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Moniliophthora perniciosa, the mushroom causing witches' broom disease of cacao: Insights into its taxonomy, ecology and host range in Brazil

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ABSTRACT

Witches' broom caused by *Moniliophthora perniciosa* is the main disease of cacao (*Theobroma cacao*) in Brazil. The fungus is known to occur on other host families and these populations have been addressed in the literature as biotypes: C (*Malvaceae*); H (*Malpighiaceae*); L (*Bignoniaceae*) and S (*Solanaceae*). No complete elucidation of the phylogenetic relationships of isolates obtained from this disparate host range appears in the literature. One member of H (ex *Heteropterys acutifolia*) has been described as a distinct species. But should other biotypes be also recognized as distinct taxa? In the present study, a survey yielding 24 isolates of *M. perniciosa* from ten hosts and covering a wide range of geographic regions in Brazil was undertaken. These isolates were compared with those from *T. cacao* using three DNA regions for the phylogenetic analyses: ITS, LSU and RPB1. Morphology was also examined. All isolates in this study were found to belong to *M. perniciosa*, including the population from *H. acutifolia*, formerly treated as *Moniliophthora brasiliensis* but reduced here to a synonym of *M. perniciosa*. This species ranged from pathogenic to a previously unreported occurrence as a non-pathogenic endophyte in the Atlantic rain-forest tree *Allophylus edulis* (*Sapindaceae*). *M. perniciosa* was recorded on a range of solanaceous hosts (16 species) over a wide variety of ecosystems. The ecological and evolutionary significance of these novel findings are discussed.

1. Introduction

Moniliophthora perniciosa (*Marasmiaceae*: *Agaricales*) is a major pathogen of *Theobroma cacao* (*Malvaceae*) in South America causing the infamous witches' broom disease of cacao (Baker and Holliday, 1957; Meinhardt et al., 2008). The common name refers to the proliferation of swollen and twisted shoots that develop on infected cacao trees: the fungus also attacks the flower cushions and pods.

Cacao is an important commodity crop – supporting small-holder farmers throughout the humid tropics – which is native to the Amazon basin (Bartley, 2005; Cornejo et al., 2018). Witches' broom disease was responsible for pod losses exceeding 90% in the Brazilian state of Rondônia during the 1970s and had a significant impact on the socio-economic development of this newly-colonised Amazonian region (Evans, 1981a). It only appeared in the main cacao-producing region of Brazil (Bahia state in eastern Brazil) in 1989 (Pereira et al. 1990, 2006), purportedly after the politically-motivated, deliberate introduction of inoculum from Amazonia (Junior, 2006), and now accepted as an act of terrorism (Evans, 2007; Caldas and Perz, 2013). The rapid spread of witches' broom disease in Bahia resulted in a dramatic decrease in Brazilian cacao production, from 378,000 tons in 1990 to less than 120,000 tons in 1999 (Gray, 2001), with devastating consequences – both

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ecological and socio-economic – for the region (Alger and Caldas, 1994; Saatchi et al., 2001; Rolim and Chiarello, 2004; Caldas and Perz, 2013). As a result, the country dropped from the second largest producer of cacao to sixth place in 15 years (Arruda et al., 2005).

The witches' broom fungus was originally described as *Marasmius perniciosus* (Stahel, 1915), and later transferred to the genus *Crinipellis* by Singer (1942), based on basidiomatal morphology. More recently, it was transferred to the (purportedly asexual) hyphomycete genus *Moniliophthora* (Seifert et al., 2011), based on molecular rather than morphological evidence (Aime and Phillips-Mora, 2005). Subsequently, the generic diagnosis of *Moniliophthora* was expanded to include species forming basidiomata (Evans et al., 2013).

In addition to *T. cacao*, *M. perniciosa* infects a variety of hosts belonging to other families, and several biotypes have been delimited based on host adaptation, namely: C-biotype infecting members of the *Malvaceae* – species in the genera *Theobroma* and *Herrania* (Evans, 1977, 1981a); S-biotype infecting members of the *Solanaceae* (Bastos and Evans, 1985; Evans and Barreto, 1996; Pereira et al., 1997; Rincones et al., 2006); B-biotype infecting *Bixa orellana*, a member of the *Bixaceae* (Bastos and Anderbrhan, 1986); L-biotype on forest lianas (Evans, 1977, 1978) – later, identified as belonging to the *Bignoniaceae* (Griffith and Hedger, 1994a) – and H-biotype infecting members of the *Malpighiaceae* (Resende et al., 2000).

It is likely that all species in the genera *Theobroma* and *Herrania* are actual or potential hosts of the C-biotype (Nyfeller et al., 2005). The S-biotype was discovered originally on *Solanum rugosum* and *Solanum lasiantherum* showing typical symptoms of brooms and parthenocarpic fruits. Both are solanaceous weeds invading the surrounding areas of cacao farms in the Brazilian Amazon (Bastos and Evans, 1985). Other solanaceous hosts have steadily been added to the list, particularly after the arrival of *M. perniciosa* in Bahia: *Athenaea pogogena* (Bastos et al., 1991); *Solanum paniculatum* (Silva et al., 1992); *Solanum cernuum* (Evans and Barreto, 1996); *Solanum lycocarpum* (Evans and Barreto, 1996; Resende et al., 1997); *Solanum gilo* and *Solanum stipulaceum* (Luz et al., 1997). Cross infectivity between the C- and S-biotypes has not been observed in experiments under controlled conditions (Bastos and Evans, 1985; Luz et al., 1997; Griffith et al., 2003).

There is only one isolated record of the B-biotype on the ubiquitous and widely planted Amazonian shrub *B. orellana* (urucum or achiote). This occurrence was reported in eastern Pará (Brazil), close to a cacao plantation with severe disease symptoms (Bastos and Andebrhan, 1986). These authors conjectured that the B-biotype is an abnormal, isolated expansion of the C-biotype due to high inoculum pressure, especially since they found that the isolate was unable to complete its life cycle on this host. Subsequently, it was reported that the isolate from *B. orellana* is genetically identical to isolates of the C-biotype from Bahia (Anderbrhan and Furtek, 1994). Based on this evidence, Meinhardt et al. (2008) did not recognise the B-biotype as a distinct subgroup of *M. perniciosa*.

Collections of liana debris and other unidentified woody tissues, lodged in or fallen from the forest canopy, and bearing basidiomata of *M. perniciosa*, have been made in the Amazon (Evans, 1977), as well as in the Atlantic rainforest of southeastern Brazil (Evans and Barreto, 1996; Evans, 2007; Tarnowski, 2009). Such host tissues showed no evidence of growth abnormalities and pathogenicity of these so-called L-biotype isolates to cacao has not been demonstrated, thus far.

The first report of *M. perniciosa* infecting a host in the *Malpighiaceae* was on *Mascagnia* cf. *Sepium* (H-biotype), showing broom symptoms in the Brazilian Amazon, and, subsequently, this isolate was shown to be pathogenic to cacao seedlings, inducing the development of swollen stems and lateral branching (Bastos et al., 1998). A separate report was made of witches' broom on *Heteropterys acutifolia* in southern Minas Gerais (Brazil) and the morphology of this isolate was regarded as identical to that of the fruit bodies on cacao (Resende et al., 2000). In addition, it was shown to produce broom symptoms when inoculated onto cacao (Resende et al., 2000). However, in a subsequent study, Arruda et al. (2005) considered that there were sufficient morphological differences in basidiospore size and shape of cystidia to justify the separation of the isolate into a distinct species. Molecular data have been used in some publications investigating the relationships among biotypes. Arruda et al. (2003) conducted a study using the intergenic spacer (IGS) region of 14 *M. perniciosa* isolates, including isolates from *T. cacao*, *S. lycocarpum* and *H. acutifolia.* The three biotypes formed two well-supported clades, one of which contained isolates from *S. lycocarpum* and *T. cacao*, and another containing isolates from *H. acutifolia*. A follow-up study, using the internal transcribed spacer (ITS) region, concluded that the *H. acutifolia* isolate was distantly related to isolates from *Theobroma*, *Herrania*, *Heteropterys nervosa* and *Solanum* and the name *Crinipellis brasiliensis* was proposed to accommodate it (Arruda et al., 2005). Recently, Niveiro et al. (2020) proposed the new combination, *Moniliophthora brasiliensis*, but added the rider that: "*M. brasiliensis* is extremely similar to *M. perniciosa* and diagnosis between the two species at present is based solely on differences in ITS sequence data".

A multi-locus phylogenetic study including isolates from a large range of biotypes and hosts is still wanting and would contribute towards elucidating the conflicting evidence regarding relationships among biotypes and the significance of supposedly host-specific lineages. Clarification of these relationships would help to indicate whether *M. perniciosa*, as it is currently defined, should be considered as a single species or a species complex.

In order to address these issues, we collected and analysed isolates of *M. perniciosa* from a range of hosts in Brazil and compared them with isolates from *T. cacao*, using both molecular and morphological data.

2. Materials and methods

2.1. Occurrence of M. perniciosa on wild and cultivated hosts in Brazil

Surveys aimed at collecting *M. perniciosa* on various hosts, as well as *ad hoc* observations and collections, were undertaken over a twenty-year period by HCE and RWB. Additionally, a literature survey of all pathogen-host associations recorded for *M. perniciosa* was performed. A distribution map of old and new records was prepared, based on this information (Fig. 1), and photographic records of relevant fungus–host associations were made.

2.2. Sample collection and isolation

Isolates of *M. perniciosa* used in this study were obtained from different hosts in Brazil in February–March of 2017 (COAD and RWB codes). One isolate from *T. cacao* was collected in the state of Espírito Santo and other isolates were obtained from various hosts collected in the state of Minas Gerais: *S. lycocarpum*, *S. cernuum*, *H. acutifolia*, *Allophylus edulis* and one or more undetermined species of liana and unknown hosts (fallen debris from the canopy bearing typical basidiomata). Isolates from other species of

solanaceous hosts were obtained from the states of Rio de Janeiro and Paraná (Table 1), as well as broom collections from a range of hosts (see Figs. 2–11).

Basidiomata of *Moniliophthora* from *Solanum* spp., *T. cacao* and *H. acutifolia*, were obtained from infected material (dry brooms) after being suspended in a glass cabinet fitted with a misting system (broom incubator or 'vassoureiro', see Fig. 2e), with a wet/dry regime (12/12 h) at 25 °C and 12-h photoperiod. The collections from *A. edulis* were obtained from the trunks of living mature trees in a remnant of Atlantic rainforest, with no symptoms of witches' broom or other diseases. Two isolates were from the basidiomata emerging from the bark and one isolate was obtained from mycelium beneath the outer bark (Fig. 10). The isolates of liana and undetermined hosts were from the fallen debris bearing basidiomata on the forest floor (Fig. 11).

Selected basidiomata were stored in Falcon tubes containing 70% ethanol for further morphological characterisation and others were used as a source of basidiospores for monosporic culture isolation. In order to obtain monosporic isolates, the basidiomata were washed in sterile distilled water and dried on filter paper. Each basidioma was then attached with petroleum jelly to the underside of Petri plate lids with the gills facing downwards above the culture medium (potato dextrose agar, PDA), and left overnight at ca 20 °C to elicit basidiospore drop (Evans, 1978). After 12 h, a single germinated basidiospore was selected from the spore print, using a stereomicroscope, and transferred to a separate PDA plate. Plates were maintained at 25 °C under fluorescent light with a 12-h photoperiod for 10 d. Pure cultures were preserved in PCA (potato carrot agar) tubes; in silica gel, as described in Dhingra and Sinclair (1996); or, in cryotubes containing 10% glycerol stored at −80 °C. Pure cultures were deposited in the culture collection of the Universidade Federal de Viçosa (COAD).

2.3. DNA extraction, PCR amplification and sequencing

Isolates of *M. perniciosa* were grown on PDA covered with a layer of sterile cellophane at 25 °C with a 12-h photoperiod for 7 d. The mycelium was scraped from the medium surface and placed in a sterilised 1.5 ml microcentrifuge tube and ground with mechanical cell disruptor L-BEADER-3 using metal microspheres (beads). DNA was extracted with a Wizard Genomic DNA purification kit by following the manufacturer's instructions.

Sequences were obtained from three loci: ribosomal internal transcribed spacer (ITS), ribosomal large subunit (LSU) and RNA polymerase II large subunit (RPB1). The amplification and sequencing were performed with the following sets of respective primers: ITS4 (TCC TCC GCT TAT TGA TAT GC) and ITS5 (GGA AGT AAA AGT CGT AAC AAG G) (White et al., 1990), LROR (GTA CCC GCT GAA CTT AAG C) (Rehner and Samuels, 1994) and LR5 (TCC TGA GGG AAA CTT CG) (Vilgalys and Hester, 1990), RPB1-Af (GAR TGY CCD GGD CAY TTY GG) (Stiller and Hall, 1997) and RPB1-Cr (CCN GCD ATN TCR TTR TCC ATR TA) (Matheny et al., 2002). PCR was performed with 12.5 μl of Dream Taq TM PCR Master Mix 2× (MBI Fermentas, Vilnius, Lithuania); 1 μl of 10 μM each forward and reverse primer; 1 μl of dimethyl sulfoxide; 5 μl of 100× (10 mg/ml) Bovine Serum Albumin; 2 μl of genomic DNA (25 ng/μl) and 2.5 μl of nuclease-free water.

The PCR conditions for ITS were: initial denaturation for 1 min at 95 °C, 38 cycles of 94 °C for 1 min, 60 °C for 50 s and 72 °C for 1 min, and an additional extension step of 72 °C for to 2 min. The conditions for LSU were: 5 min at 94 °C, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and finally 7 min at 72 °C. The conditions for RPB1 were modified from Aime and Phillips-Mora (2005): 5 min at 94 °C, then 35 cycles at 94 °C for 30 s, 50 °C for 1 min, 72 °C for 2 min, and finally 7 min at 72 °C. The PCR products were analysed by electrophoresis on 2% agarose gels that were stained with GelRed™ (Biotium Inc., Hayward, CA) in a 1× TAE buffer and visualised under UV light to check for amplification size and purity. The amplicons were purified and sequenced by Macrogen Inc., South Korea (http://www.macrogen.com).

2.4. Data editing and phylogenetic analysis

The nucleotide sequences were edited using the SeqAssem software programme (Hepperle, 2004). All the sequences were checked manually, and nucleotides with ambiguous positions were edited manually. New sequences generated in the present study were deposited in the NCBI-GenBank database and sequences obtained from other studies were retrieved from the NCBI-GenBank database (Table 2). Alignment and the concatenated tree were deposited in Treebase (accession https://www.treebase.org/treebase-web/home.html; study S26436 and S26437.). Sequence alignments were performed using MUSCLE® (Edgar, 2004) implemented in MEGA 7 (Kumar et al., 2016). In total the dataset comprised 66 sequences. The alignments were checked and edited manually. Gaps were treated as missing data. The models for phylogenetic analyses were selected independently for each locus using MrModeltest v. 2 (Nylander, 2004) under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004) implemented in both PAUP V4.0b10.

Phylogenetic reconstructions of concatenated and individual gene-trees were performed using Bayesian (BI) Markov Chain Monte Carlo and Maximum Likelihood (ML) methods. The BI analysis was performed using the CIPRES web portal (Miller et al., 2010) using MrBayes programme v. 3.2.3 (Ronquist and Huelsenbeck, 2001). Four MCMC chains were run simultaneously, starting from random trees for 10, 000, 000 generations. The trees were sampled every 1,000th generation for a total of 10,000 trees. The first 2500 trees were discarded as the burn-in phase of each analysis. The posterior probabilities were determined from a majority-rule consensus tree that was generated from the remaining 7500 trees. The ML analyses were estimated in the CIPRES Science Gateway Platform using RAXML-HPC v.8 (Stamatakis, 2014). For the concatenated dataset, all free modal parameters were estimated by RAxML with ML estimate of 25 per site rate categories. The concatenated dataset was partitioned by loci in RAxML platform. The RAxML software accommodated the GTR model of nucleotide substitution with the additional options of modeling rate heterogeneity (Γ) and proportion invariable sites (I). Phylogenetic trees were visualised using FigTree (http://tree.bio.ed.ac.uk/software/figtree/), exported to the graphic programme Corel Draw and rooted with *Chaetocalathus liliputianus* (MCA485).

2.5. Ancestral Character State Reconstruction

Aiming for a better understanding about the evolutionary pathways of pathogenicity within the lineages of *Moniliophthora* studied herein, we conducted Ancestral Character State Reconstruction in Mesquite (Maddison and Maddison, 2018). We coded each taxon based on its ecology: pathogenic (or symptomatic) endophytes (red); asymptomatic or non-pathogenic (green) endophytes, using Maximum likelihood model MK1, as implemented in Mesquite 3.51 (Maddison and Maddison, 2018). Nodes representing probabilities of <70% are displayed as dashed lines (Fig. 12).

2.6. Morphology

Macromorphological character observations were made based on an examination of basidiomata of 23 isolates from *T. cacao*

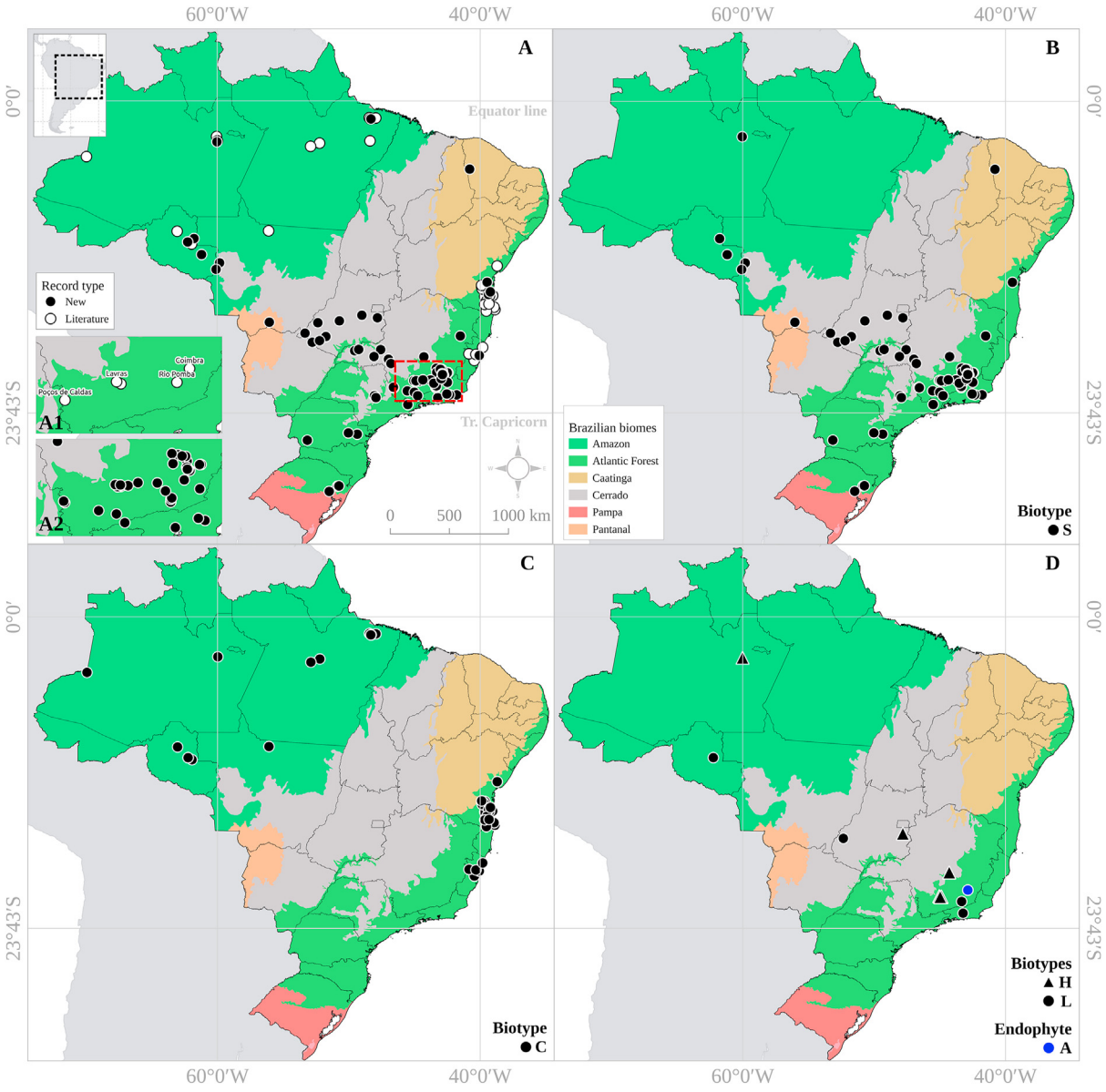

Fig. 1. Maps of distribution of records of *Moniliophthora perniciosa*-host associations in Brazil. A. Published records (white dots) *vs* novel records from this study (black dots), indicating a much broader distribution and environmental tolerance of the fungus (originally regarded as being typically limited to the warm and humid tropics) revealed by the surveys (Insets – closeup of Southern area of the state of Minas Gerais, where numerous records were made. Area indicated in larger map by red-lined square); B. Combined (published and new) records of *M. perniciosa*-solanaceous (biotype S) host associations; C. Combined (published and new) records of *M. perniciosa* (cacao and related taxa – biotype C) host associations; D. Combined (published and new) records of *M. perniciosa*-other groups of hosts: H (malphigiaceous hosts), L (liana hosts – possibly bignoniaceous), A (*Allophyllus edulis* – Sapindaceae – non-pathogenic endophytic) host associations. Mini-map in the upper left corner indicating the broad survey area in South America. *Records details provided in Supplementary Table 1.

(COAD2616, Espírito Santo), *S. lycocarpum* (COAD2611, RWB1268 Minas Gerais), *Solanum gemellum* (COAD2612, Paraná), *Solanum swartzianum* (COAD540, COAD2613, RWB1065, Rio de Janeiro), *S. cernuum* (COAD2605, Minas Gerais), *A. edulis* (COAD2599, COAD2600, COAD2601, COAD2602, COAD2603, COAD2604, COAD2607, Minas Gerais), *H. acutifolia* (COAD2614, COAD2615, RWB1267, Minas Gerais), and basidiomata formed on unidentified wood debris (COAD2608, COAD2609, COAD2610) and those on lianas (COAD2598, COAD2606). The characters examined were: pileus shape, colour and diameter; stipe colour and size; lamellae colour and size; and basidiospore shape and size. The micromorphological analyses were performed based on specimens mounted in lactofuchsin under a light microscope (Olympus BX51). At least 30 individual structures of relevance (basidia and basidiospores) were examined and measured.

3. Results

3.1. 1. Occurrence of *M. perniciosa* on wild and cultivated hosts in Brazil

In our field and literature surveys, we found 29 hosts of *M. perniciosa* in Brazil. Besides *T. cacao*, *M. perniciosa* has been recorded on five other species of *Theobroma* (*T. bicolor*, *T. grandiflorum*, *T. obovatum*, *T. speciosum* and *T. subincanum*): and, doubtless, it can infect all species of the genus, as well as those of the closely-related *Herrania* (Thorold, 1975). The combination of

Table 1
Morphology of *Moniliophthora* collections associated with different hosts.

Characteristics	Host Plant								
	Theobroma	*Solanum*	*Solanum*	*Solanum*	*Solanum*	*Heteropterys*	*Allophylus*	Liana	unidentified wood
	cacao	*cernuum*	*gemellum*	*lycocarpum*	*swartzianum*	acutifolia	edulis		
Code Isolate/	COAD2616/	COAD2605/	COAD2612/	COAD2611, RWB1268/	COAD540, 2613,	COAD2614, 2615, RWB1267/	COAD2599, 2600, 2601, 2602,	COAD2598, 2606/	COAD2608, 2609, 2610
Location	Vitória-ES	Viçosa-MG	Ponta Grossa-PR	Viçosa-MG	RWB1065/ Nova Friburgo and Macaé - RJ	Itumirim-MG	2603, 2604, 2607/ Viçosa-MG	Viçosa-MG	Viçosa-MG
Morphology of cheilocystidia	pyriform, swollen, clamped at base	clavate to obclavate, scarce	None observed	Pyriform, lageniform	None observed	clavate to pyriform, swollen	None observed	Obclavate to pyriform, scarce	Obclavate to pyriform, scarce
Basidia shape and size (μm)	Clavate/17–27 (20) × 7–10 (8)	Clavate/17–27 (20) × 7–10 (8)	Clavate/18–26 (21) × 7–12 (9)	Clavate/21–25 (24) × 8–9 (8)	Clavate/17–28 (23) × 6–13 (8)	Clavate/25–30 (27) × 6–8 (7)	Clavate/18–28 (23) × 6–13 (8)	Clavate/17–27 (20) × 7–10 (8)	Clavate/17–28 (22) × 7–10 (8)
Basidiospores shape and size (μm)	elliptical, apiculate/ 8–10 (9) × 3–7 (5)	elliptical, apiculate/ 11.5–13.0 (12.2) × 6.0–7.0 (6.8)	elliptical, apiculate/ 10.0–12.0 (11.0) × 6.0–7.0 (6.6)	elliptical, apiculate/ 12.0–14.0 (13.0) × 6.0–8.0 (6.9)	elliptical, apiculate/ 10.0–11.0 (10.5) × 4.5–5.5 (5.2)	elliptical, apiculate/ 10.0–11.0 (10.0) × 5.0–6.0 (5.5)	elliptical, apiculate/ 9.0–11.0 (10.1) × 5.0–6.0 (5.1)	elliptical, apiculate/ 11.5–13.0 (12.2) × 6.0–7.0 (6.8)	elliptical, apiculate/ 9.0–10.5 (9.8) × 4.0 –6.0
	Q = 1.80	Q = 1.80	Q = 1.67	Q = 1.88	Q = 2.02	Q = 1.82	Q = 1.98	Q = 1.80	(4.9)Q = 2.00
Pileus shape, colour and diameter (mm)	Conic, broadly umbonate/dark crimson in centre fading at margins/ 0.7–2.0 (1.3)	Conic, broadly umbonate/dark crimson in centre fading at margins/ 0.9–1.0 (0.9)	Conic, umbonate/ dark crimson in centre fading at margins/1.1–2.0 (1.4)	Conic, umbonate/ dark crimson in centre fading at margins/0.8–1.1 (0.9)	Conic, sometimes uplifted/dark crimson in centre fading at margins/1 –2.7 (1.6)	Conic, broadly umbellate/Pale crimson centre to light rose or cream margin/0.8–2.0 (1.4)	Conic, broadly umbulate/crimson/ 1.4	Conic, broadly umbulate/crimson/ 1.4	Conic, umbulate/ crimson/1.0
Stipe colour and size (mm)	Dark crimson base to cream at apex/ 0.5–1.0 (0.8)	Dark crimson base to crean at apex/0.7	Dark crimson base to cream at apex/ 0.6–1.2 (0.9)	crimson base to cream at apex/0.6 –0.8 (0.7)	Dark crimson base to cream at apex/ 0.5–1.3 (1.1)	Dark crimson base to cream at apex/ 0.5–1.7 (1.1)	Dark crimson base to cream at apex/ 0.7	Dark crimson base to cream at apex/ 0.7	Dark crimson base to cream at apex/ 0.7
Lamellae colour and size (mm)	Cream/0.2–0.5 (0.3)	Grey/0.4–0.5 (0.4)	Grey/0.4–0.5 (0.4)	Grey/0.3	Cream/0.6–1.2 (0.8)	Cream/0.5–1.0 (0.7)	Cream/0.7	Cream/0.7	Cream/0.5

Fig. 2. *Moniliophthora perniciosa*: symptoms on *Theobroma cacao* (C-biotype); original L-biotype; and production method for basidiomata a. Green flower cushion broom; b. Necrotic lateral broom on flower cushion; c. Parthenocarpic pods or 'chirimoyas'; d. Basidiomata on necrotic brooms from western Ecuador, dark crimson morphotype originally designated as a separate variety – var. *ecuadoriensis*, central broom has citron yellow basidiomata, also recognised as a separate variety – var. *citriniceps* (Pegler, 1978); e. Incubation chamber ('vassoureiro') with hanging brooms during 12-h misting period; f. Large, uniformly crimson basidiomata of L-biotype on forest liana from western Ecuador – included within var. *ecuadoriensis* by Pegler (1978) – host now identified as *Arrabidaea verrucosa* (*Bignoniaceae*) and this non-pathogenic, endophytic biotype is heterothallic.

previous observations made by HCE and RWB and the literature records included 14 different species of *Solanum*, as well as other solanaceous hosts (*Athenaea pogogena* and *Capsicum annuum* – from experimental inoculations); *Bixa orellana* (*Bixaceae*); *Heteropteris acutifolia* and *Magascania sepium* (*Malpighiaceae*); and unidentified lianas, possibly belonging to the *Bignoniaceae*, with no broom symptoms.

Representing additional hosts for *M. perniciosa* not known prior to this work were: *Solanum baturitense, Solanum cladotrichum*, *S. gemellum*, *S. grandiflorum*, *S. mauritianum*, *S. subumbellatum, S. swartzianum* in forest or semi-forest situations; *S. gilo* and *S. melongena*, in crop situations; and *Cestrum* sp. in a ruderal situation. *Allophylus edulis* (*Sapindaceae*), a new host-family record (Fig. 10). *Vernonia diffusa* (*Asteraceae*) was also reported to be infected by *M. perniciosa* (Pereira et al., 2000) but this record requires confirmation.

As shown in Fig. 1, the situation between the known distribution of *M. perniciosa* – causing witches' broom disease on various hosts – in 1989 and in 2018 has changed dramatically. It appears from these new records that the natural distribution and host range of this fungus is probably one of the widest recorded for any obligate plant pathogen, especially the latitudinal range reached by *M. perniciosa*, mainly on solanaceous hosts, including the most meridional record from Montenegro municipality (29° 41' S), state of Rio Grande do Sul on *S. mauritianum* (Supplementary Table S1).

3.2. Sample collection and isolation

The samples of witches' brooms collected on various host species (mostly members of the *Solanaceae*) over the years and their involvement with or connection to *M. perniciosa sensu lato* was confirmed either through direct observation of typical basidiomata present on brooms in the field, or those forming on pre-treated

Fig. 3. *Moniliophthora perniciosa* (S-biotype) on *Solanum cernuum* (*Solanaceae*): an understorey tree in Atlantic rainforest, particularly common around forest edges a. Lateral brooms densely covered in hairs - typical of the shoots of this tree, hence the common name 'sloth's arm' - formed along woody branch; b. Basidiomata produced amongst the dense hairs on large, terminal necrotic broom; c. Dissected broom to show inner cavities filled with the white dikaryotic mycelium (arrow); d. Basidiospores, guttulate, elliptical and apiculate, slightly larger than other morphotypes (11.5–13.0 × 6.0–7.0 μm). Scale bar: d = 10 μm.

brooms in the 'vassoureiro', or by isolating mycelium bearing typical clamp-connections from the pith of broomed tissues. Unfortunately, *M. perniciosa* is notoriously difficult to preserve in culture and most of the cultures lost viability in storage over the years. This required a more recent effort at recollecting witches' brooms on various hosts for inclusion in the phylogenetic study. However, it was not possible to recollect several relevant isolates. Nevertheless, a total of 24 isolates was obtained (Table 2). Infected material from six hosts produced basidiomata: *T. cacao*, *S. cernuum*, *S. lycocarpum*, *S. gemellum*, *S. swartzianum* and *H. acutifolia* (Table 2). Isolates were also obtained from hosts, collected in a local fragment of Atlantic rainforest (Mata do Paraíso, Viçosa, Minas Gerais), bearing no witches' broom symptoms but forming typical basidiomata on: unidentified lianas; unknown wood debris; and on living, healthy stems of *A. edulis*.

3.3. Phylogenetic analyses

The combined phylogenetic analysis included 66 isolates of *M. perniciosa*, with *C. liliputianus* (MCA485) as the outgroup (Fig. 13). The dataset of three gene/gene regions [ITS (1–885), LSU (886–2232), RPB1 (2233–3603)] comprised 3603 characters including the alignment gaps. Of these, 2397 characters were constant, 1088 variable characters were parsimony-uninformative and 656 characters were parsimony informative.

The analyses using multilocus dataset revealed that all isolates grouped with members of the *M. perniciosa*, with posterior probability (pp) = 1 and bootstrap (mlb) = 99%, and some isolates were subdivided into subclades (Fig. 13). The isolates from *Solanum* and *Theobroma* hosts stayed close together. In the other subclade, liana isolates (COAD2598, COAD2606) and those from unidentified wood debris (COAD2608, COAD2609, COAD2610), grouped together with pp = 0.99 and mlb = 83%. *A. edulis* isolates (COAD2599, COAD2600, COAD2601, COAD2602, COAD2603, COAD2604, COAD2607) grouped together with mlb = 91%. The isolates of *H. acutifolia* (COAD2614, COAD2615, RWB1267) grouped together with *C. brasiliensis* UB2553 (pp = 1 and mlb = 98%), as expected – these were obtained from the same original host population and type locality along the margins of the Rio Capivari (state of Minas Gerais). But both are within the clade of *M. perniciosa*, which shows that they are the same species as the other isolates. The isolates from liana, unidentified wood debris, *A. edulis* and *H. acutifolia*, were grouped together with an isolate of *M. perniciosa* (DIS70) from brooms on the well-known, hallucinogenic liana species *Banisteriopsis caapi* (*Malpighiaceae*), ayahuasca, caapi or yagé, collected in Amazonian Ecuador (pp = 0.98 and mlb = 81%).

3.4. Evolutionary transitions between non-pathogenic and pathogenic lineages of *moniliophthora*

Our Ancestral Character State Reconstruction could not provide strong support for the ancestral state of the genus *Moniliophthora*, with 55% for a non-pathogenic (symptomless) ancestral state versus 45% for a pathogenic origin. The results support the hypothesis that *Moniliophthora roreri*, a pathogen of cacao pods, and *Moniliophthora aurantiaca*, purportedly a non-pathogenic endophyte, share a common ancestor that parasitised its hosts (79.8%, Fig. 12, Node A). In addition, the common ancestor of *M. roreri*, *M. aurantiaca* and *M. perniciosa* was also a parasite (70.4% probability, Fig. 12, Node B). Furthermore, *M. perniciosa*, recorded both in its pathogenic and non-pathogenic morphs, is strongly supported

Fig. 4. *Moniliophthora perniciosa* (S-biotype) on *Solanum gemellum* (*Solanaceae*): a small relatively uncommon shrub in Atlantic rainforest borders at highland situations. Green terminal broom; b. Nectotic terminal broom; c-d. Basidiomata, groups produced on necrotic broom, showing the deep crimson colour, prominent umbonate form and broad lamellae; e. Basidioma, close-up showing the dense central hairs; f. Basidiospores, relatively short and wide giving a low Q index (1.67) and a broad elliptical shape. Scale bar: f = 10 μm.

(~100%) to have originated from a parasitic lineage (Fig. 12, Node C) and its non-pathogenic lineage arose later, from a parasitic lineage (92.3%, Fig. 12, Node D), endophytic in the sapindaceous host *Allophylus edulis* and lianas in Brazil. Thus, our results suggest that the genus *Moniliophthora* arose as a non-pathogenic endophyte. Further, along its evolutionary pathway, one lineage (Fig. 12, Node B) switched to a pathogenic life-style with two reversions back to a non-pathogenic, endophytic life-style on *M. aurantiaca* and two lineages on lianas (probably *Bignoniaceae*) and *Allophylus edulis*. In part, these results also agree with the proposal that: "All available evidence suggests that endophytes arose from plant pathogenic fungi" (Carroll, 1988).

3.5. Morphology

The morphology of the basidiomata and its components largely agreed with Singer's description of the species (Singer, 1976) (Table 1). Hyphae bearing clamp connections, characteristic of dikaryotic mycelia and indicative of a homothallic condition (Griffith and Hedger, 1994b), were present in all the isolates examined.

Pileus diameter ranged from 0.7 to 2.0 mm, with larger pilei produced by COAD2613, COAD1065 and COAD540 (*S. swartzianum*), which ranged from 1.0 to 2.7 mm. The pileus colour from isolates of *T. cacao* and *Solanum* spp. was dark crimson centrally, fading at margins and the caps were initially conic-shaped becoming convex and broadly umbonate with age,

Fig. 5. *Moniliophthora perniciosa* (S-biotype) on *Solanum lycocarpum* (*Solanaceae*): a common, prickly and invasive shrub in pastures and along roadsides in Minas Gerais, often heavily infected a-b. Large terminal and lateral brooms in canopy; c Parthenocarpic, necrotic pods; d-f. Basidiomata, produced in 'vassoureiro' on necrotic brooms bearing spines, showing dense production of primordia, pale crimson pileus and abundant basidiospore drop on broom surface; g. Basidiospores, long compared to width giving a narrow ellipsoidal shape (Q = 1.88). Scale bar: g = 10 μm.

except for *S. swartzianum* which had uniformly conic-shaped pilei, sometimes uplifted. The pilei of the basidiomata formed on *A. edulis*, liana and unidentified wood debris were crimson and conic-shaped, sometimes uplifted. The pilei of the fungus on *H. acutifolia* (COAD2614, COAD2615, RWB1276) were pale crimson centrally to light rose or cream at the margin and conic-shaped and broadly umbonate (Fig. 9). The colour of the stipe in older specimens was dark crimson at the base becoming cream at apex for all isolates and the size of stipe did not vary between them. The colour of the lamellae in specimens from all hosts ranged from cream to grey.

Basidia in specimens from all hosts were clavate and had similar sizes, except hosts, such as *T. cacao* (averaging 20 × 8 μm). Basidiospores from all hosts were elliptical and apiculate, whilst those from *T. cacao* were smaller (averaging 9 × 5 μm), than those from solanaceous hosts, in particular, which averaged 11–13 × 6.5–7 μm. The Q factor (ratio of length to breadth) ranged from 1.80 to 2.02; falling within the ellipsoidal shape, typical of *M. perniciosa* (Singer, 1976; Pegler, 1978); although, in the isolate from *S. gemellum*, Q was distinctly lower with a broader ellipsoidal shape (Table 1; Fig. 4). Cheilocystidia were variable in shape and size among the isolates, appearing of little use for taxonomic separation.

Fig. 6. *Moniliophthora perniciosa* (S-biotype) on *Solanum swartzianum* (*Solanaceae*): a medium-sized shrub in degraded Atlantic rainforest a. Terminal green broom; b. Basidiomata, flushes produced in 'vassoureiro'; c-d. Basidiomata, large and uniformly crimson with crowded lamellae; e. Yellow-pink mycelial pads on exposed broom surface; f. Basidiospores, guttulate with the classic ellipsoidal shape (Q = 2.02). Scale bar: f = 10 μm.

3.6. Nomenclatural novelty

M. perniciosa = syn. nov. *M. brasiliensis* (M.C.C. Arruda, G.F. Sepúlveda, R.N.G. Miller, M.A. Ferreira & M.S. Felipe) Niveiro, Lodge & Aime in MycoKeys 66: 47 (2020).

Note: For a complete description of *C. brasiliensis* see Arruda et al. (2005). *M. brasiliensis* (originally described as *C. brasiliensis*) was isolated from *H. acutifolia* collected in the gallery forest at Itumirim, southern Minas Gerais, where this riparian liana is common along both banks of the Rio Capivari. This species was

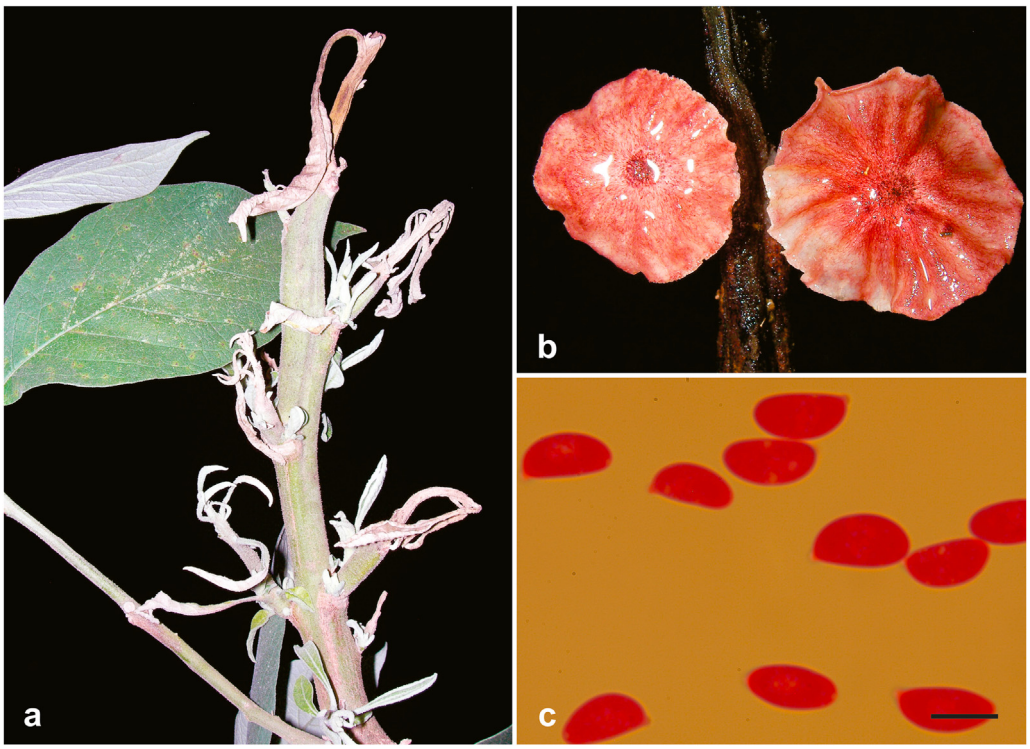

Fig. 7. *Moniliophthora perniciosa* (S-biotype) on *Solanum mauritianum* (*Solanaceae*), understorey tree in Atlantic rainforest a. Broom on terminal inflorescence; b. Basidiomata on necrotic broom, with prominent dark crimson hairs in centre; Basidiospores, guttulate c. and ellipsoidal, above average size 12–14 x 6–7 μm (Q = 2.00). Scale bar: c = 10 μm.

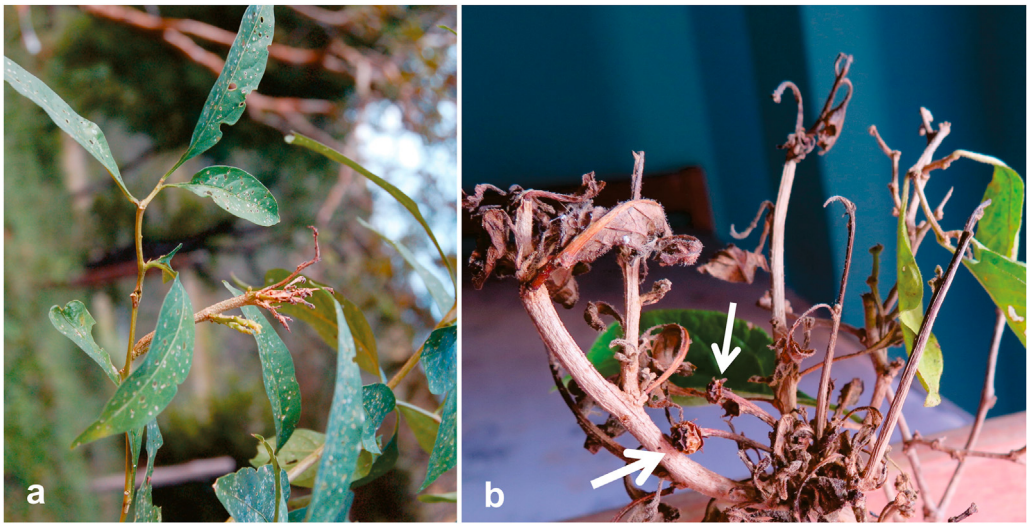

Fig. 8. *Moniliophthora perniciosa* (S-biotype) brooms on *Solanaceae* hosts in contrasting ecosystems a. Lateral broom on species of *Cestrum*, an understorey shrub in moist Atlantic rainforest at elevated locality in Rio de Janeiro state; b. Broom on inflorescence of *Solanum baturitense*, in the semi-arid Caatinga ecosystem of north-east Brazil (Ceará state) – with parthenocarpic fruits (arrows).

identified based on morphological characters and the neighbor-joining (NJ) analysis of sequence ITS. The isolates from this study (COAD2614, COAD2615 and RWB1267) obtained from *H. acutifolia* collected at the type locality and on the type host of *C. brasiliensis* grouped with this species with (pp) = 1 and mlb = 98%. Our phylogenetic analysis, based on three concatenated gene regions (ITS, RPB1 and LSU), showed that all these isolates of *H. acutifolia* belong to *M. perniciosa*. In addition, our observation of clavate to pyriform cheilocystidia produced by isolates from *H. acutifolia* contradicts that of Arruda et al. (2005), who indicated that the H-biotype (*H. acutifolia*) produces lageniform cheilocystidia. Resende et al. (2000) also affirmed that the morphology of the *H. acutifolia* isolate matched, in all aspects, the *T. cacao* isolate. The newly proposed synonymy with *M. perniciosa*, therefore, is justified. A recent publication (Niveiro et al., 2020) accounted for the recombination of *C. brasiliensis* into *Moniliophthora*, an appropriate nomenclatural decision, but failed to recognise the conspecificity of *M. brasiliensis* and *M. perniciosa*, elucidated herein.

Fig. 9. *Moniliophthora perniciosa* (H-biotype) on *Heteropterys acutifolia* (*Malpighiaceae*): riparian understorey woody liana, becoming tree-like a. Large, inflorescence broom hanging from canopy; b. Necrotic broom producing basidiomata and white mycelial pads around flower scars in 'vassoureiro'; c-d. Basidiomata, pale crimson with broad, prominent lamellae; e. Basidiospores, guttulate and ellipsoid, similar to C-biotype (Q = 1.82). Scale bar: e = 10 μm.

4. Discussion

The kingdom *Fungi* is a heterogeneous group of organisms composed of species exhibiting a myriad of ecologies. Such ecological plasticity probably played a crucial role in their diversification and domination of all environmental types (Rundle and Nosil, 2005; James et al., 2006). The ability to switch across different host orders and kingdoms occurred multiple times, notably in insect and plant-associated fungi (Nikoh and Fukatsu, 2000; Spatafora et al., 2007; Kepler et al., 2012; Chaverri and Samuels, 2013; Araújo and Hughes, 2019).

Our results indicate that the genus *Moniliophthora* also evolved through multiple switches between host families and ecologies, i.e. non-pathogenic and pathogenic life-styles (Fig. 12). According to our analyses, its origin was reconstructed as being an endosymbiont, particularly colonising woody plants. Essentially, all endophytes derive their nutrients for all or part of their life-cycles from the host plant and, therefore, the life-style is parasitic *sensu stricto*. This endophytic association may be non-pathogenic – causing no apparent harm to the host in the form of visible damage, often termed commensalism – or pathogenic, in which, at some point, the coloniser became an invader, or pathogenic, manifested by damage to host tissues and external symptoms (disease). It could be argued that if the commensalist sequestered nutrients from the host without 'payment in kind', then, by definition, the association would be parasitic rather than neutral or unharmful and must impact on host fitness. This is when the terminology of commensalism, in particular, and of symbiosis, in general, becomes ambiguous (see Kirk et al., 2008; Hardoim et al., 2015). There is also ambiguity with the definition of fungal endophytes as proposed by Carroll (1988), as "inapparent infections within leaves and stems of healthy plants". Infection implies parasitism, but without

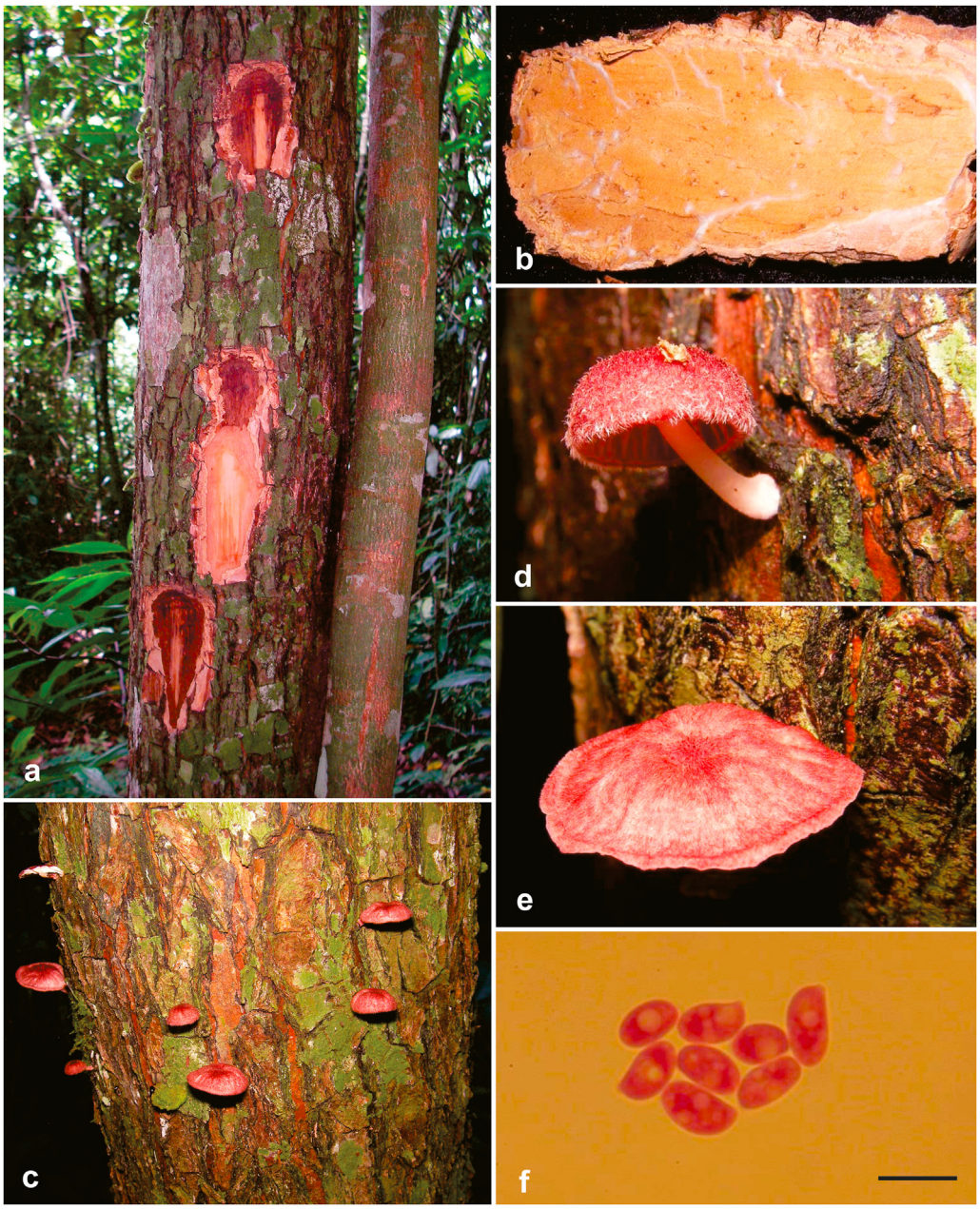

Fig. 10. *Moniliophthora perniciosa* (non-pathogenic, endophytic biotype) on *Allophyllus edulis* (*Sapindaceae*), an upperstorey tree in Atlantic rainforest a. Main trunk with bark removed from areas producing basidiomata; b. Dikaryotic mycelium between outer and inner bark; c. Trunk showing flush of maturing basidiomata; d. Young basidioma, densely covered in hairs; e. Mature basidioma; f. Basidiospores, markedly guttulate and ellipsoidal, 9–11 x 5–6 μm (Q = 1.98). Scale bar: f = 10 μm.

manifesting symptoms. All the biotypes of *M. perniciosa* reported here fall within this definition and only after the initial colonisation phase – varying from one to two months, depending on the tissue type and age – do the pathogenic strains show symptoms. Thus, we have classified them either as non-pathogenic or pathogenic endophytes. For most species of the genus *Moniliophthora* described, thus far, the endophytic association is non-pathogenic, with no visible external or internal symptoms, and we assume that it is beneficial to the host plant, rather than neutral, and, therefore, that the relationship may be one of mutualistic symbiosis. As yet, however, there have been no studies to support this hypothesis for *Moniliophthora*, but there is ample and ever increasing evidence for other fungal genera (Hardoim et al., 2015) and, in particular, the endophytic clade of the genus *Trichoderma* (Samuels, 2006; Bailey and Melnick, 2013).

The switch from a non-pathogenic (asymptomatic) to a pathogenic (symptomatic) nutritional mode apparently promoted the radiation of *Moniliophthora*, including broad diversification of the lineages *M. perniciosa* and *M. roreri*: the two most economically-important pathogens of cacao in the Neotropics (Evans, 1981a, 1981b). After the transition from the non-pathogenic to the pathogenic nutritional mode, it appears that two reversions occurred back to a non-pathogenic life-style, with *M. aurantiaca* and two lineages within *M. perniciosa* on lianas, probably belonging to the *Bignoniaceae*, and on the sapindaceous tree *Allophylus edulis*. However, it should be noted that a more robust and conclusive

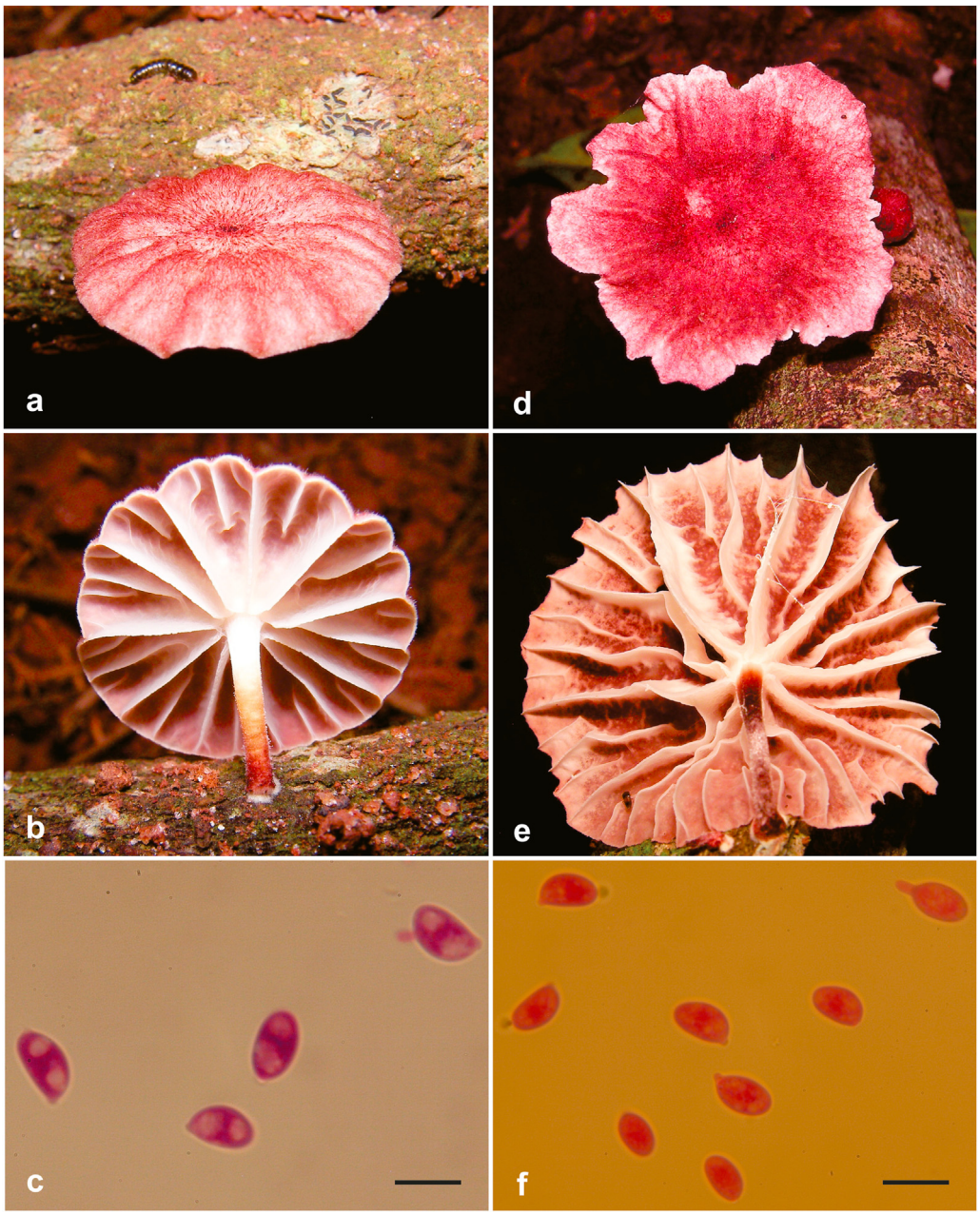

Fig. 11. *Moniliophthora perniciosa* (L-biotypes) on hanging liana and fallen branch, showing variation in basidiomatal form, in Atlantic rainforest a-b. Basidioma, from above and below, on dead liana (*Bignoniaceae*) hanging in lower understorey; c. Basidiospores, densely guttulate, large 11.5–13 x 6–7 μm (Q = 1.80); d-e. Basidioma, from above and below, on fallen branch on forest floor, with markedly irregular, frilled pileus and denser hair layer with more prominent, crowded lamellae; f. Basidiospores, not markedly guttulate and smaller than liana biotype, 9–10.5 x 4–6 μm (Q = 2.00). Scale bar: f-e = 10 μm.

hypothesis regarding the evolution of *Moniliophthora* and its lineages is still needed – especially as our study is based only on three genomic regions – and this will require a broader and more inclusive sampling set, as well as more genomic regions. Notable absences are related species still placed within the *Iopodinae* section of the genus *Crinipellis* – such as *Crinipellis siparunae* and *C. eggersii* – which are purportedly non-pathogenic endophytes of forest trees in the Amazon basin (Singer, 1976); and the endophytic, asymptomatic *M. perniciosa* on the bignoniaceous liana, *Arrabidaea verrucosa* in western Ecuador (Griffith and Hedger, 1994a). The latter, in particular, could be crucial to any analysis because of its unique heterothallic condition identified by Griffith and Hedger (1994b); contrasting with the exclusively homothallic (dikaryotic) isolates of *M. perniciosa* included in the present study. With the wholesale and increasing loss of forests on both sides of the Andes, however, collection of these isolates for phylogenetic studies will be problematic.

From the results of this study, and others (Artero et al., 2017), we now know that the natural range of *M. perniciosa* in Brazil is immense, extending from the Amazon basin in the north – across the entire region, from Pará state in the east to Rondônia and Acre in the far west, bordering Bolivia, predominantly as a pathogen of *Theobroma*, but also recorded on solanaceous hosts (*S. lasiantherum*, *S. rugosum*) – to the deep south, abutting Uruguay, mainly associated with and pathogenic on a range of species in the *Solanaceae* (*S. cernuum*, *S. lycocarpum*, *S. mauritianum*, *S. gemellum*,

Table 2
GenBank accession numbers of DNA sequences of *Marasmiaceae* used in the phylogenetic analysis.

Species	Isolate	Host	Locality	GenBank accession number		
				ITS	LSU	RPB1
Chaetocalathus liliputianus	MCA485	Saprotroph	Puerto Rico	AY916682	AY916680	AY916683
Crinipellis campanella	DAOM17785		Guyana	–	U11916	-
Crinipellis maxima	DAOM196019			–	AF042630	-
Crinipellis setipes	RV 83194		USA	–	AY916689	-
Crinipellis sp.	MCA1527		Guyana	AY916701	AY916699	AY916702
Crinipellis sp.	MCA2240		Guyana	–	AY916695	–
Crinipellis sp.	OKM26890		Thailand	AY916698	AY916696	–
Crinipellis sp.	RV98/75			–	AF261348	-
Crinipellis stipitaria	PB302		Germany	–	AY570997	-
Crinipellis zonata	OKM25450		USA	AY916692	AY916690	AY916693
Marasmius cladophyllus	MCA1837		Guyana	AY916705	AY916704	AY916706
Marasmius delectans	U11922			–	U11922	–
Marasmius plicatulus	MCA1409		USA	–	AY916708	–
Marasmius sp.	JEJPR256			–	AF261342	–
Marasmius sp.	MCA1506		Guyana	AY916733	AY916731	AY916734
Marasmius sp.	MCA1577		Guyana	AY916711	AY91679	AY916712
Marasmius sp.	MCA1594		Guyana	–	AY916728	–
Marasmius sp.	MCA1611		Guyana	AY916725	AY916723	AY916726
Marasmius sp.	MCA1708		Guyana	AY916720	AY916718	AY916721
Marasmius sp.	MCA1711		Guyana	–	AY916714	–
Marasmius sp.	MCA1719		Guyana	–	AY916715	–
Marasmius sp.	MCA2060		Guyana	–	AY916716	–
Marasmius sp.	MCA2523		Ecuador	–	AY916736	–
Marasmius sp.	MCA2526		Ecuador	–	AY916730	–
Marasmius sp.	MCA2546		Ecuador	–	AY916717	–
Marasmius rotula	JEJVA595			–	AF261345	–
Moniliophthora aurantiaca	UTC253824	Woody debris	Samoa - Polynesia	JN692482	JN692483	-
Moniliophthora canescens	DED7518	Unknown dicot host	Malaysia	FJ167668	–	–
Moniliophthora mayarum	DJL BZ511	*Ceiba pentandra*	Belize	MT162718	–	–
Moniliophthora perniciosa	COAD540	*Solanum swartzianum*	Macaé - RJ	MK785139	–	MK792251
Moniliophthora perniciosa	COAD2598	Liana	Mata do Paraíso - Viçosa MG	MK785140	MK785236	MK792252
Moniliophthora perniciosa	COAD2599	*Allophyllus edulis*	Mata do Paraíso - Viçosa MG	MK785141	MK785237	MK792253
Moniliophthora perniciosa	COAD2600	*Allophyllus edulis*	Mata do Paraíso - Viçosa MG	MK785142	MK785238	MK792254
Moniliophthora perniciosa	COAD2601	*Allophyllus edulis*	Mata do Paraíso - Viçosa MG	MK785143	MK785239	MK792255
Moniliophthora perniciosa	COAD2602	Liana (*Bignoniaceae*)	Mata do Paraíso - Viçosa MG	MK785144	MK785240	MK792256
Moniliophthora perniciosa	COAD2603	Unknown host	Mata do Paraíso - Viçosa MG	MK785145	MK785241	MK792257
Moniliophthora perniciosa	COAD2604	*Allophyllus edulis*	Mata do Paraíso - Viçosa MG	MK785146	MK785242	MK792258
Moniliophthora perniciosa	COAD2605	*Solanum cernuum*	Mata do Paraíso- Viçosa MG	MK785147	MK785243	MK792259
Moniliophthora perniciosa	COAD2606	Liana (*Bignoniaceae*)	Mata do Paraíso - Viçosa MG	MK785148	MK785244	MK792260
Moniliophthora perniciosa	COAD2607	*Allophyllus edulis*	Mata do Paraíso - Viçosa MG	MK785149	MK785245	MK792261
Moniliophthora perniciosa	COAD2608	Unknown host	Mata do Paraíso - Viçosa MG	MK785150	MK785246	MK792262
Moniliophthora perniciosa	COAD2609	Unknown host	Mata do Paraíso - Viçosa MG	MK785151	MK785247	MK792263
Moniliophthora perniciosa	COAD2610	Unknown host	Mata do Paraíso - Viçosa MG	MK785152	MK785248	MK792264
Moniliophthora perniciosa	COAD2611	*Solanum lycocarpum*	Cristais - Viçosa MG	MK785153	MK785249	MK792265
Moniliophthora perniciosa	COAD2612	*Solanum gemellum*	Vila Velha - PR	MK785154	MK785250	MK792266
Moniliophthora perniciosa	COAD2613	*Solanum swartzianum*	Nova Friburgo - RJ	MK785155	MK785251	MK792267
Moniliophthora perniciosa	COAD2614	*Heteropterys acutifolia*	Itumirim - MG	MK785156	MK785252	MK792268
Moniliophthora perniciosa	COAD2615	*Heteropterys acutifolia*	Itumirim - MG	MK785157	MK785253	MK792269
Moniliophthora perniciosa	COAD2616	*Theobroma cacao*	Linhares - ES	MK785158	MK785254	MK792270
Moniliophthora perniciosa	DIS70	*Banisteriopsis caapi*	Ecuador	MK785163	AY916737	MK792274
Moniliophthora perniciosa	DIS71	*Theobroma cacao*	Eastern Ecuador	–	AY916738	AY916740
Moniliophthora perniciosa	MCA2520	*Theobroma cacao*	Western Ecuador	AY916743	AY916742	-
Moniliophthora perniciosa	RWB1065	*Solanum swartzianum*	Nova Friburgo - RJ	MK785159	–	MK792250
Moniliophthora perniciosa	RWB1205	*Cestrum* sp.	Nova Friburgo - RJ	MK785160	–	MK792271
Moniliophthora perniciosa	RWB1267	*Heteropterys acutifolia*	Itumirim - MG	MK785161	–	MK792272
Moniliophthora perniciosa	RWB1268	*Solanum lycocarpum*	Mata do Paraíso - Viçosa MG	MK785162	–	MK792273
Moniliophthora perniciosa	UB2053	*Heteropterys acutifolia*	Itumirim - MG	AY317137	-	-
Moniliophthora roreri	C21	*Theobroma cacao*	Costa Rica	AY916746	AY916744	AY916747
Moniliophthora roreri	C22	*Theobroma cacao*	Costa Rica	-	AY916749	-
Moniliophthora roreri	MCA2521	*Theobroma cacao*	Ecuador	-	AY916750	-
Moniliophthora sp.	MCA2500	*Bouteloua eriopoda*	USA	AY916754	AY916752	AY916755
Moniliophthora sp.	MCA2501		USA	MT162719	MT162719	–
Moniliophthora sp.	GJS00-165	*Bouteloua eriopoda*	USA	–	AY916751	–
Moniliophthora ticoi	NY00511157		Bolivia	MT162721	MT162717	–
Moniliophthora ticoi	Niveiro 2249	*Myrcianthes pungens*	Argentina	MT162720	MT162716	–

Note: Isolates obtained in this study are highlighted in bold. COAD, Coleção Octávio Almeida Drummond at the Universidade Federal de Viçosa.

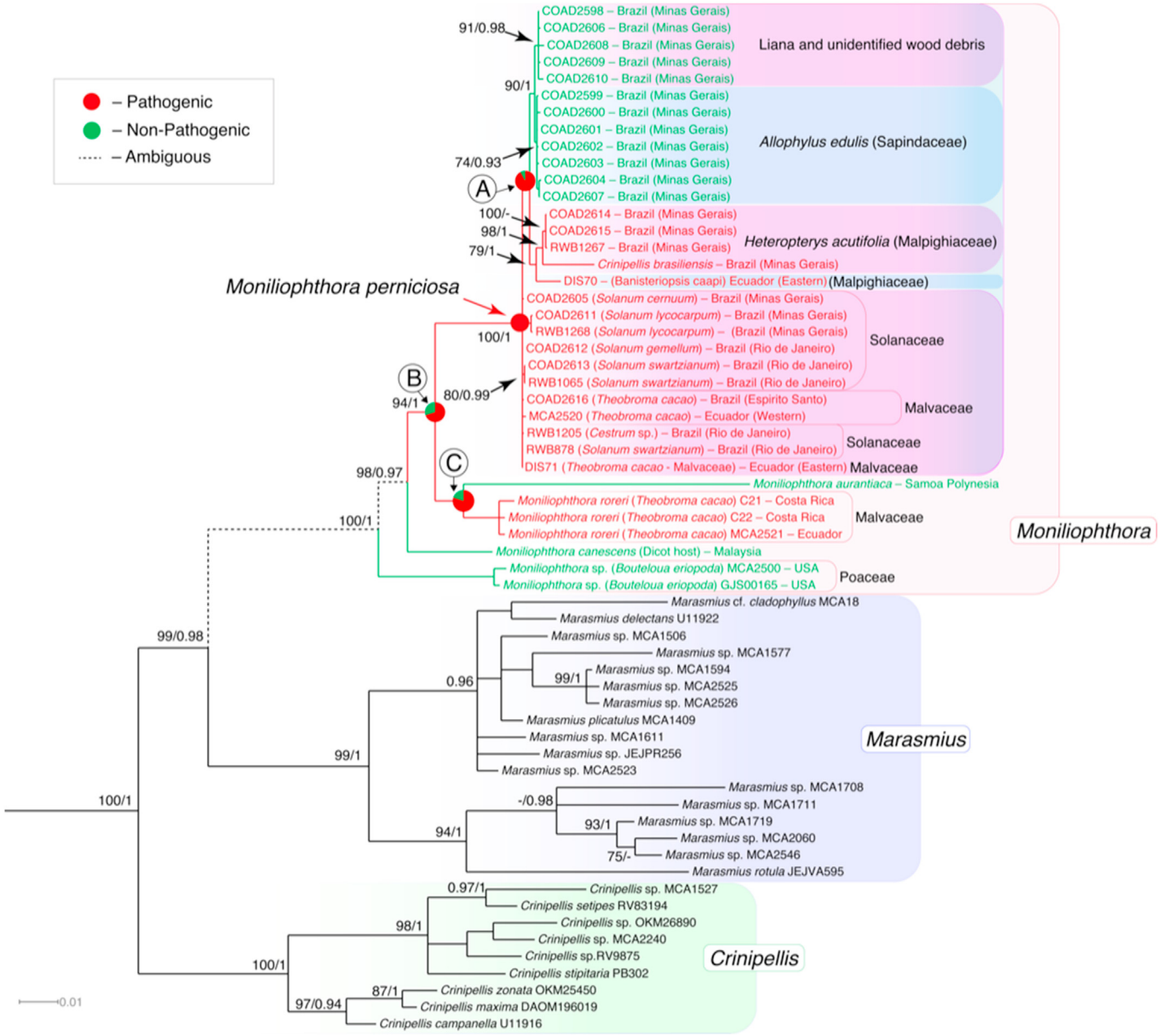

Fig. 12. Maximum likelihood tree obtained from RAxML analyses with a concatenated 3-loci dataset (ITS < tef, RPB1) and Ancestral Character State Reconstrcution (ACSR) analyses. ACSR is based on the ecology (red for pathogenic or green for non-pathogenic endophytic life-style). Pie-charts indicate probabilities for the ancestral state ecology for the nodes and Bootstrap results are shown on the left (>70), Posterior Probabilties on the right (>0.9).

S. swartzianum and *Cestrum* sp.). In all these hosts, the presence of the fungus is manifested by the formation of spectacular witches' brooms, as well as by parthenocarpic fruits, initially as distorted green shoots that necrose and eventually produce the typical pink mushrooms or basidiomata.

M. perniciosa stands out amongst plant pathogens not only because of the spectacular disease symptoms – with seemingly one of the widest natural distributions, on a disparate range of host families – but also the fact that it is a 'mushroom': the *Agaricales* being "rarely parasitic on plants" (Kirk et al., 2008). Needless to say, that *M. perniciosa* also attacks a plant that produces probably the world's favourite food – and the most ecologically-benign crop in the humid tropics, supporting the livelihoods of some of its poorest farmers – and which has the potential to cause even more devastating losses in the future (Meinhardt et al., 2008; Marelli et al., 2019).

Although the first confirmed report of the witches' broom disease was in Surinam around the turn of the 19th century (Ritzema Bos, 1901), there is compelling evidence that it had been described more than a century earlier in the Brazilian Amazon, and given the Portuguese name 'lagartão', presumably with reference to the lizard-like form of the distorted green brooms (Viera, 1942; Silva, 1987). Undoubtedly, the witches' broom pathogen is endemic throughout the Amazon basin – occurring on cacao and related *Theobroma* species, as well as on *Herrania*, in the forest understorey (Pound, 1938, 1943; Evans, 1977, 1981a) – but, because cacao was only a minor crop in the region until the mass-colonisation of the Brazilian Amazon in the 1970s, the disease 'slipped under the radar'. In contrast, when the pathogen breached the Andean barrier in the 1920s – purportedly, with human assistance from the eastern or Amazonian region of Ecuador (Rorer, 1926; Baker and Holliday, 1957) – and reached the susceptible 'Cacao Nacional'

plantations of western Ecuador, it contributed to the demise of the industry and to the loss of Ecuador's place as the leading producer of cacao, that had been held for almost half a century (Pound, 1938; Bartley, 2005).

It was in western Ecuador that the fungus was first confirmed on hosts outside the family *Malvaceae* when basidiomata were found on unknown lianas, shrubs and tree branches in the forest understorey, with subsequent findings on similar hosts in the Amazonian regions of both Ecuador and Brazil (Evans, 1977, 1978). These isolates proved to be non-pathogenic to cacao and other species of *Theobroma* (Evans, 1978). However, although brooms or other growth abnormalities were never associated with these forest records, it was posited that the so-called liana pathotype is parasitic in living lianas rather than simply saprophytic in necrotic tissues (Evans, 1977). The principal liana host in western Ecuador was identified later as *A. verrucosa* (*Bignoniaceae*), and an investigation of the breeding strategy of this isolate – later termed the L-biotype – revealed that it is out-crossing and heterothallic, contrasting with the established homothallic nature of the cacao- or C-biotype (Griffith and Hedger, 1994a, 1994b). Earlier, in a taxonomic study of these Ecuadorian isolates, Pegler (1978) concluded that: "Anatomically, the saprophytic form collected in the forests is indistinguishable from the pathogenic strains found in cocoa plantations". However, he confirmed that both of these Ecuadorian strains differed from cacao isolates from Surinam and Trinidad – principally, in the darker red colour and larger size of their basidiomata, as noted previously by Stahel (1924) – and he proposed the new combination *Crinipellis perniciosa* var. *ecuadoriensis* (Stahel) Pegler.

The first record of *M. perniciosa* forming witches' brooms on hosts outside the *Malvaceae* was from the Brazilian Amazon on two solanaceous weeds, *S. rugosum* and *S. lasiantherum* (Bastos and Evans, 1985). The authors designated this isolate as a new pathotype; producing growth abnormalities, rather than true brooms, in inoculated tomato plants but not in cacao. This was followed by a succession of new host records within the *Solanaceae* from outside the Amazon, in the main cacao-producing region of Brazil in Bahia state (Bastos et al., 1991; Silva et al., 1992). However, host range tests produced ambiguous results: the former isolate proved to be non-pathogenic to cacao whilst that from *S. paniculatum* produced typical broom symptoms in cacao seedlings; with the conclusion that this was an alternative host and thus posed a danger to cacao plantations (Silva et al., 1992). This was confirmed subsequently by Lopes et al. (2001) and led to the speculation that the strain of *M. perniciosa* from *S. paniculatum* jumped to cacao and caused the devastating disease outbreaks in 1989: a theory that had been posited earlier (Barreto and Evans, 1996; Evans and Barreto, 1996). This, of course, preceded the later revelation that broom inoculum had deliberately been introduced into Bahia from the Amazon region (specifically, from the state of Rondônia), in the first recorded case of agro-terrorism (Junior, 2006; Evans, 2007; Evans and Waller, 2010). This act of agro-terrorism – so-called because it had a political agenda – and the socio-economic and ecological consequences have been well documented since, both in print (Alger and Caldas, 1994; Saatchi et al., 2001; Rolim and Chiarello, 2004; Evans and Waller, 2010; Caldas and Perz, 2013) and on film (Araújo, 2012).

Until the finding of basidiomata on brooms of the solanaceous tree *S. cernuum* in a small fragment of Atlantic rainforest in Minas Gerais state (Evans and Barreto, 1996), it was thought that *M. perniciosa* was restricted in its distribution to its supposed endemic range in the Amazon and to the main cacao region in Bahia. It was concluded that *S. cernuum* is a natural host – since this has never been a cacao-growing area – and that the fungus is endemic in Minas Gerais; having been overlooked because it is on a host of no economic significance (Evans and Barreto, 1996). Shortly afterwards, this conclusion was corroborated when two new wild solanaceous hosts were reported from Minas Gerais; the pathogen being abundant and widespread, forming particularly prominent brooms on the weedy woody shrub, *S. lycocarpum* (Barreto and Evans, 1996; Pereira et al., 1997), and even extending to the western state of Goias on the same host (Resende et al., 1997).

As survey trips and *ad hoc* collections progressed, it became clear that witches' broom disease caused by *M. perniciosa* is prevalent throughout Brazil – on a wide range of hosts and in a variety of different ecosystems – and that the natural distribution of the pathogen is not, as previously thought, geographically and ecologically restricted to the humid tropical forests of Amazonia (Baker and Holliday, 1957). More new host records in the *Solanaceae* were added (Luz et al., 1997; Pereira et al., 1997), as well as hosts in other plant families, most notably on lianas in the *Malpighiaceae* (Bastos et al., 1998; Bezerra et al., 1998; Resende et al., 2000; Arruda et al., 2005; Evans et al., 2013). Interestingly, a new host record was identified in the Amazon or Oriente region of Ecuador when conspicuous brooms were found on the woody, malpighiaceous liana *Banisteriopsis caapi* (Holmes et al., 2004): the source of the hallucinogen, ayahuasca or yagé.

Although *M. perniciosa* is a well-studied pathogen, covering more than a century of research, there are still gaps in our knowledge of the phylogenetic relationships between the biotypes and the significance of specific host strains. Clarification of these relationships would help to resolve whether *M. perniciosa*, as it is currently defined, should be considered as a single species or a species complex. Phylogenetic studies have been conducted over recent years, but these are incomplete due to a lack of sequences in public databases. Most of the studies were based only on sequences of the ITS region (Arruda et al. 2003, 2005; Kerekes and Desjardins, 2009; Marelli et al., 2009; Kropp and Albee-Scott, 2012; Artero et al., 2017). A more complete study, using five genes, was performed by Aime and Phillips-Mora (2005), but for many of the *M. perniciosa* isolates there were no sequences for all the five regions. The phylogenetic analysis presented here is the most complete for *M. perniciosa sensu lato*, thus far, including three genome regions and covering the broadest diversity of host sources.

The analyses using multilocus dataset resolved that all isolates belong to a single species, *M. perniciosa*, which represents a sister clade to *M. roreri*, *M. aurantiaca* and *M. canescens*, but with various genotypes according to the host range. For example, in our analyses it was not possible to separate the isolates that infect *Solanaceae* from those pathogenic on *Theobroma* (*Malvaceae*) hosts. On solanaceous hosts, typical symptoms are brooms on vegetative shoots, and stem swellings that can lead to plant death, similar to the disease symptoms on cacao. *M. perniciosa* on *Solanaceae* hosts has long been recognised as having no significant morphological differences from populations associated with witches' broom disease on cacao; although incompatibility was demonstrated in dual cultures (Bastos and Evans, 1985). Marelli et al. (2009) concluded that the S- and C-biotypes are not genetically distinct and found that they share a similar disease expression: "indicating a degree of conservation of pathogenicity mechanisms between the two biotypes"; whilst Tarnowski (2009) firmly placed these isolates in the same clade, but as distinct lineages. Based on our morphological and phylogenetic results, the isolates obtained from solanaceous hosts (S-biotype) and *T. cacao* (C-biotype) cannot be separated; confirming the results of a recent study (Artero et al., 2017).

Bastos et al. (1998) demonstrated the non-pathogenicity of *Solanaceae* isolates to cacao, and similar results have been reported with isolates of *S. cernuum* and *S. lycocarpum* (Evans et al., 2013). However, as discussed previously, some cross-infectivity studies have shown ambiguous results, especially with *S. paniculatum*, for

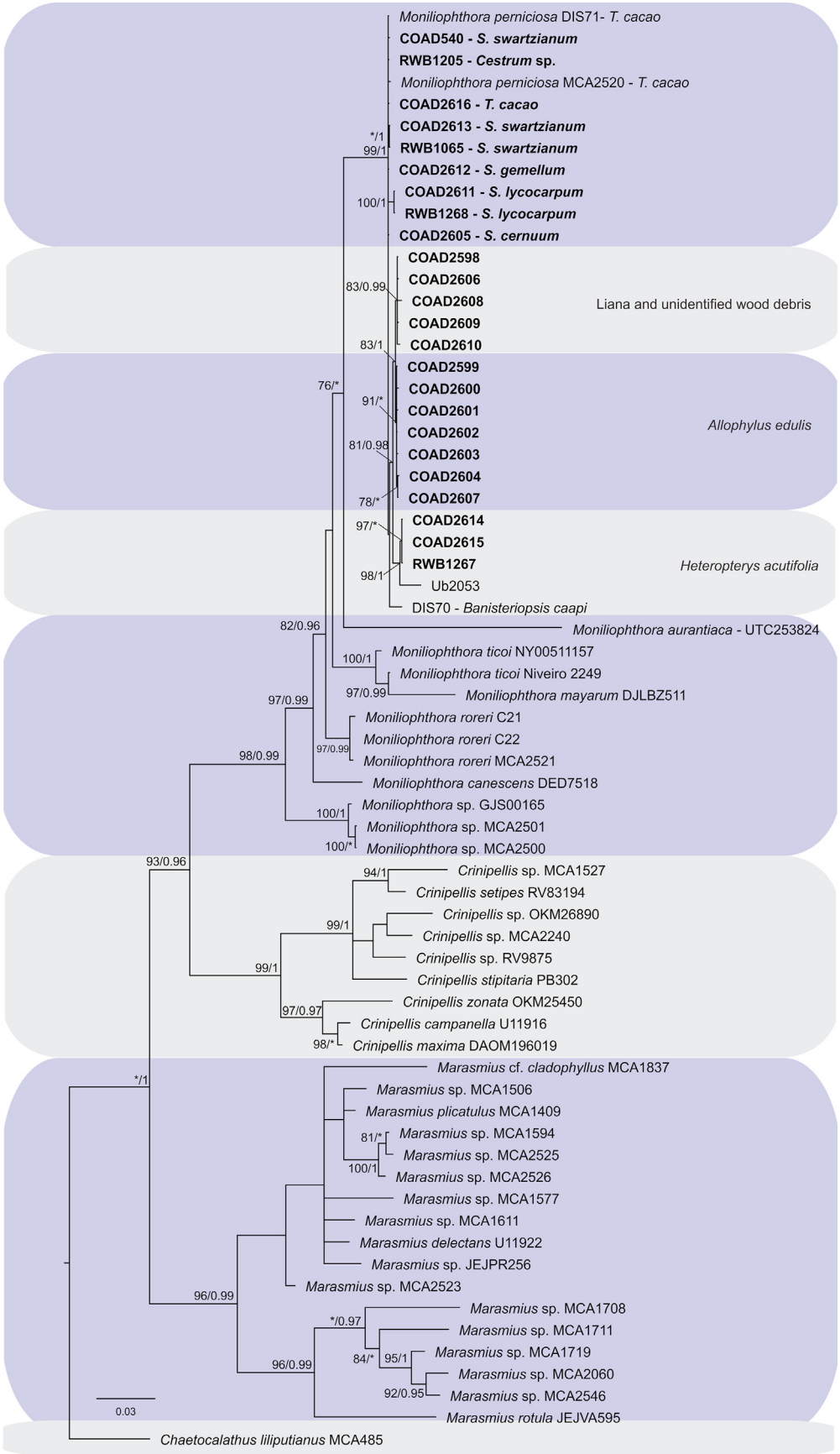

Fig. 13. The Bayesian phylogenetic tree inferred from DNA sequence data from the multigene alignment (ITS, LSU and RPB1). Bootstrap values of the maximum likelihood support (ML $\geq$ 75) and Bayesian posterior probabilities (BI $\geq$ 0.95) are shown at the first and second position, respectively. * Bootstrap values < 75% and posterior probabilities value < 95%. The species of this study are highlighted in bold. The tree was rooted with *Chaetocalathus liliputianus* MCA485.

which an isolate from this host was shown to produce brooms on cacao (Silva et al., 1992): corroborated later by Lopes et al. (2001), who also screened other species of *Theobroma* with conflicting results. Ironically, the isolate produced only minor symptoms (localised swelling) on its original host, *S. paniculatum* (Lopes et al., 2001). The only explanation for these results is that the isolate was a C-biotype strain which jumped onto this solanaceous host in an area of high inoculum pressure, similar to that reported for the jump from cacao to *B. orellana* (Bastos and Andebrhan, 1986; Anderbrhan and Furtek, 1994). Pierre et al. (2017) reported that a C-biotype from eastern Amazonia (Pará state) induced stem fasciation and swellings in inoculated tomato plants.

The isolates associated with unidentified wood debris, unknown species of liana (probably *Bignoniaceae*) and the sapindaceous tree *Allophylus edulis*, grouped in a subclade; indicating that, although belonging to *M. perniciosa*, there is a degree of genetic distinction within *M. perniciosa*. Thus, these represent an ecologically distinct group, seemingly adapted to a non-pathogenic endophytic life-style, corresponding to the L-biotype, as characterised by the heterothallic, non-pathogenic strain isolated from *A. verrucosa* (*Bignoniaceae*) in western Ecuador (Griffith and Hedger, 1994a, 1994b).

Interestingly, the broom-forming isolate from the liana *B. caapi* (*Malpighiaceae*) – collected in the Ecuadorian Amazon – clusters with strains isolated from brooms produced on another malpighiaceous liana host, *H. acutifolia*, in southern Brazil, both assignable to the H-biotype group. However, no significant morphological differences were detected between these isolates and those from cacao and solanaceous hosts. The newly collected isolates from *H. acutifolia* had a high homology with *C. brasiliensis* (pp = 1 and mlb = 98%) and, according to our multilocus phylogenetic analysis, we conclude that *C. brasiliensis sensu* Arruda et al. (2005) – recently transferred to the genus *Moniliophthora* (Niveiro et al., 2020) – is synonymous with *M. perniciosa*. Our observation of clavate to pyriform cheilocystidia produced by isolates from *H. acutifolia* contradicts that of Arruda et al. (2005), who indicated that the H-biotype (*H. acutifolia*) produced lageniform cheilocystidia. Resende et al. (2000) also confirmed that the morphology of the *H. acutifolia* isolate matched, in all aspects, with the cacao isolate: moreover, they also reported that it was pathogenic to cacao; although it is not clear that full symptoms were expressed (Griffith et al., 2003). However, a later pathogenicity study, using the same cacao clone (Catongo) as Resende et al. (2000) and the *M. perniciosa* isolate from *H. acutifolia* – as well as isolates from a wide range of solanaceous hosts – failed to produce full broom symptoms; although a range of abnormalities were recorded, especially with the H-biotype isolate resulting in host dwarfing and rhizomania (Evans et al., 2013).

There is mounting evidence that the species pertaining to the genus *Moniliophthora* are obligate endophytes: the majority would appear to be plant colonisers rather than invaders or pathogens; the exceptions being those causing major diseases of cacao, *M. perniciosa* and *M. roreri*. It is highly probable that all the species in the section *Iopodinae* (Singer, 1976, 1986), are obligate endophytes and should be transferred to *Moniliophthora*. One such species is *C. siparunae* described from basidiomata produced in annual flushes on the Brazilian forest tree *Siparuna* (*Siparunaceae*) in a tropical greenhouse in Leningrad (Singer, 1942), and which Singer suspected – and later confirmed (Singer, 1976) – had been introduced into Russia with its host from South America. This mimic the annual flushes of basidiomata of *M. perniciosa* reported here on the sapindaceous forest tree *Allophylus edulis* in its Atlantic rainforest habitat. Similarly, flushes of *C. siparunae*-like fruit-bodies have been observed several times on trunks of upperstorey forest trees in both the Brazilian and Ecuadorian Amazon (HCE, pers. obs.); providing further evidence that species originally included in the *Iopodinae* section of *Crinipellis* are non-pathogenic, obligate endophytes of forest trees in South America. For these species to sporulate on the trunks of healthy forest trees, as reported here and by Niveiro et al. (2020), implies an intimate association with its host from the seedling stage since penetration by the basidiospores of *M. perniciosa*, and initial colonisation of the plant, has been shown to be restricted to actively-growing meristematic tissues (Evans, 1978). Thus, sporulation occurs seasonally when the systemic intercellular mycelium switches to the saprophytic intracellular phase as the outer bark senesces (Fig. 10b), as observed for *A. edulis* over a three-year period. It is possible therefore, that sporulation occurs throughout the tree bole and even into the canopy. For *Allophylus* in the Atlantic forest, it was only feasible to follow the basidiomata up to 3–4 m and for *C. sipuranae* on its host tree in the Amazon forest, a similar distance was recorded (HCE, pers. obs.). This extensive host colonisation suggests a sophisticated systemic life-style and one in synchrony with its plant host which is why our interpretation is of a mutually beneficial association (mutualism) rather than neutral (commensalism). Erroneously, Niveiro et al. (2020) described *Moniliophthora mayarum* sp. *nov.* and *Moniliophthora ticoi comb. nov.* as "Neotropical tree pathogens", despite the absence of symptoms.

Within the genus *Moniliophthora*, Aime and Phillips-Mora (2005) also included an unknown species isolated as an endophyte from a graminaceous host in the USA; whilst Kropp and Albee-Scott (2012) conjectured that the new species they described from a Polynesian island, *M. aurantiaca*, was not indigenous and had arrived in driftwood – presumably as an endophyte, and possibly from the Americas – since it only occurred on the shoreline and not in the native forest. The grouping of *M. aurantiaca* with South American strains of *Moniliophthora* (Fig. 12) would seem to endorse this interpretation but, clearly, this can only be confirmed pending a more comprehensive molecular study.

The geographic distribution, and resilience to seemingly adverse climatic conditions of *M. perniciosa* – a purportedly tropical rainforest species, "of the South American equatorial belt" (Baker and Holliday, 1957) – on the *Solanaceae* hosts reported here is unexpectedly wide; reaching the most meridional of Brazilian states of Rio Grande do Sul, in a sub-tropical locality on *S. mauritianum*. It was also found in cool highland situations, such as: Nova Friburgo (state of Rio de Janeiro) 1100 masl, on *Solanum schwartzianum* and *Cestrum* sp.; and Serra da Piedade (state of Minas Gerais) 1700 masl, on *S. subumbellatum*; as well as in a semi-arid habitat in north-east Brazil (Serra das Almas, Crateús, state of Ceará), on *S. baturitense* (Fig. 1).

In conclusion, since the seminal publications of Stahel (1915, 1919), a considerable body of work has been compiled on witches' broom disease caused by *M. perniciosa*. Nevertheless, there are still many gaps to fill in our knowledge of the fungus. The present study has grouped all the isolates or biotypes – both pathogenic and non-pathogenic, and from an eclectic range of hosts – within a single species. The significance of differentiating populations of *M. perniciosa* based on the hosts from which isolates were obtained as a form of "pseudotaxa" is, therefore, weakened by our results. Whether or not this species should be sub-divided into varieties, on the basis of basidiomatal morphology – as suggested by Pegler (1978) – or into pathotypes, based on host-range testing, is also highly debatable, especially in view of the often ambiguous results from cross-inoculation studies. Although the present work increases our understanding of the natural distribution of *M. perniciosa* in Brazil and of the taxonomy of this important pathogen of cacao, the title given by Evans and Barreto (1996) to their publication – "*C. perniciosa*: a much investigated but little understood fungus" – still remains valid.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2020.09.001.

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