Fischer Scientific, Waltham, MA) following the manufacturer's instructions and sent for sequencing to GENEWIZ (Morrisville, North Carolina) and Penn State University (University Park, Pennsylvania). Chromatograms were examined with BioEdit 7.2.5 (Hall 1999), and forward and reverse sequences were aligned and consensus sequences assembled. DNA sequences of *P. frigida* for *TEF1* α , *RAS*, and *RAS* intron 1, and *P. bisheria* for *RAS* intron 1, were obtained from DNA samples provided by Dr. Michael D. Coffey (University of California, Riverside, California).

Phylogenetic analysis.—Sequences of isolates obtained for all loci were used to verify their identities using the

BLAST tool on the National Center for Biotechnology Information (NCBI) nucleotide database (www.ncbi.nlm. nih.gov). Sequences from closely related clade 2 Phytophthora species (Martin et al. 2014) were obtained from NCBI, including ex-type sequences when available, and used in the analysis. Accession numbers for sequences generated and used in this study are included in SUPPLEMENTARY TABLE 1. Multiple sequence alignments were produced for each locus using Clustal W (Thompson et al. 1994). Phylogenetic analysis were performed for 10 isolates of the new species along with closely related clade 2 Phytophthora species using maximum parsimony (MP) and maximum likelihood (ML) analyses in PAUP* 4.0b10 (Sinauer Associates, Sunderland, Massachusetts). Bootstrap support values were calculated from 1000 replications. Phytophthora boehmeriae was used as outgroup.

Pathogenicity tests.—The unidentified *Phytophthora* isolates were inoculated onto 1-y-old black wattle plants for experiments to prove Koch's postulates. A mycelial plug from a 1-wk-old isolate grown on CA was placed on a stem wound made in *A. mearnsii* plants with a 6-mm-diam cork borer, and wounds were sealed with a strip of parafilm. Plants were kept under greenhouse conditions at temperatures ranging from 22 to 32 C. Control plants were inoculated with sterile CA plugs only. Ten seedlings trees were inoculated for each isolate and control. After 4 wk, trees were evaluated for presence of lesions at inoculation points and the presence or absence of gum exudation. Isolations were made from symptomatic trees, and the identities of the resulting isolates were confirmed by ITS sequencing.

RESULTS

Molecular phylogeny.—The aligned DNA sequences from seven loci provided phylogenetically informative characters to infer relationships among the isolates of *Phytophthora* obtained from black wattle analyzed in this study. The size of the PCR amplicons ranged from 350 bp (*RAS* intron 1) to1120 bp (*TEF1* α); however, the number of characters included in the phylogenetic analyses was smaller with the removal of ambiguously aligned bases (TABLE 1). The fragment sizes and tree statistics for the seven loci analyzed are shown in TABLE 1. Representative DNA sequences from ITS, β -*TUB*, *TEF1* α , *RAS*, *RAS* intron 1, COXI, and COXII genes were submitted to GenBank (SUPPLEMENTARY TABLE 1).

The phylogeny inferred from ITS sequence data including our new *Phytophthora* species and closely related *Phytophthora* species in the NCBI database, including

Locus	Number of characters	Syn	Aut	Number MPTs	MPT length	CI	RI
ITS	861	104	75	1	237	0.848	0.909
COXI	673	62	25	2	125	0.792	0.855
COXII	791	31	69	1	120	0.908	0.776
RAS intron 1	234	26	69	1	110	0.936	0.794
RAS	459	51	88	5	165	0.964	0.915
TEF1a	845	27	64	1	117	0.863	0.636
β-TUB	699	33	84	3	155	0.858	0.681

Table 1. Loci sequenced, number of characters and characteristics, and tree statistics for the individual loci.

Note. MPTs = most parsimonous trees; CI = consistency index; RI = retention index; Syn = synapomorphy or parsimony-informative character; Aut = autapomorphy or parsimony-uninformative character.

P. frigida, P. bisheria, P. elongata, P. multivesiculata, and *P. multivora,* revealed that our isolates formed a monophyletic clade, designated as a new species described below as *P. acaciae. Phytophthora acaciae* is phylogenetically distinct (100% bootstrap support in both MP and ML analyses) from all previously described species in the clade (FIG. 1). The tree inferred from COXI data also shows that the group of *P. acaciae* isolates formed a single well-supported clade. In both the ITS and COXI trees, the closest sister species was *P. frigida.*

The trees inferred from the *TEF1a*, β -*TUB*, COXII, RAS, and *RAS* intron 1 data sets also grouped all isolates designated as *P. acaciae* into a well-supported clade for each locus, and bootstrap support was very high (\geq 98% in MP and ML analyses) (FIG. 2). These trees show either *P. bisheria* or *P. frigida*, or both *P. bisheria* and *P. frigida*, or *P. multivesiculata* to be close sister species of *P. acacia*, depending on the locus examined. Alignments and phylogenetic trees of the ITS, COXI, COXII, RAS, RAS intron 1,

TEF1 α , and β *-TUB* were submitted to TreeBASE (study no. S20136).

Pathogenicity tests.—All isolates of *P. acaciae* that were inoculated into black wattle plants in the nursery produced gummosis symptoms. Necrotic lesions up to 4 cm long were observed 4 wk after inoculation, with either the presence or absence of gum, consistent with observation initially made in the field. The stems of the control plants inoculated with sterile CA plugs only showed small, dark brown spots at the inoculation points and no lesions. *Phytophthora acaciae* was reisolated from all infected stems; thus, Koch's postulates were completed.

TAXONOMY

Phytophthora acaciae A.F. dos Santos, T.C.A. Alves, D.J. Tessmann, K.L. Ivors & J.B. Ristaino, sp. nov. Figs. 3–6



Figure 1. Phylogenies of *P. acaciae* obtained with maximum parsimony of DNA sequences from ITS and COXI. Type strain labeled in bold. Maximum parsimony and maximum likelihood bootstrap support values >50% from 1000 replicates are shown above internodes. *Phytophthora boehmeriae* was the outgroup.



Figure 2. Phylogenies of *P. acaciae* obtained with maximum parsimony of DNA sequences from COXII, *RAS, RAS* intron 1, *TEF1a*, and β -*TUB*. Type strain labeled in bold. Maximum parsimony and maximum likelihood bootstrap support values >50% from 1000 replicates are shown above internodes. *Phytophthora boehmeriae* was the outgroup.



Figure 3. Colony morphological types of *P. acaciae* grown on CA medium after 5 d at 20 C. A–B. Rosaceous pattern.

MycoBank MB823684

Typification: BRAZIL. RIO GRANDE DO SUL: Triunfo, 29°54′21.34″S, 51°42′27.05″W, 36 m alt, dried culture isolated from black wattle trunk, 12 Nov 1999, *A. F. Santos* (holotype MBM 415188).

GenBank accessions: mitoITS = KX396303; COXI = KX396267; COXII = KX396279; RAS = KX396313; RAS intron 1 = KX396291; $TEF1\alpha$ = KX396326; β -TUB = KX396338.

Etymology: "acaciae" referring to the genus of the host plant.

Colonies on CA growing well, aerial mycelium moderate, rosaceous or slightly rosaceous (FIG. 3). Hyphal swellings not observed (FIG. 4A). All isolates producing abundant sporangia in 10% soil water extract under constant light. Sporangiophores aerial, single or loosely sympodial (FIG. 4B–C). Sporangia noncaducous, papillate, predominantly ellipsoidal, ovoid, obpyriform or of distorted shape (FIG. 4D–M); persistent with a basal attachment to the sporangiophore. Sporangia $28-85 \times 21-50 \ \mu m$ (avg. $51 \pm 7 \times 31 \pm 5 \ \mu m$), L/B 1.4:1–1.9:1 (avg. ≥ 1.6), with a broad exit pore 3–12 μm diam, papilla 6–7 μm deep, exit pore 3–12 μm diam (TABLE 2). Chlamydospores abundant in 10% soil water extract, globose, terminal or intercalary, 15–55 μm diam (avg. $32 \pm 6 \ \mu m$) (FIG. 5A–D), walls 1–5 μm thick (avg. $2 \pm 0.6 \ \mu m$) (TABLE 2).

Heterothallic, producing abundant gametangia after 5 d on CA. Oogonia were produced in pairings of *P. acacia* A1 and A2 mating type cultures. Oogonia terminal, spherical, smooth, 20–34 µm diam (avg. 25 \pm 4 µm) (TABLE 2; FIG. 5E–H). Oospores globose, aplerotic, 17–30 µm diam (avg. 22 \pm 3 µm). Oospore walls thick 2–3 µm diam. Antheridia amphigynous, elongated, cylindrical or spherical to ellipsoidal.

Cardinal temperatures: Minimum 6 C, optimum 18–24 C, maximum 36 C (FIG. 6).

Habitat: Trunk lesions accompanied by gummosis or no gum exudation, from the trunk base to ~1.5 m of 2–6-y-old black wattle commercial plantations. *Distribution*: Piratini and Triunfo, in Rio Grande do Sul State, Brazil.

Additional material examined: 16 additional strains from Triunfo, 8 from Piratini (SUPPLEMENTARY TABLE 1).

Notes: From the 25 isolates examined, 8 were A1 and 17 were A2 mating types.

DISCUSSION

A new species of Phytophthora, which we have named Phytophthora acaciae, was found associated with gummosis in black wattle plantations in the subtropical, humid, south of Brazil. Classical morphophysiological characters were combined with multilocus DNA sequence data to evaluate the taxonomic status of 25 Phytophthora isolates associated with gummosis on black wattle. The multilocus genealogies inferred from partial DNA sequences of five protein-coding genes and ITS showed that the isolates form a wellsupported monophyletic clade. This clade was recognized as a distinct lineage using the genealogical concordance phylogenetic species recognition concept (Taylor et al. 2000) and can be recognized as a phylogenetic species. However, the phylogenetic trees for each separate gene analyzed did not always infer the same relationships with the closest sister species. In the trees from ITS, COXI, and RAS, P. frigida is the closest sister species of P. acaciae (FIG. 1). However, in the trees inferred from COXII and $TEF1\alpha$, P. bisheria and P. multivesiculata are the sister species of P. acaciae, respectively. In the trees from RAS intron 1 and β -TUB, the sister species is the clade including *P. frigida* and P. bisheria. Phytophthora bisheria was not included in the tree for RAS because there were no such sequences available for that species and no culture was available (FIG. 2). In fact, considering the high bootstrap support (91%) for the clade grouping P. acaciae and P. frigida in the ITS phylogeny and the lack of support for any other competing relationships in phylogenies of other gene regions, it is reasonable to infer that P. frigida is the closest sister species of P. acacia. Phytophthora frigida was only recently reported in the same region of Brazil (Alves et al. 2016). We included only P. frigida, P. bisheria, P. multivesiculata, and P. multivora in our analysis because they were the known species that had the highest ITS sequence identity with the new species, P. acaciae.

Morphologically, *P. acaciae* is very similar to *P. frigida* and *P. nicotianae*. All species are assigned to the Group II of Waterhouse (1963) because of their noncaducous, papillate sporangia and amphigynous antheridia. Noncaducous sporangia are a character of species belonging to Group I or II of Waterhouse, but amphigynous antheridia are known only in Group II



Figure 4. *Phytophthora acaciae*. A. Primary hyphae. B–C. Loosely sympodial sporangia morphology. D–F. Ellipsoid papillate sporangia. G. Ovoid sporangium. H. Obpyriform sporangium. I–M. Sporangia irregular with distorted shapes. Bars = 10 μm.

(Stamps et al. 1990). Noncaducous, papillate sporangia were also observed in *Phytophthora* isolates from black wattle in Brazil identified as *P. nicotianae* by Santos et al. (2005) and as *P. frigida* by Alves et al. (2016).

Phytophthora boehmeriae, identified previously by Santos et al. (2006) on black wattle in Brazil, also belongs to Group II of Waterhouse but, in contrast to *P. acaciae*, has caducuous sporangia. In addition,



Figure 5. *Phytophthora acaciae*. A–C. Terminal globose chlamydospore. D. chlamydospore on a 6-wk-old culture. E–H. Spherical oogonia; plerotic oospores; amphigynous antheridia. Bars = 10 μm.



Figure 6. Radial growth rate of *Phytophthora acaciae* and *P. frigida* on CA medium at temperatures ranging from 6 to 36 C for 7 d.

P. boehmeriae is a homothallic species, whereas *P. acaciae*, *P. nicotianae*, and *P. frigida* are heterothallic. This could increase the probability of sexual recombination, which could increase genetic diversity in these pathogens (Goodwin 1997; Islam et al. 2005).

Phytophthora acaciae can be distinguished morphologically from *P. frigida* based upon sporangial shape. *Phytophthora acaciae* predominantly produces ellipsoidal sporangia with L/B ratios varying from 1.4:1 to 1.9:1 (avg. \geq 1.6), whereas *P. frigida* has ovoid sporangia and L/B ratios

varying from 1.3:1 to 1.5:1 (avg. <1.5). *Phytophthora acaciae* also grows faster at lower temperatures than *P. frigida*. *Phytophthora nicotianae* also differs from *P. acaciae* based on cardinal temperatures. Optimal growth temperature for *P. acaciae* is between 18 and 24 C, whereas the optimal growth temperatures for *P. nicotianae* range from 27 to 32 C (Erwin and Ribeiro 1996) or 24 to 32 C (Santos et al. 2005) for isolates from black wattle in Brazil. None of the *P. acaciae* isolates shared high DNA sequence identity with *P. nicotianiae*.

The species *P. acaciae, P. frigida*, and *P. nicotianae* cause similar symptoms on black wattle trunks, and they cause dark brown, irregular or necrotic lesions, accompanied by gummosis or no gum exudation, that occur at the trunk base up to approximately 1.5 m above the soil. In contrast, *P. boehmeriae* causes symptoms on trunks that include lesions without gum exudation, and these lesions are found in trees at higher levels up to 10 m high above the soil (Santos 2016a, 2016b).

Many other studies of *Phytophthora* use ITS (Cooke et al. 2000) and COXI gene (Robideau et al. 2011), *TEF1* α , β -*TUB*, and COXI genes (Kroon et al. 2004), and COXII gene to differentiate species (Martin and Tooley 2003). In addition, DNA sequences from *RAS* (Schena et al. 2007; Gómez-Alpizar et al. 2008) and *RAS* intron 1 (Gómez-Alpizar et al. 2007) are also used in oomycete molecular taxonomy to resolve

Table 2	. Morphological	characteristics of	Phytophthora	acacia sp. nov.	., P. frigida, P	P. <i>boehmeriae</i> , and	P. nicotianiae	found in	Brazil.
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		P. frigida	P. boehmeriae	P. nicotianiae
Character	P. acacia, sp. nov.	(Alves et al., 2016)	(this study and Santos et al. 2006)	(Santos et al. 2006)
No. of isolates	25	24	5	3
Main hyphae	Coralloid	Coralloid	—	—
Hyphal swellings	Spherical	Spherical	—	—
Mean width (µm)	_	_	—	—
Sporulation	Líquid media	Liquid media	Liquid media	Liquid media
Sporangia	Papillate	Papillate	Papillate	Papillate
Range (µm)	28-85 × 21-50	29–71 × 20–53	37–55 × 27 × 41	33–56 × 24–35
Lengh:breadth ratio (mean)	≥1.6	<1.5	<1.3	1.4
Lengh $ imes$ breadth mean	51 × 31	46 × 33	47 × 37	42 × 29
Shapes observed	Predominantly ellipsoid, ovoid, obpyriform	Ovoid, obpyriform	Ovoid, spherical	Ovoid
Distorted shapes	+	+	-	-
Caducity	Noncaducous	Noncaducous	Caducous	Noncaducous
Pedicel (µm)	—	_	<5	—
Sporangiophores	Loose sympodium	Loose sympodium	_	—
Exit pore (µm)	Broad (3–12)	Broad (3–9)	4–6	6
Chlamydospores	Abundant	Abundant	Infrequent	Abundant
Range (µm)	15–55	21–55	24–42	25–40
Mean diam (µm)	32	32	34	33
Wall diam (µm)	2	2	1.6	—
Oogonia				
Range diam (µm)	20–34	22–37	24–34	—
Mean diam (µm)	25	30	29	—
Oospores				
Range diam (µm)	17–30	18–31	18–31	23–38
Mean diam (µm)	22	24	25	29
Antheridia	Amphigynous	Amphigynous	Amphigynous	Amphigynous
Sex	Heterothallic	Heterothallic	Homothallic	Heterothallic

Note. + and -, presence and absence, respectively.

species, and both these DNA regions were informative in *Phytophthora* phylogeny. Interestingly, the trees inferred from β -*TUB* and *RAS* intron 1 depict a clade grouping *P. frigida* and *P. bisheria* together, but the morphological characters of these species are very different. *Phytophthora frigida* is heterothallic, with papillate sporangia, amphigynous antheridia, and maximum mycelial growth at 36 C (Alves et al. 2016), whereas *P. bisheria* is homothallic and has semipapillate sporangia, paragynous antheridia, and the maximum temperature for growth is 32 C (Abad et al. 2008).

Black wattle is native to Australia and was introduced to Brazil in the 1920s. It is unknown whether the Phytophthora species complex associated with black wattle in Brazil were introduced along with the host or whether they are naturally occurring species that have the ability to infect black wattle. The first hypothesis seems remote because Phytophthora species are not found attacking black wattle in Australia, and the second hypothesis seems more likely because P. nicotianae has many plant hosts in Brazil (Santos et al. 2014), in contrast to P. frigida and P. boehmeriae, which are only found attacking black wattle so far (Santos et al. 2006; Alves et al. 2016). Although oospores were produced in matings between P. acaciae and P. frigida during the initial mating type determination, such oospores are not necessarily of hybrid origin. In fertile matings of P. citrophthora and P. capsici, Mchau and Coffey (1994) found that the P. citrophthora isolate often failed to form gametangia and merely stimulated the production of oospores in *P. capsici*. The possibility that a similar phenomenon occurred during our interspecies matings between *P. acaciae* and *P. frigida* cannot be ruled out. Consequently, sexual compatibility and the possibility of interspecies hybridization cannot be assessed with such direct mating assays.

This and other studies indicate that gummosis of black wattle in Brazil is caused by a complex of *Phytophthora* species that now includes at least four species, including the newly named *P. acaciae*, in addition to *P. nicotianae*, *P. boehmeriae*, and *P. frigida*. Further studies to expand our knowledge on the ecology of these species, determine their migration patterns with the global movement of trees, and learn how they compete in nature and which species is potentially most destructive to black wattle plantations are needed. Such information will be useful for developing improved management strategies for the disease in plantations in Brazil.

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