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Genetic diversity of *Bursaphelenchus cocophilus* in South America

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Summary – Molecular characterisation of *Bursaphelenchus cocophilus*, the causal agent of 'red ring disease', is imperative for efficient identification procedures in Brazil and Colombia, because quarantine species such as *B. xylophilus* and *B. mucronatus* are already listed in both countries. ITS-1/2 region and D2-D3 segment of LSU rDNA were used to characterise isolates of *B. cocophilus* obtained from coconut plantations in Brazil and Colombia. Results from ITS-1/2 and LSU rDNA regions showed that all isolates of *B. cocophilus* from Brazil and Colombia formed a monophyletic group. The LSU rDNA region indicated that all isolates formed a single monophyletic group with high Bayesian posterior probability (100%). This is the first study on ITS-1/2 for the characterisation of *B. cocophilus* populations. A species-specific primer was designed for identification of *B. cocophilus*.

Keywords – Aphelenchoididae, Brazil, coconut palm, Colombia, diagnostics, ITS, LSU, phylogeny, red ring disease, species-specific primers.

Bursaphelenchus Fuchs, 1937, a genus including more than 100 described species worldwide, belongs to the family Aphelenchoididae (Kanzaki & Giblin-Davis, 2012). The most important species within this genus are the facultative plant-parasitic nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 responsible for pine wilt disease (PWD) in *Pinus* spp. forests (Mota & Vieira, 2008; Vicente *et al.*, 2012), *B. sycophilus* Kanzaki, Tanaka, Giblin-Davis & Davies, 2014, a putative plant-parasitic of *Ficus variegata* (Kanzaki *et al.*, 2014), and *B. cocophilus* (Cobb, 1919) Baujard, 1989, the causal agent of red ring disease (RRD) in coconut (*Cocos nucifera* L.), oil palm (*Elaeis guineensis* Jacq.) and peach palm (*Bactris gasipaes* Kunth). RRD is of significant economic importance in coconut (losses up to 60% in plantations) and oil palm industry in Colombia, Brazil and other countries of Latin America (Costa Rica, Ecuador, Mexico, Nicaragua, Panama, Peru, Surinam and Venezuela) (FAO, 2011). Thus, it is imperative for South American countries to be on the alert for quarantine nematode species of *Bursaphelenchus* such as *B. xylophilus* as an A1 quarantine pest (EPPO/European Plant Protection Organisation). In addition to *B. cocophilus*, the only two other *Bursaphelenchus* species described from Brazil are *B. mucronatus* Mamiya & Enda, 1979, and the mycophagous species *B. fungivorus* Franklin & Hooper, 1962 (Oliveira *et al.*, 2011; Pereira *et al.*, 2013). In Trinidad, *B. gerberae* Giblin-Davis, Kanzaki, Ye, Center & Thomas, 2006, has been found associated with the American palm weevil (Giblin-Davis *et al.*, 2006).

Considered an obligate parasite, *B. cocophilus* has not yet been successfully cultivated *in vitro*, as reported for

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B. sycophilus (Kanzaki *et al.*, 2014). Recently, numerous attempts to grow the nematode on a variety of fungal species, as well as using tissue culture, have failed (R. Souza, pers. comm.). In addition, the host range of this nematode is restricted to the family Arecaceae, parasitising more than 17 species. The life cycle of *B. cocophilus* is completed within 9-10 days (Singh *et al.*, 2013) and time of appearance of external symptoms can vary depending on the host. *Bursaphelenchus cocophilus* is mainly vectored by the American palm weevil *Rhynchophorus palmarum* L. (Griffith, 1968), the sugarcane weevil *Metamasius* sp. (Silva, 1991) and the palm weevil *Dynamis borassi* (F.) (Gerber *et al.*, 1990). It can also be transmitted through oviposition on healthy plants, faeces or direct contact of roots from a healthy to an infected plant (Araújo, 1990).

The use of molecular techniques for identification of this nematode would potentially increase accuracy of diagnosing infected palms in Brazil and Colombia, thereby avoiding misidentification with other members of *Bursaphelenchus* not yet present or reported in these countries. In recent years, much work has been done on the molecular characterisation of different *Bursaphelenchus* species (Burgermeister *et al.*, 2005, 2009; Mota *et al.*, 2006; Ye *et al.*, 2007; Zhang *et al.*, 2008; Li *et al.*, 2009; Cardoso *et al.*, 2012; Valadas *et al.*, 2012, 2013; Marek *et al.*, 2014). Within this genus, the LSU (D2-D3) and the ITS-1/2 ribosomal regions have been targeted for diversity studies (Mota *et al.*, 2006; De Luca *et al.*, 2011; Subbotin *et al.*, 2011; Cardoso *et al.*, 2012; Sultana *et al.*, 2013) and for diagnosis purposes (Burgermeister *et al.*, 2005, 2009). Both regions are used since they provide a valuable tool in the identification of nematodes (Palomares-Rius *et al.*, 2010, 2013) and reconstruction of phylogenetic relationships (Kaplan *et al.*, 2000; Ye *et al.*, 2007; Coomans *et al.*, 2012). Although molecular studies for identification of *B. xylophilus* are abundant (Cao *et al.*, 2005; Zhou *et al.*, 2007; Jung *et al.*, 2010; Cardoso *et al.*, 2012; Valadas *et al.*, 2013; Ye & Giblin-Davis, 2013), little is known about *B. cocophilus*. To date, only four sequences (SSU, LSU and mtCOI regions) of *B. cocophilus* collected in Costa Rica (one isolate) and Honduras (one isolate) from coconut and African oil palm are available in the NCBI database (Ye *et al.*, 2007). Molecular characterisation of the LSU rDNA and ITS-1/2 regions of the plant-parasitic nematode *B. cocophilus* from different geographic populations was the main purpose of this study to further explore intraspecific phylogenetic relationships of RRN populations, and design a species-specific primer for rapid identification of *B. cocophilus*.

Materials and methods

Sample collection and nematode extraction

In total, 13 coconut plantations were visited in the main producing regions of Brazil and Colombia for sample collection (Table 1). Transverse portions (discs) of infected coconut stems were cut and carried to the laboratory for nematode extraction. Stem fragments of red ring-diseased palms were chopped into small pieces. Both coloured and discoloured red ring-diseased tissues were mixed and *ca* 30 g of tissue per sample was placed in a modified nematode extraction tray with a plastic net and paper tissue. Nematodes were allowed to migrate from tissue into the water overnight. Each nematode suspension was sieved through 20 μm pore size sieves, and individually handpicked to Eppendorf tubes with DESS (dimethyl sulphoxide, disodium EDTA, and saturated NaCl) preserving solution for DNA extraction and molecular analysis (Yoder *et al.*, 2006).

DNA extraction

DNA extraction was done using a PureLink® Genomic DNA extraction kit (Life Technologies, Thermo Fisher Scientific). For each geographic region, 20 nematodes with mixed developmental stages were handpicked and transferred from the DESS solution to deionised water for 1 h to remove preserving solution. Nematodes were then placed in a cavity slide with 20 μl of PureLink® Genomic Digestion Buffer (Life Technologies), and cut using a blade. The remaining steps were followed according to the manufacturer's protocol. DNA samples were quantified in a Nanodrop2000, and then stored at -20°C until use.

DNA sequencing

Amplification of the D2-D3 segment in LSU rDNA was done using the following set of primers: forward D2A (5′-ACAAGTACCGTGAGGGAAAGT-3′) and reverse primer D3B (5′-TGCGAAGGAACCAGCTACTA-3′) (Nunn, 1992). For amplification of ITS-1/2, the forward F194 (5′-CGTAACAAGGTAGCTGTA-3′) (Ferris *et al.*, 1993) and reverse primer 5368 (5′-TTTCACTCGCC GTTACTAAGG-3′) (Vrain, 1993) were used. PCR reaction mixture (25 μl) consisted of 2.5 μl of 10× PCR Buffer (Fermentas), 0.5 μl of each primer (10 mM), 0.25

Table 1. Geographic locations and origins of coconut host of *Bursaphelenchus cocophilus* isolates used in the study.

Country	State	Collection locations	GenBank accession number	
			D2-D3	ITS
Brazil	Pernambuco	Petrolina	KT156771	
Brazil	Pernambuco	Petrolina	KT156770	KT156784
Brazil	Alagoas	Maceió	KT156769	KT156782
Brazil	Alagoas	Maceió	KT156774	KT156785
Brazil	Alagoas	Maceió	KT156773	KT156783
Brazil	Espírito Santo	São Mateus	KT156772	
Colombia	Nariño	Aguaclara	KT156778	
Colombia	Nariño	Aguaclara	KT156775	KT156787
Colombia	Nariño	Aguaclara	KT156776	KT156788
Colombia	Nariño	Tumaco	KT156777	KT156786
Colombia	Nariño	Tumaco	KT156781	
Colombia	Nariño	Tumaco	KT156779	
Colombia	Nariño	Tumaco	KT156780	

units of Taq DNA polymerase (Fermentas), 2.0 μl of 10 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas) and 3 μl of DNA template (*ca* 3-5 ng/μl). Amplification of the D2-D3 segment was done with an initial denaturation step at 95°C for 5 min, followed by 35 reaction cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 2 min, with a final extension at 72°C for 10 min. ITS-1/2 amplification was done using an initial denaturation step at 94°C for 3 min, followed by 40 reaction cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 2 min. PCR products were electrophoresed in a 1% agarose gel stained with SYBR Safe DNA gel stain (Life Technologies, Thermo Fisher Scientific), followed by purification with Exonuclease I (Exo I, Thermo Scientific) and FastAP (Thermosensitive Alkaline Phosphatase, Thermo Scientific). Purified D2-D3 and ITS-1/2 fragments were quantified and then sequenced by Macrogen.

Molecular phylogenetics

Consensus sequences were assembled from forward and reverse sequences using SeqAssem version 07/2008 (SequentiX Digital DNA Processing). Additional sequences of *Bursaphelenchus* species were retrieved from GenBank (NCBI). *Aphelenchoides besseyi* (EU325682 and JF826519) was used as outgroup in both analyses. Sequences were aligned by MAFFT version 7.212 (Katoh & Standley, 2013). Base substitution models in ITS-1/2 and D2-D3 were determined using Kakusan4 (Tanabe, 2011). Bayesian analysis was performed using MrBayes version 3.2 (Ronquist & Huelsenbeck, 2003) running, respectively, 2×10^5 (ITS-1/2) and 3.5×10^5 (D2-D3) generations and setting the burn in at 25 000 (ITS-1/2) and 50 000 (D2-D3). Convergence of runs was checked with Tracer version 1.6 (Rambaut & Drummond, 2007). Estimations of posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) were determined using the MCMC (Markov chain Monte Carlo) methods in MrBayes. The phylogenetic trees were visualised using FigTree version 1.4.2 (Rambaut, 2008).

All sequences reported in this study were deposited in GenBank under the accession numbers listed in Table 1.

Analysis of the genetic distance of populations

Genetic distances between populations were assessed by comparing the number of pairwise differences, using MEGA 5 (Tamura *et al.*, 2011). Sequences of the D2-D3 segment from phylogenetic analysis were used for the analysis of population genetic structure of *B. cocophilus*.

Species-specific PCR for the identification of *B. cocophilus*

Based on the D2-D3 and ITS-1/2 region alignment, unique regions for *B. cocophilus* were identified and selected for primer design. Primers were designed with PRIMER 3 software (Rozen & Skaletsky, 1999). NCBI blast was used to compare primers sequences and verify

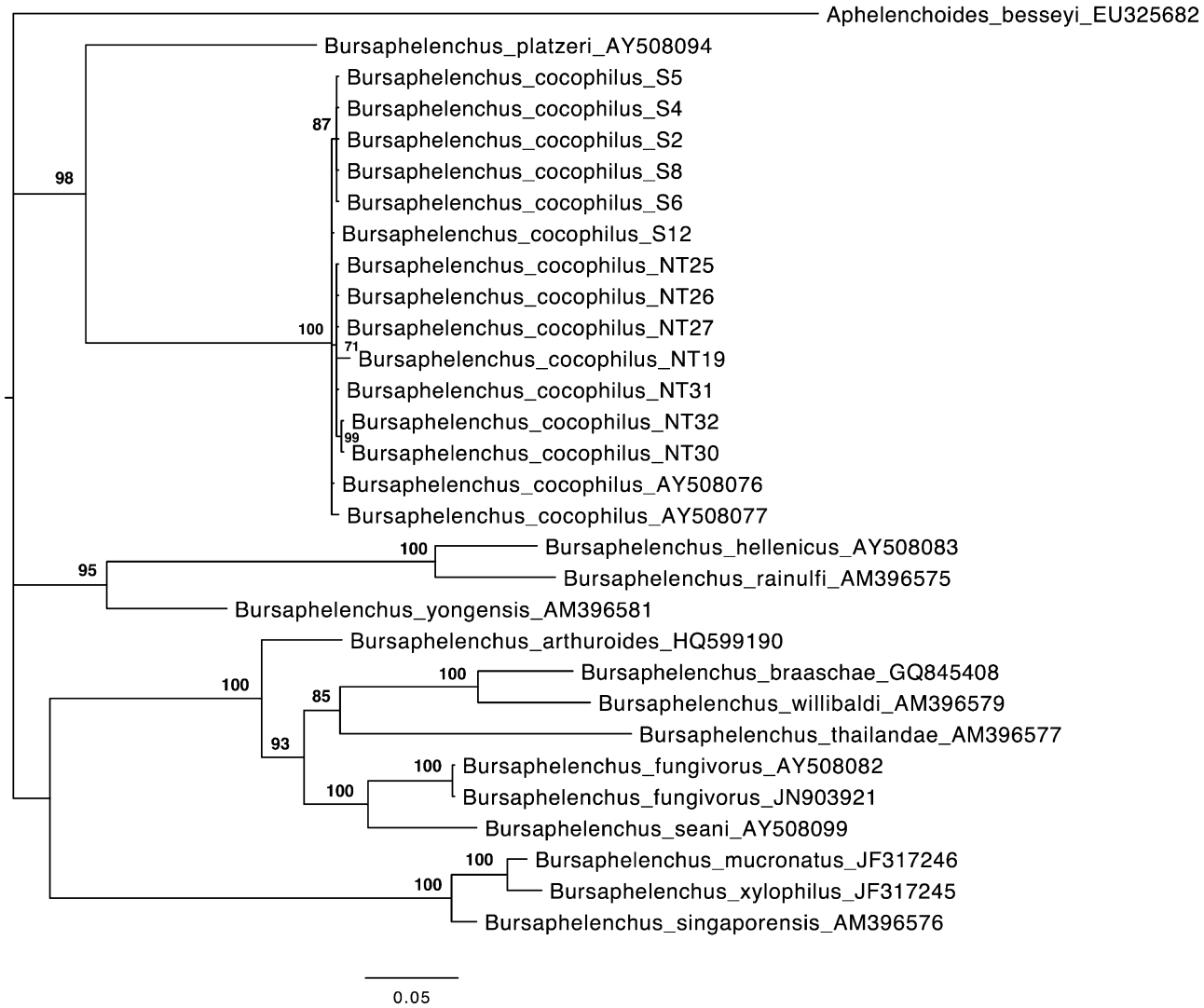

Fig. 1. Phylogenetic tree from Bayesian analysis of the D2-D3 region of 28S of *Bursaphelenchus cocophilus* and other species of *Bursaphelenchus*. The best-fit substitution model was estimated using Kakusan4. The HKY85 (Hasegawa *et al.*, 1985) Gamma model ($-\ln L = 4434.887$; $\ln Pr = 45.528$; TL = 2.257; r(A ↔ C) = 3.9^{-2}; r(A ↔ G) = 0.259; r(A ↔ T) = 0.115; r(C ↔ G) = 3.3^{-2}; r(C ↔ T) = 0.429; r(G ↔ T) = 0.125); pi(A) = 0.165; pi(C) = 0.205; pi(G) = 0.342; pi(T) = 0.288; $\alpha = 0.426$) was then used for Bayesian analysis. Bayesian posterior probability values (BPP), exceeding 50% and given in the appropriate clade, were estimated after 3.5×10^5 generations.

their homology with other previously deposited sequences in GenBank. Designed primers were used in PCR reactions with *B. cocophilus*, *B. xylophilus* and *B. fungivorus* DNA in order to validate specificity of the designed primers. For the ITS-1/2 forward primer BC1F (5′-AA CTACCGTCTTCCGCTGTCG-3′) and reverse BC1R (5′-TTGAGCACCAACACGCCGTCA-3′) were used. The PCR reaction (25 μl) was performed by using 2.5 μl of PCR Buffer (10×); 0.5 μl of each primer (10 mM); 0.25 units of Taq DNA polymerase; 2.0 μl of $MgCl_2$ (10 mM); 0.5 μl of dNTPs (mM) and 3 μl of DNA template. Amplification was carried out according to the following program: initial denaturation at 92°C for 2 min, 30 reaction cycles at 94°C for 1 min, annealing 55°C for 45 s, extension 72°C for 2 min and a final extension at 72°C for 5 min.

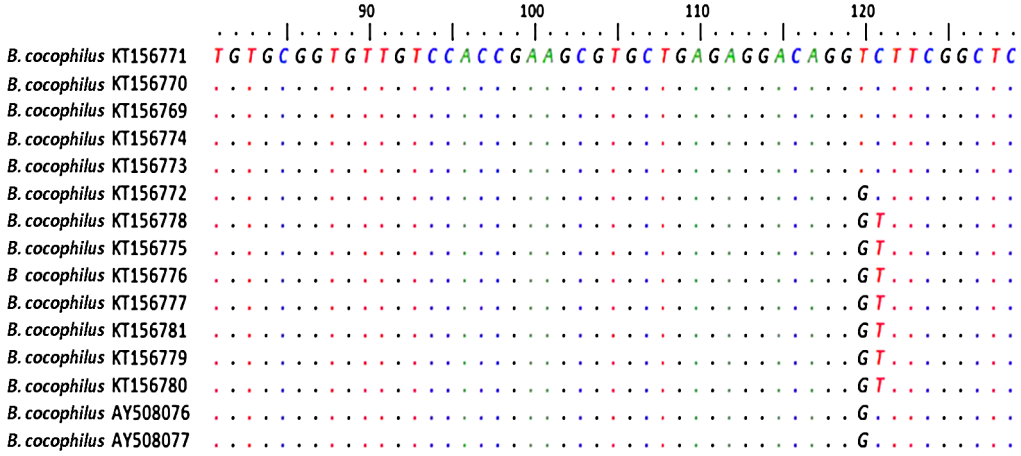

Fig. 2. Alignment of sequences of the D2-D3 region of the 28S gene isolates of *Bursaphelenchus cocophilus* from Brazil and Colombia showing transition mutation of nucleotide (C-T). This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

Table 2. Genetic distances among *Bursaphelenchus cocophilus* population based on D2-D3 28Sr RNA gene region sequence data.

Accession number	Brazil						Colombia							Costa Rica	Honduras
	71	70	69	74	73	72	78	75	76	77	81	79	80	076	077
KT156771	–	0	0	0	0	1	6	2	2	3	4	2	4	1	3
KT156770	0.000	–	0	0	0	1	6	2	2	3	4	2	4	1	3
KT156769	0.000	0.000	–	0	0	1	6	2	2	3	4	2	4	1	3
KT156774	0.000	0.000	0.000	–	0	1	6	2	2	3	4	2	4	1	3
KT156773	0.000	0.000	0.000	0.000	–	1	6	2	2	3	4	2	4	1	3
KT156772	0.002	0.002	0.002	0.002	0.002	–	5	1	1	2	3	1	3	0	2
KT156778	0.010	0.010	0.010	0.010	0.010	0.008	–	1	0	2	3	1	3	2	4
KT156775	0.003	0.003	0.003	0.003	0.003	0.002	0.006	–	0	1	2	0	2	1	3
KT156776	0.003	0.003	0.003	0.003	0.003	0.002	0.006	0.000	–	1	2	0	2	1	3
KT156777	0.005	0.005	0.005	0.005	0.005	0.003	0.008	0.002	0.002	–	1	1	1	2	3
KT156781	0.006	0.006	0.006	0.006	0.006	0.005	0.010	0.003	0.003	0.002	–	1	0	3	5
KT156779	0.003	0.003	0.003	0.003	0.003	0.002	0.006	0.000	0.000	0.002	0.003	–	0	1	3
KT156780	0.006	0.006	0.006	0.006	0.006	0.005	0.010	0.003	0.003	0.002	0.000	0.003	–	3	5
AY508076	0.002	0.002	0.002	0.002	0.002	0.000	0.008	0.002	0.002	0.003	0.005	0.002	0.005	–	2
AY508077	0.005	0.005	0.005	0.005	0.005	0.003	0.011	0.005	0.005	0.006	0.008	0.005	0.008	0.003	–

* Above diagonal shows the number of different sites and below diagonal genetic distances.

Results

A total of 13 isolates were used for molecular characterisation of *B. cocophilus* collected in Brazil and Colombia. The phylogenetic analyses of the D2-D3 segment of rDNA LSU sequences, using Bayesian analysis and a comprehensive data set of species within *Bursaphelenchus* showed that populations from Brazil, Colombia and Costa Rica and Honduras (*B. cocophilus* AY508076 and AY508077) formed a single clade with high Bayesian posterior probability (100%) and distinct from other species (Fig. 1). The genetic diversity analysis of the D2-D3 segment of LSU revealed that Brazilian isolates are identical except for isolate KT156772 which had a single nucleotide substitution (T) (Fig. 2). The Colombian populations display more diversity (Table 2), isolate KT156778 having the highest number of substituted nucleotides.

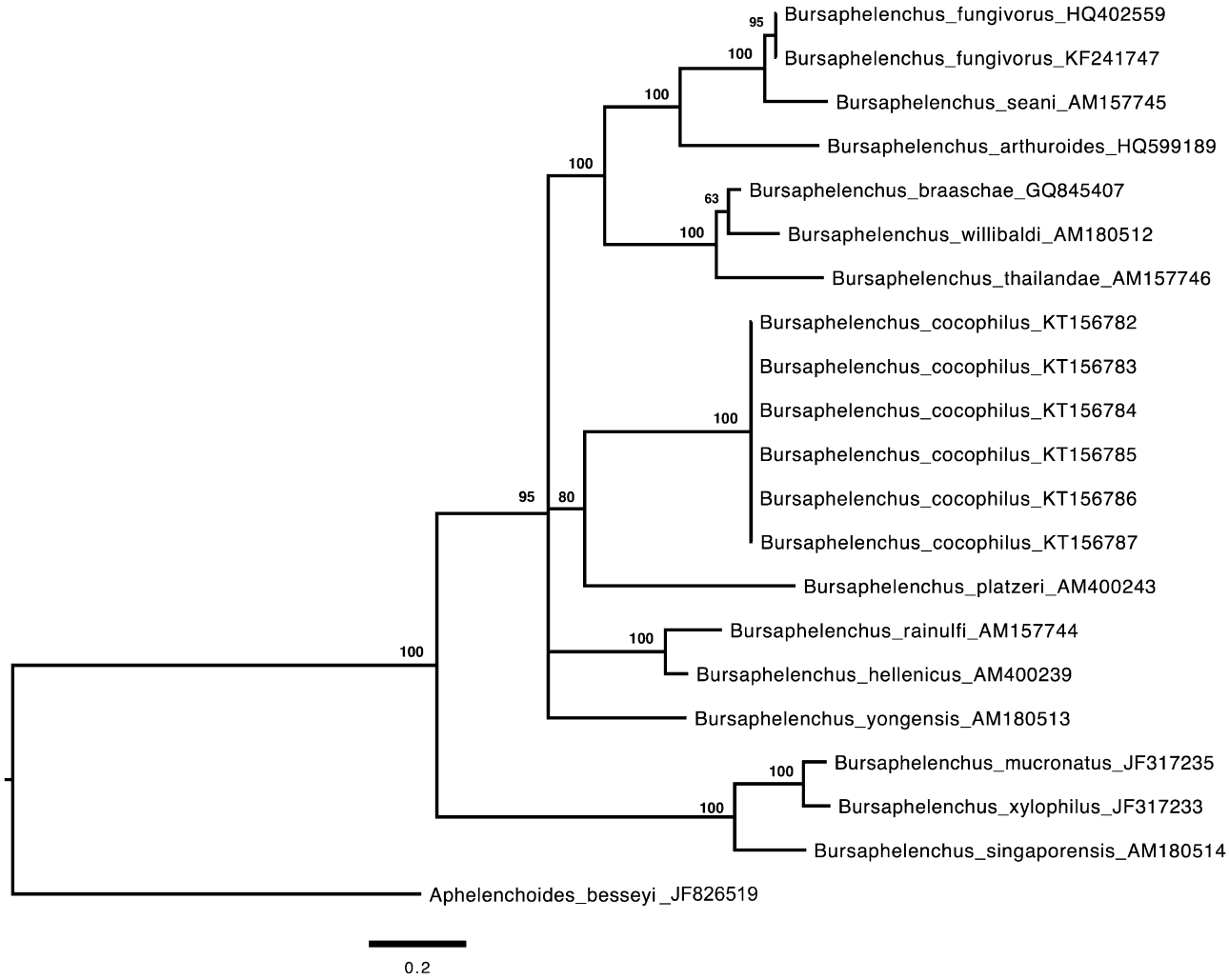

Fig. 3. Phylogenetic tree from Bayesian analysis of the ITS-1/2 region of *Bursaphelenchus cocophilus* and other species of *Bursaphelenchus*. The best-fit substitution model was estimated using Kakusan4. The GTR (Generalised time-reversible) Gamma model ($-\ln L = 5804.434$; $-\ln Pr = 12.575$; TL = 5.352; kappa = 3.621; pi(A) = 0.214; pi(C) = 0.192; pi(G) = 0.252; pi(T) = 0.343; $\alpha = 0.461$) was then used for Bayesian analysis. Bayesian posterior probability values (BPP), exceeding 50% and given in the appropriate clade, were estimated after 2×10^5 generations.

Regarding the ITS-1/2 region, *B. cocophilus* isolates from Brazil and Colombia formed a single monophyletic group, with 100% Bayesian posterior probability, and distinct from other species of the *Bursaphelenchus* (Fig. 3). From the ITS-1/2 alignment of *B. cocophilus*, a specific primer set was designed using sequence differences between other sequences of *Bursaphelenchus* species. The specific DNA sequence of the *B. cocophilus* fragment amplified with this primer set showed no similarity to other sequences in GenBank. The BC1F and BC1R primers were able to detect the target species specifically and providing PCR products with the expected size of 528 bp fragment (Fig. 4). Non-specific amplifications did not occur in any of the *B. cocophilus* isolates, and no amplification was observed for the isolates of *B. xylophilus*.

Based upon the overall topologies of trees, the sister species of *B. cocophilus* was *B. platzeri* AY508094. Analysis of both regions ITS-1/2 and D2-D3 of LSU rDNA separated *B. cocophilus* from other species listed in GenBank.

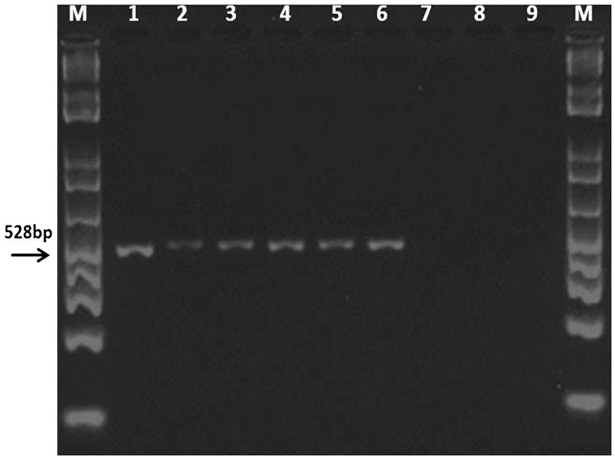

Fig. 4. Amplification of PCR products using BC1F/R species-specific primers of *Bursaphelenchus cocophilus* populations. Lane order: Lanes 1-6: KT156771; KT156770; KT156769; KT156774; KT156773; KT156772; Lane 7: *B. xylophilus*; Lane 8: *B. fungivorus*; and Lane 9: negative control. Nematode codes are as abbreviated in Table 1. Lane M represents a DNA marker of known molecular weights. The amplicon sizes are indicated by arrows.

Discussion

Although the ITS-1/2 region did not distinguish *B. cocophilus* isolates, this region has been used to analyse genetic diversity of worldwide *B. xylophilus* populations (Canada, China, Japan, Portugal, Russia and the USA), allowing group segregation depending upon country of origin. Also, this region has shown high polymorphism between *B. xylophilus* and *B. mucronatus* populations, both inter- and intra-specific (Futai & Kanzaki, 2002; Cardoso *et al.*, 2012). Likewise, De Luca *et al.* (2011) reported high variability in populations of *Pratylenchus* spp. based on this molecular marker. In addition to the ITS-1/2 region, the D2-D3 region of rDNA LSU has also been studied for plant-parasitic nematode taxonomy and species identification (Kaplan *et al.*, 2000; de la Peña *et al.*, 2007; Ye *et al.*, 2007; Múnera Uribe *et al.*, 2010; Mekete *et al.*, 2011; Oliveira *et al.*, 2011). Based on the LSU region, we could clearly distinguish Brazilian and Colombian populations. Comprehensive sequence analysis identified a single nucleotide (T) exchange specific to the Colombian populations and absent in Brazilian, Costa Rica and Honduras populations.

The north-eastern South American coast is considered to be the potential centre of origin of *B. cocophilus*, where the first palm tree symptomatic of the disease was observed and reported to occur in Trinidad (Cobb, 1919; Gerber *et al.*, 1990; Giblin-Davis *et al.*, 2013). Our analysis supports low genetic diversity between *B. cocophilus* populations from Brazil and Colombia. The variability of the D2-D3 region and ITS-1/2 for *B. cocophilus* is in accordance with that observed in *B. niphades*, where more variable sites (nucleotides alteration) were observed in D2-D3 than ITS-1/2, which showed a single-nucleotide alteration (Tanaka *et al.*, 2014). Nevertheless, this region has been useful as a DNA marker to distinguish intra- and inter-specific isolates of *B. xylophilus* and *B. mucronatus* because of their high sequence polymorphism (Futai & Kanzaki, 2002).

Other molecular markers such as 18S and COI (data not shown) were attempted but were difficult to amplify with conventional universal primers, as also observed by Ye *et al.* (2007) for a population from Costa Rica, perhaps because of the low sequence homology at the primer annealing site (Vossen *et al.*, 2014).

Moreover, we also showed that *B. cocophilus* and *B. platzeri* might have shared a common ancestor, grouping distantly from other mycophagous and plant-parasitic nematode. This last observation corroborates the inferred phylogeny studies of Ye *et al.* (2007) and Kanzaki *et al.* (2014).

Nuclear and mitochondrial regions have accumulated mutations over time, some regions being more prone to change than others. The ITS-1/2 sequence can provide useful genetic markers for species, due to their low intraspecific variation (Gasser, 2001). The ITS-1/2 region was used for the design of species-specific primers in other species of nematodes such as *Xiphinema*, for conventional PCR (Oliveira *et al.*, 2005) and real-time PCR (Van Ghelder *et al.*, 2015); *B. xylophilus* nested PCR-based (Takeuchi *et al.*, 2005) and real-time PCR (Cao *et al.*, 2005; Ye & Giblin-Davis, 2013); *Meloidogyne hapla*, *M. chitwoodi* and *M. fallax* multiplex PCR (Zijlstra, 1997) and *Pratylenchus coffeae* (Saeki *et al.*, 2003). Until now, *B. cocophilus* has been identified based only on morphological characters or by using molecular markers (28S, 18S and mtCOI) (Ye *et al.*, 2007).

Accurate and quick detection of this plant-parasitic nematode is very important to prevent the introduction and dispersal of the pest to new sites (Ye & Giblin-Davis, 2013; Van Ghelder *et al.*, 2015). The design of species-specific primers is necessary for *B. cocophilus*, since there are no indisputable morphological diagnostic characters for this species in the described members of the genus. The presence of a precloacal papilla, bursal and vulval

flap shape, and ratio a are not exclusively diagnostic for *B. cocophilus* (Giblin-Davis *et al.*, 1989).

The present study focused on the molecular characterisation of *B. cocophilus* populations from Brazil and Colombia using the most informative ribosomal DNA genetic loci currently used for nematode phylogeny (Fouly *et al.*, 1997; Kaplan *et al.*, 2000; De Luca *et al.*, 2011; Subbotin *et al.*, 2011; Marek *et al.*, 2014) and allowing the design of a species-specific primer pair for accurate identification of *B. cocophilus*. Here we characterised the genetic diversity of several *B. cocophilus* populations from Brazil and Colombia. Knowledge of the intra-specific diversity of *B. cocophilus* will enable a better understanding of ITS-1/2 population dynamics. More studies should be done with a larger number of isolates from different regions of Brazil and from other countries using other genomic loci, thus generating additional information about the genetic/population structure of *B. cocophilus*.

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