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NOVEL MOLECULAR MARKERS FOR INFERENCE OF PHYLOGENETIC RELATIONSHIP AND DIFFERENTIAL DETECTION OF *Moniliophthora perniciosa* AND *Moniliophthora roreri*

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Dissertação apresentada à Universidade de Brasília como requisito parcial para obtenção do título de Mestre em Fitopatologia pelo Programa de Pós-Graduação em Fitopatologia.

Orientador: Prof. Dr. Danilo Batista Pinho

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SUMÁRIO

LISTA DE FIGURAS

LISTA DE TABELAS

GENERAL ABSTRACT

Magalhães, Eduardo Henrique Porto. **Novel molecular markers for inference of phylogenetic relationship and differential detection of *Moniliophthora perniciosa* and *Moniliophthora roreri***. 46 p. Dissertation (Master in Plant Pathology) – Universidade de Brasília, Brasília, DF, Brazil.

Cocoa (*Theobroma cacao*) is a plant species native to the Amazon rainforest, widely cultivated in tropical areas for chocolate production. The fungi *Moniliophthora perniciosa* and *M. roreri* are the main pathogens that affect cocoa production in South and Central America, where they are responsible for significant yield losses. These pathogens pose a real threat to other regions of cocoa production. The quarantine fungus *M. roreri* was detected in Brazil on July 2021 close to the Peruvian border. Prevention is the main strategy to protect cacao from *M. perniciosa* and *M. roreri*. Therefore, accurate and rapid detection methods are essential to prevent the entry and spread of diseases. A single molecular marker is insufficient for accurate identification of fungi. Selecting proper genomic regions of homologous single-copy genes is essential to avoid species overlapping and enable precise molecular identification of *Moniliophthora* species. In addition, the validation of a Polymerase Chain Reaction (PCR) using species-specific primers allows obtaining fast, accurate and effective results. Based on these premises, this work aimed to (i) develop and validate new molecular markers for robust phylogenetic studies of *Moniliophthora* spp.; (ii) indicate the optimal molecular markers, and (iii) develop and validate species-specific primers for quick and easy detection of *M. perniciosa* and *M. roreri*. *Moniliophthora roreri* isolates were collected in Manabí-Ecuador, while *M. perniciosa* isolates were obtained from the states of Amazonas, Bahia and Pará-Brazil for sequencing the molecular markers ITS, LNS2, MCM7, RPB1, RPB2, TEF1- α, TEF3 and TOPI. Combinations of primers were tested to amplify the ITS and LNS2 regions. Eight sequenced genomic regions did not have intraspecific variation among isolates. The developed species-specific primers from the ITS region successfully amplified *M. perniciosa* and *M. roreri* isolates, while the ones designed from the LNS2 region only amplified *M. roreri*. Therefore, a protocol based on the primers designed by this work would be able to effectively identify and detect *M. perniciosa* and *M. roreri*, which is essential to mitigate risks caused by witches' broom and cocoa frosty pod rot. These tools can be included in international surveillance programs for plant products, and in contingency plans for monitoring plant health.

Keywords: quarentine fungus, diagnosis, plant pathology, species-specific primers, plant diseases

RESUMO GERAL

Magalhães, Eduardo Henrique Porto. **Novos marcadores moleculares para o relacionamento filogenético e detecção de *Moniliophthora perniciosa* e *Moniliophthora roreri***. 2021. 46 p. Dissertação (Mestrado em Fitopatologia) – Universidade de Brasília, Brasília, DF, Brasil.

O cacaueiro (Theobroma cacao) é uma espécie nativa da floresta Amazônica cultivada principalmente na América do Sul e Central, Sudeste Asiático e África Ocidental para a produção do chocolate. Os fungos *Moniliophthora perniciosa* e *M. roreri* são os principais patógenos que afetam a produção de cacau na América do Sul e Central. Nessas regiões, a produção foi reduzida drasticamente devido a introdução desses fungos, ocasionando o abandono de áreas ou o desmatamento para a implementação de novos plantios. A introdução desses dois patógenos em outras regiões é uma ameaça para a produção de cacau. O fungo quarentenário *M. roreri* foi detectado em julho de 2021 em áreas no estado do Acre próximas a fronteira com o Peru. Como a prevenção é a principal estratégia para proteger o cacaueiro de *M. perniciosa* e *M. roreri*, protocolos de biossegurança e métodos de detecção precisos e rápidos são essenciais para evitar a entrada e a disseminação de doenças e garantir a rápida erradicação do foco nas áreas. Como um único marcador molecular é insuficiente para a identificação precisa de fungos, a escolha de diferentes regiões genômicas de genes homólogos de cópia única é essencial para evitar a sobreposição de espécies, e consequentemente, permitir a identificação molecular precisa das espécies de *Moniliophthora*. Além disso, a validação de uma Reação em Cadeia da Polimerase (PCR) usando iniciadores espécie-específicos possibilita a obtenção de resultados mais rápidos, precisos e eficazes para o monitoramento das espécies de *Moniliophthora* no cacaueiro. Baseado nessas premissas, esse trabalho visa (i) desenvolver e validar novos marcadores moleculares para estudos filogenéticos de *Moniliophthora* spp.; e (ii) desenvolver e validar iniciadores espécie-específicos para rápida e fácil detecção de *M. perniciosa* e *M. roreri*. Os isolados de *M. roreri* foram coletados em Manabí-Equador enquanto os isolados de *M. perniciosa* foram obtidos dos estados do Amazonas, Bahia e Pará-Brasil para o sequenciamento dos marcadores moleculares ITS, LNS2, MCM7, RPB1, RPB2, TEF1-α, TEF3 e TOPI. Após o alinhamento das sequências de cada região genômica, três e quatro combinações de iniciadores foram testadas para amplificar a região ITS e LNS2, respectivamente. As oito regiões genômicas analisadas não possuem variação intraespecífica entre os isolados e possibilitam a separação de *M. perniciosa* e *M. roreri*. Os iniciadores espécie-específicos da região ITS amplificaram isolados de *M. perniciosa* e *M. roreri*, enquanto os iniciadores espécie-específicos da região LNS2 amplificaram somente isolados de *M. roreri*. Um protocolo baseado nos primers desenvolvidos nesse trabalho permitem uma identificação rápida e precisa de *M. perniciosa* e *M. roreri*, auxiliando na prevenção dos prejuízos causados pela vassoura de bruxa e monilíase do cacaueiro. Portanto, essas ferramentas podem ser utilizadas em programas de vigilância internacional e planos de contingenciamento para o monitoramento da sanidade do cacaueiro.

Palavras-chave: fungo quarentenário, diagnose, fitopatologia, iniciadores espécie-específicos, doenças de plantas.

MANUSCRIPT

According to the guidelines of Tropical Plant Pathology

Novel molecular markers for inference of phylogenetic relationship and differential detection of *Moniliophthora perniciosa* and *Moniliophthora roreri*.

Novel molecular markers for inference of phylogenetic relationship and differential detection of *Moniliophthora perniciosa* and *Moniliophthora roreri*.

ABSTRACT

Cocoa (*Theobroma cacao*) is a plant species native to the Amazon rainforest, widely cultivated in tropical areas for chocolate production. The fungi *Moniliophthora perniciosa* and *M. roreri* are the main pathogens that affect cocoa production in South and Central America, where they are responsible for significant yield losses. These pathogens pose a real threat to other regions of cocoa production. The quarantine fungus *M. roreri* was detected in Brazil on July 2021 close to the Peruvian border. Prevention is the main strategy to protect cacao from *M. perniciosa* and *M. roreri*. Therefore, accurate and rapid detection methods are essential to prevent the entry and spread of diseases. A single molecular marker is insufficient for accurate identification of fungi. Selecting proper genomic regions of homologous single-copy genes is essential to avoid species overlapping and enable precise molecular identification of *Moniliophthora* species. In addition, the validation of a Polymerase Chain Reaction (PCR) using species-specific primers allows obtaining fast, accurate and effective results. Based on these premises, this work aimed to (i) develop and validate new molecular markers for robust phylogenetic studies of *Moniliophthora* spp.; (ii) indicate the optimal molecular markers, and (iii) develop and validate species-specific primers for quick and easy detection of *M. perniciosa and M. roreri*. *Moniliophthora roreri* isolates were collected in Manabí-Ecuador, while *M. perniciosa* isolates were obtained from the states of Amazonas, Bahia and Pará-Brazil for sequencing the molecular markers ITS, LNS2, MCM7, RPB1, RPB2, TEF1- α, TEF3 and TOPI. Combinations of primers were tested to amplify the ITS and LNS2 regions. Eight sequenced genomic regions did not have intraspecific variation among isolates. The developed species-specific primers from the ITS region successfully amplified *M. perniciosa* and *M. roreri* isolates, while the ones designed

from the LNS2 region only amplified *M. roreri*. Therefore, a protocol based on the primers designed by this work would be able to effectively identify and detect *M. perniciosa* and *M. roreri,* which is essential to mitigate risks caused by witches' broom and cocoa frosty pod rot. These tools can be included in international surveillance programs for plant products, and in contingency plans for monitoring plant health.

Keywords: quarentine fungus, diagnosis, plant pathology, species-specific primers, plant diseases

INTRODUCTION

Cocoa (*Theobroma cacao*) is a native plant of the Amazon rainforest, cultivated mainly in South and Central America, Southeast Asia and West Africa to produce chocolate (Argout et al. 2011). The international trade of chocolate moves around 103 billion dollars annually, but cocoa production is often affected by diseases (Ploetz 2007; Bailey and Meinhardt 2018; Marelli et al. 2019).

There are several pathogens affecting cocoa worldwide. *Phytophthora palmivora* is the only pest widely reported in all cocoa growing areas (Ali et al. 2016; Wang et al. 2020; Decloquement et al. 2021). *Phytophthora megakarya* and Cacao swollen shoot virus have their occurrence limited to West Africa, while *Ceratobasidium theobromae* is present only in Southeast Asia. This restricted pest range is probably caused by the introduction of cocoa in new areas other than its origin center (Domfeh et al. 2019; Marelli et al. 2019). The fungi *Moniliophthora perniciosa* and *M. roreri* occur naturally in cocoa areas in the Amazon rainforest (Phillips-Mora and Wilkinson 2007; Meinhardt et al. 2008; Evans et al. 2013). These two pathogens have spread to new areas in the American continent, and they represent a continuous threat to cocoa cultivation (Bailey and Meinhardt 2018; Sousa Filho et al. 2021).

The witches' broom disease caused by *M. perniciosa* is the main responsible for yield losses on Brazilian cocoa production (Evans et al. 2013; Sousa Filho et al. 2021). Brazil was the world's second largest producer of cocoa in the 1980s. However, the introduction of *M. perniciosa* in the state of Bahia (the main producing state) in 1989 had drastically reduced the production to less than a third over the following 10 years (Pereira et al. 1989; Pimenta Neto et al. 2018). This event has significantly changed the situation of cocoa, turning Brazil into a cocoa importing country (Peres Filho 1998).

In July 2021, the frosty pod rot caused by *M. roreri* was found in cocoa pods and cupuaçu fruits in urban Brazilian areas near the border with Peru (MAPA 2021a). The *M. roreri*

causes bigger losses compared to the witches' broom disease. It also is a harder pest to control, and, therefore, currently represents the major threat to Brazil's cocoa production (Bailey et al. 2018; Fidelis et al. 2018; Pimenta Neto et al. 2018).

The fungus *M. perniciosa* infects meristematic tissues of *Theobroma* and *Herrania* species (Malvaceae), *Solanum* spp. (Solanaceae), *Bixa orellana* (Bixaceae) and plant species belonging to Bignoniaceae and Malpighiaceae (Lisboa et al. 2020). This pathogen induces leaf hypertrophy and hyperplasia through the accumulation of auxiliary shoots and the thickening of meristematic tissues. Infected flower cushion produces a green broom structure and an abnormal formation of dried flowers. Infected young fruits are parthenocarpic with a relatively small size, abnormal morphology and mummified appearance. The late infection of fruits causes premature yellowing and necrotic irregular lesions that become depressed and/or surrounded by chlorotic halos (Oliveira and Luz 2005; Santos et al. 2017).

The infection of *M. roreri* is limited to pods from *Theobroma* spp. and *Herrania* spp. (Malvaceae), while *M. perniciosa* targets all meristematic tissues from species belonging to Bignoniaceae, Bixaceae, Malpighiaceae, Malvaceae and Solanaceae (Bailey et al. 2018). The frosty pod rot symptoms are similar to those caused by witches' broom disease, except for the intense sporulation on the pod surface. Young pods may remain asymptomatic for up to 90 days and then show lateral swelling on their surface.

The necrotrophic phase is characterized by the premature yellowing on the pods. The symptoms rapidly change to irregular, necrotic and coalescent lesions that can cover the entire pod surface. Late infections can cause necrotic, restricted, and depressed lesions on the pods. The lesions can evolve quickly and show a white colored mycelial growth that will later develop into a dense and powdery mass of spores called pseudostroma. It can cover the entire pod (Oliveira and Luz 2005).

Preventing the introduction and establishment of *M. roreri* and *M. perniciosa* in disease-free areas are the only efficient strategy to reduce the potential damage caused by these phytopathogens (Bailey et al. 2018; Sousa Filho et al. 2021). The frosty pod rot infection in cocoa can cause losses of up to 100% and this situation often leads to the cocoa fields abandonment (Phillips-Mora and Wilkinson 2007; Bailey et al. 2018). To reduce the damage caused by frosty pod rot, the removal of infected pods from the cultivated areas is the main control strategy. (Tirado-Gallego et al., 2016).

The likely pathway for *M. roreri* to enter Brazil is associated with infested plant products, especially cocoa pods and seedlings. However, other non-host plant species may constitute pathways for introducing this pathogen in the country (Fidelis et al. 2018).

Illegal transit of plant material between Brazil's border areas and Bolivia, Colombia, Peru and Venezuela is the main potential source of witches' broom and frosty pod rot spreading to new areas (Moraes et al. 2012; Marelli et al. 2019). Therefore, effective detection methods are essential to prevent the diseases entry and spread. Furthermore, a rapid detection increases the chances of success in eradicating a possible outbreak, preventing the disease from moving to areas that are considered disease-free (Moraes et al. 2012; Luchi et al. 2020).

The current protocol for *Moniliophthora* spp. detection is based only on sequencing of the internal transcribed spacer (ITS) and its comparison to databases (MAPA 2021b). The ITS region is frequently used in phylogenetic studies of *M. perniciosa* and *M. roreri*, and many studies are based exclusively on this genomic region (Arruda et al. 2003, 2005; Kerekes and Desjardin 2009; Maridueña-Zavala et al. 2016; Artero et al. 2017; Niveiro et al. 2020).

The first multilocus analysis of *Moniliophthora* spp. was performed using five genomic regions (LSU, SSU, ITS, RPB1 and TEF1) by Aime and Phillips-Mora (2005). However, most isolates of *Moniliophthora* spp. deposited the GenBank still only have sequences from the ITS region (Lisboa et al., (2020). A molecular approach using three genomic regions (ITS, LSU and

RPB1) from different *M. perniciosa* biotypes and hosts confirmed the presence of several *M. perniciosa* genotypes (Lisboa et al. 2020). Other studies using different molecular tools also revealed the presence of different genotypes of *M. perniciosa* and *M. roreri* (Arruda et al. 2003; Ali et al. 2015; Jaimes et al. 2016; Artero et al. 2017; Barbosa et al. 2018). Thus, new molecular markers should be tested to clarify the intraspecific variability and reveal the possible occurrence of cryptic species in *Moniliophthora* spp.

A single molecular marker may be insufficient for the accurate identification of fungi (Lücking et al. 2020). Different genomic regions are chosen to avoid overlapping species, and, consequently, allow a correct molecular identification of *Moniliophthora* species. The growing number of sequenced fungal genomes, combined with algorithms to search for homologous single-copy genes, have enabled the selection of new phylogenetic markers that are more informative and selective than traditionally used markers (Aguileta et al. 2008; Walker et al. 2012; Vialle et al. 2013; Luchi et al. 2020).

Other studies tested molecular markers of the ortholog genes LNS2, MCM7, RPB2, TEF3 and TOPI, in addition to the often-used markers (ITS, RPB1 and TEF1-α), revealing satisfactory outcomes and making these markers potential candidates for *Moniliophthora* spp. phylogeny (Aguileta et al. 2008; Schmitt et al. 2009; Feau et al. 2011; Stielow et al. 2015).

Although the sequencing of target regions is efficient for the accurate identification of *Moniliophthora* species, the process for obtaining sequences in most Brazilian laboratories is time-consuming and costly. In addition, it requires expensive equipment, training for operators and in-depth knowledge of phylogenetic analysis (Lücking et al. 2020). Therefore, new protocols and effective tools for the detection of *M. perniciosa* and *M. roreri* urgently need to be developed.

The establishment of a new test to detect *M. perniciosa* and *M. roreri* must be compatible with laboratories infrastructure, especially those located in risk areas. The assay

must have a minimum level of specificity, sensitivity, cost and a reasonable speed for its execution. Detection methods based on species-specific primers have been widely used in disease diagnosis. This method is considered a reliable and fast technique for detection of several phytopathogens (Cho et al. 2016). Therefore, the validation of a Polymerase Chain Reaction (PCR) using species-specific primers will allow obtaining successful results for monitoring *Moniliophthora* species in cocoa.

Considering the above, this work aimed to (i) develop and validate new molecular markers for robust phylogenetic studies of *Moniliophthora* spp.; and (ii) develop and validate species-specific primers for quick and easy detection of *M. perniciosa* and *M. roreri.*

MATERIAL AND METHODS

Isolates

The isolates of *M. roreri* were collected from symptomatic cocoa pods in the Manabí province, western Ecuador. The isolates of *M. perniciosa* were obtained from cocoa tissues in the Bahia, Pará e Amazônia states, Brazil. The isolates were preserved in the Culture Collection at the Universidade de Brasília (CCUB) and are listed in Table 1.

Table 1. Thirty-one *Moniliophthora* isolates collected from Brazil and Ecuador.

Species	Internal code	Place of sampling
Moniliophthora roreri	3126	Manabí (Ecuador)
Moniliophthora roreri	3127	Manabí (Ecuador)
Moniliophthora roreri	3128	Manabí (Ecuador)
Moniliophthora roreri	3129	Manabí (Ecuador)
Moniliophthora roreri	3130	Manabí (Ecuador)
Moniliophthora roreri	3131	Manabí (Ecuador)
Moniliophthora roreri	3132	Manabí (Ecuador)
Moniliophthora roreri	3133	Manabí (Ecuador)
Moniliophthora roreri	3261	Manabí (Ecuador)
Moniliophthora roreri	3262	Manabí (Ecuador)

Moniliophthora roreri	3263	Manabí (Ecuador)
Moniliophthora roreri	3277	Manabí (Ecuador)
Moniliophthora roreri	3264	Manabí (Ecuador)
Moniliophthora roreri	3265	Manabí (Ecuador)
Moniliophthora roreri	3266	Manabí (Ecuador)
Moniliophthora perniciosa	3136	Bahia (Brazil)
Moniliophthora perniciosa	3137	Bahia (Brazil)
Moniliophthora perniciosa	3138	Bahia (Brazil)
Moniliophthora perniciosa	3139	Bahia (Brazil)
Moniliophthora perniciosa	3140	Bahia (Brazil)
Moniliophthora perniciosa	3141	Bahia (Brazil)
Moniliophthora perniciosa	3142	Bahia (Brazil)
Moniliophthora perniciosa	3143	Bahia (Brazil)
Moniliophthora perniciosa	3144	Bahia (Brazil)
Moniliophthora perniciosa	3145	Bahia (Brazil)
Moniliophthora perniciosa	3260	Bahia (Brazil)
Moniliophthora perniciosa	3727	Pará (Brazil)
Moniliophthora perniciosa	3728	Pará (Brazil)
Moniliophthora perniciosa	3729	Amazonas (Brazil)
Moniliophthora perniciosa	3730	Amazonas (Brazil)
Moniliophthora perniciosa	3731	Pará (Brazil)

The isolates were cultivated for 24 hours in Petri dishes with water agar solution (1.5%). Then, the purification of the cultures was made by cutting the hyphae tips of each isolate and placing it on new Petri dishes with water agar (1.5%). The pure cultures were maintained for five days at 25 °C and then, stored at 18 °C in sterilized water, glycerol (10%) and mineral oil.

DNA extraction, PCR and sequencing for selection the molecular markers.

The DNA extraction was done using the Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.). About 40 mg of mycelia were transferred to 1.5 mL microcentrifuge tube containing four metal beads of 2 mm, 600 µL of Nuclei Lysis Solution (Wizard® Genomic DNA Purification Kit - Promega Corporation, WI, U.S.A.) and 100 mg of PVP - Polyvinylpyrrolidone (Sigma–Aldrich Co.). The samples were mixed using the L-Beader

(Loccus Biotecnologia) and the DNA extraction was made using the protocol described by PINHO et al., 2012.

Thirty-one isolates of *M. perniciosa* (n=16) and *M. roreri* (n=15) were submitted to amplification using twenty-three primers sets from eleven genomic regions. These regions have been extensively used in phylogenetic studies (Schmitt et al. 2009; Feau et al. 2011; Vialle et al. 2013; Stielow et al. 2015).

The PCR mixtures consisted of 6.25 µL of MyTaq PCR Master Mix (2x), 0.3 µL of each primer, 1.0 µL of genomic DNA (25 ng/µL) and 4.65 µL of ultrapure water. The primers employed and PCR conditions for each molecular marker are shown in Table 2. Each amplification was repeated at least twice in separated assays.

The amplified products were evaluated for verifying the bands presence or absence. The individual PCR products were purified and bidirectionally Sanger-sequenced using the corresponding primer sets. The new sequences were assembled and manually edited using Geneious Prime version 2020.2.2.

New primers were designed for genes MCM7, RPB1 and RPB2 amplification. This primer development was done through *Moniliophthora roreri* sequence analysis available at Mycocosm (https://mycocosm.jgi.doe.gov/) (Meinhardt et al. 2014). The primers were tested at gradient PCR of 52 to 60 °C to set out the optimal annealing temperature (Table 2).

Phylogenetic Analyses

The partial nucleotide sequences from each genomic region from the isolates were searched at the NCBI-GenBank nucleotide database. Then, Bayesian phylogenetic trees were individually inferred from each genomic region (ITS, LNS2, RPB1, RPB2, TEF1-α, TEF3, MCM7 and TOPI). The Agaricomycetes *Agaricus bisporus* was used as outgroup (Morin et al. 2012). The best nucleotide substitution model for Bayesian inference was settled on

MrModeltest. The MrBayes v3.2.133. was run through CIPRES portal. The Markov Chain Monte Carlo (MCMC) analysis was made for a total of one million generations, with a sampling process taking place for every 100 generations. The convergence of the log likelihoods was confirmed using TRACER v1.7.134. The first 25% of the sampled trees were discarded, with the posterior probability (PP) values calculated for the remaining trees. The phylogenetic trees visualization and edition were carried out using FigTree v1.4.4 and MacSVG softwares.

Primer design and testing for *Moniliophthora* spp. detection

The ITS and LNS2 sequences of *M. perniciosa* and *M. roreri* were selected and aligned for searching species-specific primers using Primer3 Plus and Primer-BLAST (Ye et al. 2012; Hung and Weng 2016). Additionally, divergent regions within the ITS and LNS2 sequences were selected for manual primers development. The specificity of the primers sequences made from ITS and LNS2 regions was silico-tested before synthesis by searching similar DNA sequences on the NCBI database. Each specific primer were checked for verifying the following parameters: primer length, primer melting temperature, GC content, GC clamp, primer secondary structures (hairpins, self-dimer, and cross dimer), repeats, runs and 3′ end stability.

The specific primers were designed and screened against ten isolates from *M. perniciosa* (n=5) and *M. roreri* (n=5). The screening also added eight fungal genera (*Fusarium* sp., *Colletotrichum* sp., *Cercospora* sp. *Aspergillus* sp., *Lasiodiplodia* sp. *Macrophomina* sp., *Trichoderma* sp., and *Phytophthora* sp.) that may occur on cocoa plants or be found as contaminants. Each amplification was repeated at least twice in separated assays.

The PCR parameters were set to start at 95˚C for 90 seconds, followed by 35 cycles at 95˚C for 20 seconds, 52˚C for 45 seconds, and 72˚C for 60 seconds, ending with a final extension at 72˚C for 5 minutes. Amplification products were visualized on 2% agarose gel stained with EtBr. After the initial screening, the validated primers were tested on all thirty-one

isolates. Thus, the individual PCR amplicons were purified and bidirectionally Sanger-sequenced using the corresponding primer sets.

Table 2. Primers selected for phylogenetic analysis and detection of *Moniliophthora perniciosa* and *Moniliophthora roreri*.

Genomic region	Primers	Primer sequence (5'to 3')	Annealing (temperature / duration)	Amplicon length	Reference
ITS	ITS1 / ITS4	TCCGTAGGTGAACCTGCGG / TCCTCCGCTTATTGATATGC	53 ºC / 45"	750 bp [C]	(White et al. 1990)
TEF-1α	EF1F / EF2R	TGCGGTGGTATCGACAAGCGT / AGCATGTTGTCGCCGTTGAAG	PCR amplification failed	-	(Jacobs et al. 2004)
TEF1-α	983F / 2218R	GCYCCYGGHCAYCGTGAYTTYAT / ATGACACCRACRGCRACRGTYTG	56ºC / 45"	500 bp [A] 1000bp [B]	(Rehner 2001)
TEF1-α	EF1-1018F / EF1-1620R	GAYTTCATCAAGAACATGAT / GACGTTGAADCCRACRTTGTC	PCR amplification failed	-	Rehner, 2001
TEF3	EF3 3185F / EF3_3538R	TCYGGWGGHTGGAAGATGAAG / YTTGGTCTTGACACCNTC	56ºC / 45"	500 bp [C]	(Stielow et al. 2015)
TEF3	EF3_3188F / EF_3984R	GGHGGHTGGAAGATGAAG / TCRTAVSWGTTCTTGAACTT	PCR amplification failed	-	Stielow et al., 2015
TOPI	TOP1_501-F / TOP1-501-R	TGTAAAACGACGGCCAGTACGATACT GCCAAGGTTTTCCGTACHTACAACGC / CAGGAAACAGCTATGACCCAGTC CTCGTCAACWGACTTRATRGCCCA	54ºC / 45"	1000 bp [C]	Stielow et al., 2015
LNS2	LNS2_468-F / LNS2_468-R	GGCCATGTGCTGAACATGATCGGHCGWGA YTGGAC / CGGTTGCCRAAKCCRGCATAGAAKGG	54ºC / 45"	750 bp [C]	Stielow et al., 2015

MCM7	709f / 1348r	ACIMGIGTITCVGAYGTHAARCC / GAYTTDGCIACICCIGGRTCWCCCAT	PCR amplification failed	-	(Schmitt et al. 2009)
MCM7	MCM7-709F_Moni / MCM7-1348R_Moni	ACCCGTGTATCGGAAGTMAAGCC / GATTTGGCAACACCAGGGTCRCCCAT	58-56ºC / 60”	750 bp [C]	This study
MCM7	MCM7-Moni_3F / MCM7-Moni_2R	CATTACTGTGCGTGGCATCG / GTCTCGTGTGGGTTTGTCGAG	60-52ºC / 60”	1100 bp [C]	This study
MCM7	MCM7-Moni_937F / MCM7-Moni_2R	CAGCAGAACACTCGTCGATA / GTCTCGTGTGGGTTTGTCGAG	60-56ºC / 60”	1500 bp [A] 1250 bp [B]	This study
MCM7	MCM7-Moni_640F / MCM7-Moni_2R	GGRTTYCCGGATCACTTACTT / GTCTCGTGTGGGTTTGTCGAG	60-56ºC / 60”	1000 bp [C]	This study
RPB1	Af / Ac/Ar	GARTGYCCDGGDCAYTTYGG / CCNGCDATNTCRTTRTCCATRTA	PCR amplification failed	-	(Brandon Matheny et al. 2002)
RPB1	RPB1-Moni_Af / RPB1-Moni_Cr	GAGTGTCCAGGTCAYTTCGG / CCAGCRATGTCGTTATCCATATA	60-56ºC / 60”	1400 bp [C]	This study
RPB1	RPB1-Moni_167F / RPB1-Moni_1026R	TGGATCCTCGGATGGGAACT / CTTTAGACGCGCACGAATGG	60-56ºC / 60”	1400 bp [C]	This study

RPB2	5F2 / 7cR_P8	GGGGWGAYCAGAAGAAGGC / CCCATRGCTTGYTTRCCCAT	PCR amplification failed	-	(Liu et al. 1999; Sung et al. 2007)
RPB2	RPB2-Moni_5F / RPB2-Moni_7cR	GATGAYCGYGACCACTTYGG / CCCATGGCTTGTTTACCCAT	60-56ºC / 60”	1150 bp [C]	This study
RPB2	RPB2-Moni_5F2 / RPB2-Moni_7cR	GGGGYGACCARAAGAAATC / CCCATGGCTTGTTTACCCAT	56-52ºC / 60”	1000 bp [C]	This study
RPB2	RPB2-Moni_7R / RPB2-Moni_6F	GTTATGATCSGGGAAAGG / TGGGGAATGGTGTGTCCTGC	58-56ºC / 60”	750 bp [C]	This study
60S	60S-506F / 60S-908R	GHGACAAGCGTTTCTCNGG / CTTVAVYTGGAACTTGATGGT	PCR amplification failed	-	Stielow et al., 2015
TUB2	2a / Bt2b_P6	GGTAACCAAATCGGTGCTGCTTTC / ACCCTCAGTGTAGTGACCCTTGGC	PCR amplification failed	-	(Glass and Donaldson 1995)
MS204	E1F1 / E5R1	AAGGGCACCCTGGAGGGCCAC / GATGGTGACGGYGTTGATGTA	PCR amplification failed	-	(Walker et al. 2012)

[A] *Moniliophthora* roreri amplicon length. [B] *Moniliophthora perniciosa* amplicon length. [C] Amplicon length found for both species.

ITS: Internal transcribed spacer, T**EF-1α**: Translation elongation factor 1-alpha, **TEF3**: Translation elongation factor 3, **TOPI**: DNA Topoisomerase I, **LNS2**: Protein LNS2, **MCM7**: minichromosome maintenance complex (DNA helicase) **RPB1**: The largest subunit of RNA polymerase II, **RPB2**: The second largest subunit of RNA polymerase II, **60S**: small ribosomal protein necessary for t-RNA docking, **TUB2**: β-tubulins and **MS204**: Guanine nucleotide-binding protein subunit beta-like protein.

RESULTS

PCR and Phylogenetic Analyses

The tested primers successfully amplified the genomic regions ITS, TEF1-α (983F and 2218R), TEF3 (EF3 3185F and EF3 3538R), TOPI and LNS2 from *M. perniciosa* and *M. roreri.* On the other hand, the primers tested for TUB2, MS204, RPB1, RPB2, 60S and MCM7 did not amplify any isolates. Although unexpected, the primers set used for RPB1 PCR-amplifications failed for all samples. Thus, nine new primers sets were designed to the genomic regions MCM7 (n=4), RPB1 (n=2) and RPB2 (n=3).

Thirty-one isolates from *M. perniciosa and M. roreri* were selected for amplification and sequencing of the ITS, LNS2, MCM7, RPB1, RPB2, TEF-1α, TEF3 and TOPI regions, which were 630, 400, 730, 950, 880, 1080, 380 and 880 bp in length, respectively.

The ITS, LNS2, MCM7, RPB1, RPB2, TEF-1α, TEF3 and TOPI phylogenetic trees showed distinct clades for both species. These analyses revealed the existence of high-interspecific and low intra specific polymorphism within the *Moniliophthora* spp.

Primer design and testing for *Moniliophthora* spp. detection

Primer sequences were compared against obtained sequences in GenBank and result of BLAST (Basic Local Alignments Search Tool) showed 100% homology of primers with sequences of isolates belonging to the species for which primers were designed. The ITS (n=3) and LNS2 (n=4) primers were able to specifically amplify only isolates of *M. perniciosa* and *M. roreri* (Table 3).

The primers set MR-ITS-353F/MPR-ITS-R, specifically designed for the detection of *M. roreri,* successfully amplified a fragment of 350 bp from each *M. roreri* isolate (Figure 1,

samples 1, 2, 3, 4 and 5). The *M. perniciosa* isolates (samples 6, 7, 8, 9, and 10) and the other fungi species (samples 11 to 18) did not have any fragment amplified.

The primers set MP-ITS-354F/MPR-ITS-R, developed specifically to detect *M. perniciosa*, effectively detected this one among all other species (Figure 2). The detected amplicon length was approximately 400 bp.

The primers MR-LNS2-F/MR-LNS2-414R1 and MR-LNS2-F/MR-LNS2-261R2 from the LNS2 region amplified only one amplicon of *M. roreri* isolates of 450 bp (Figure 3) and 350 bp (Figure 4), respectively (samples 1, 2, 3, 4 and 5 of both figures).

The primer set MPR-ITS-158F/MPR-ITS-R from ITS region, and MP-LNS2-292F1/MP-LNS2-R and MP-LNS2-230F2/MP-LNS2-R from LNS2 region, amplified only one amplicon of approximately 200, 400, and 300 bp, respectively, from both isolates of *M. perniciosa* and *M. roreri* (data not showed).

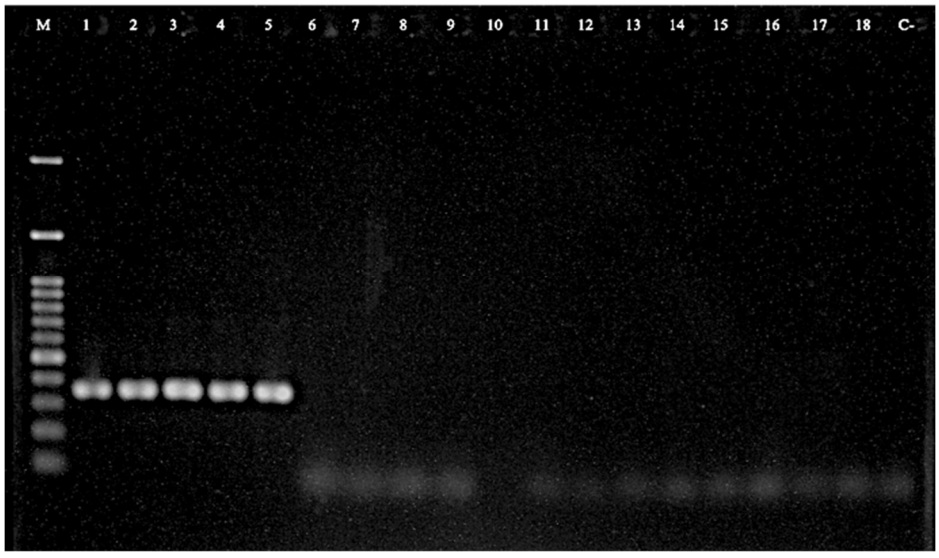

Fig 1. Amplicons of 350 bp visualized on 2% agarose gel from the amplification of isolates of *M. roreri* (1, 2, 3, 4 e 5), *M. perniciosa* (6, 7, 8, 9, e 10), *Fusarium* sp. (11), *Colletotrichum* sp. (12), *Cercospora* sp. (13), *Aspergillus* sp. (14), *Lasiodiplodia* sp. (15), *Macrophomina* sp. (16), *Trichoderma* sp. (17) and *Phytophthora* sp. (18) using MR-ITS-353F and MPR-ITS-R primers. M = molecular marker 100bp DNA Ladder

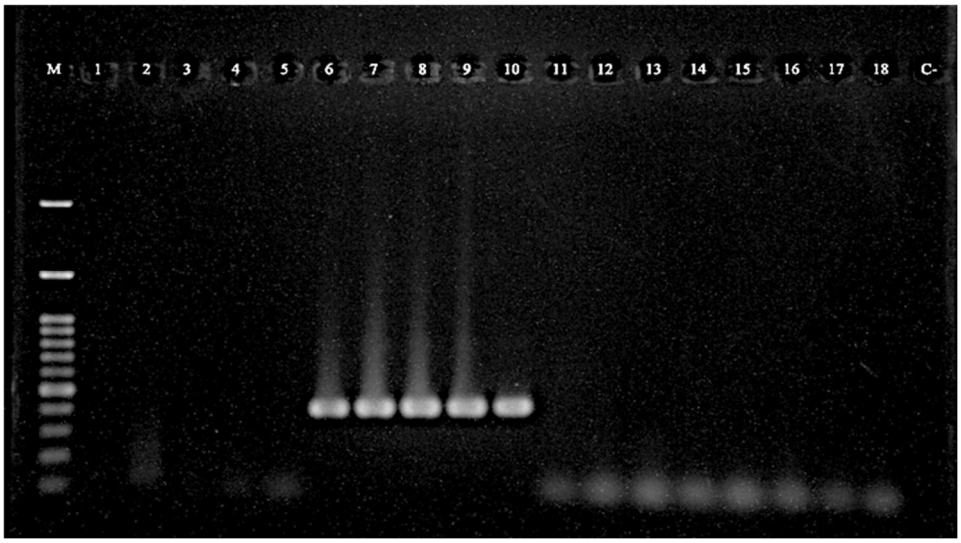

Fig. 2. Amplicons of 400 bp visualized on 1.5% agarose gel from the amplification of isolates of *M. roreri* (1, 2, 3, 4 e 5), *M. perniciosa* (6, 7, 8, 9, e 10), *Fusarium* sp. (11), *Colletotrichum* sp. (12), *Cercospora* sp. (13), *Aspergillus* sp. (14), *Lasiodiplodia* sp. (15), *Macrophomina* sp. (16), *Trichoderma* sp. (17) and *Phytophthora* sp. (18) using MP-ITS-354F and MPR-ITS-R primers. M = molecular marker 100bp DNA Ladder

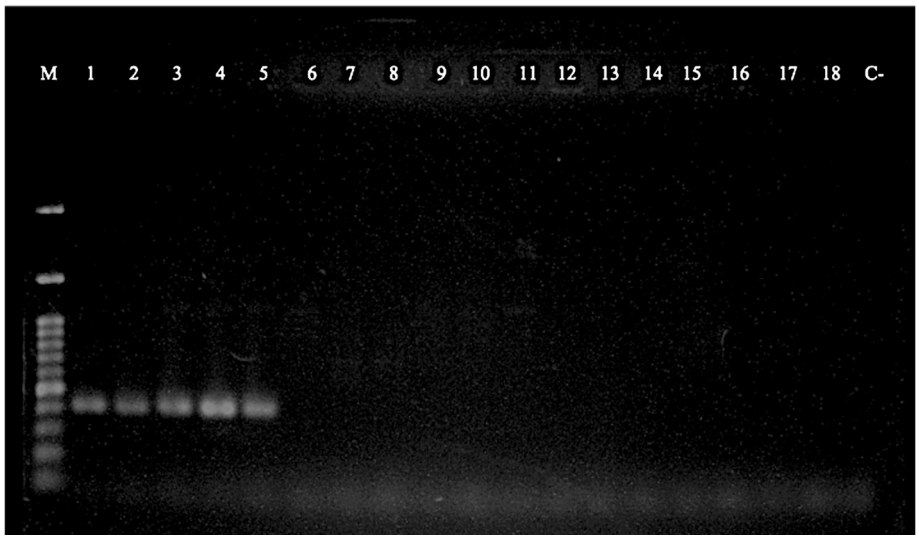

Fig. 3. Amplicons of 450 bp visualized on 1.5% agarose gel from the amplification of isolates of *M. roreri* (1, 2, 3, 4 e 5), *M. perniciosa* (6, 7, 8, 9, e 10), *Fusarium* sp. (11), *Colletotrichum* sp. (12), *Cercospora* sp. (13), *Aspergillus* sp. (14), *Lasiodiplodia* sp. (15), *Macrophomina* sp. (16), *Trichoderma* sp. (17) and *Phytophthora* sp. (18) using MR-LNS2-F and MR-LNS2-414R1 primers. M = molecular marker 100bp DNA Ladder.

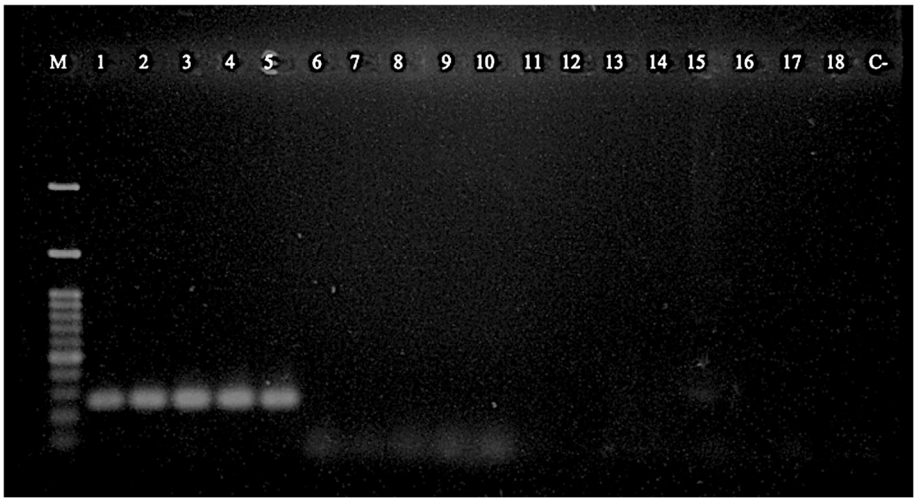

Fig. 4. Amplicons of 350 bp visualized on 1.5% agarose gel from the amplification of isolates of *M. roreri* (1, 2, 3, 4 e 5), *M. perniciosa* (6, 7, 8, 9, e 10), *Fusarium* sp. (11), *Colletotrichum* sp. (12), *Cercospora* sp. (13), *Aspergillus* sp. (14), *Lasiodiplodia* sp. (15), *Macrophomina* sp. (16), *Trichoderma* sp. (17) and *Phytophthora* sp. (18) using MR-LNS2-F_P319 and MR-LNS2-261R2_P322 primers. M = molecular marker 100bp DNA Ladder.

The amplicons were sequenced in order to confirm primer specificity. Comparison of their sequences with the regions used for primer design shown 100% homology, confirming the species-specificity of the primers. No cross-reactions were observed with the other species or genera tested.

Table 3. Primers developed and validated for the detection of *Moniliophthora* spp.

Gene	Primer	Primer sequence (5'to 3')	Expected amplicon length
ITS	MR-ITS-353F / MPR-ITS-R	CCAAACCGAAGTGTTAGCTAGG / CAACTTTCAGCAACGGATCTCTTGG	350 bp
ITS	MP-ITS-354F / MPR-ITS-R	CGAAGTGTTGAGACCTAATTAAAGAGCC / CAACTTTCAGCAACGGATCTCTTGG	400 bp
LNS2	MR-LNS2-F / MR-LNS2-414R1	GGACAAGAACCGATACCTAGCATG / AAGGCTGAGATGTTACCTTCCA	450 bp
LNS2	MR-LNS2-F / MR-LNS2-261R2	GGACAAGAACCGATACCTAGCATG / TCAGCTTCGAGTTCCATCTTCG	350 bp

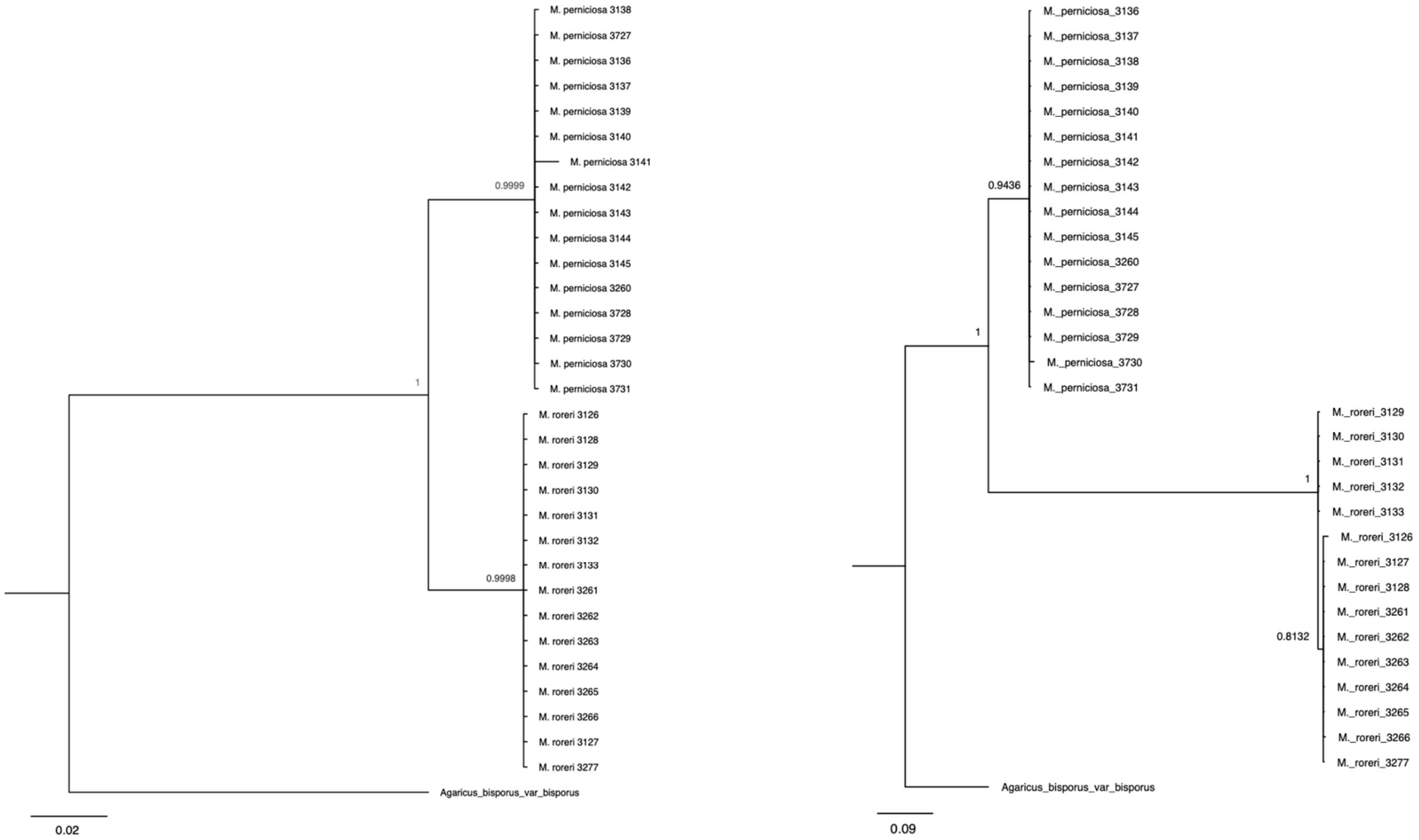

Fig. 5. Phylogenetic trees inferred from Bayesian analysis of ITS (left) and LNS2 (right) sequences of *Moniliophthora* species. Bayesian posterior probabilities are indicated next to the nodes. Specimen numbers are indicated after species names and locality in parentheses. The scale bar represents the number of expected changes per site.

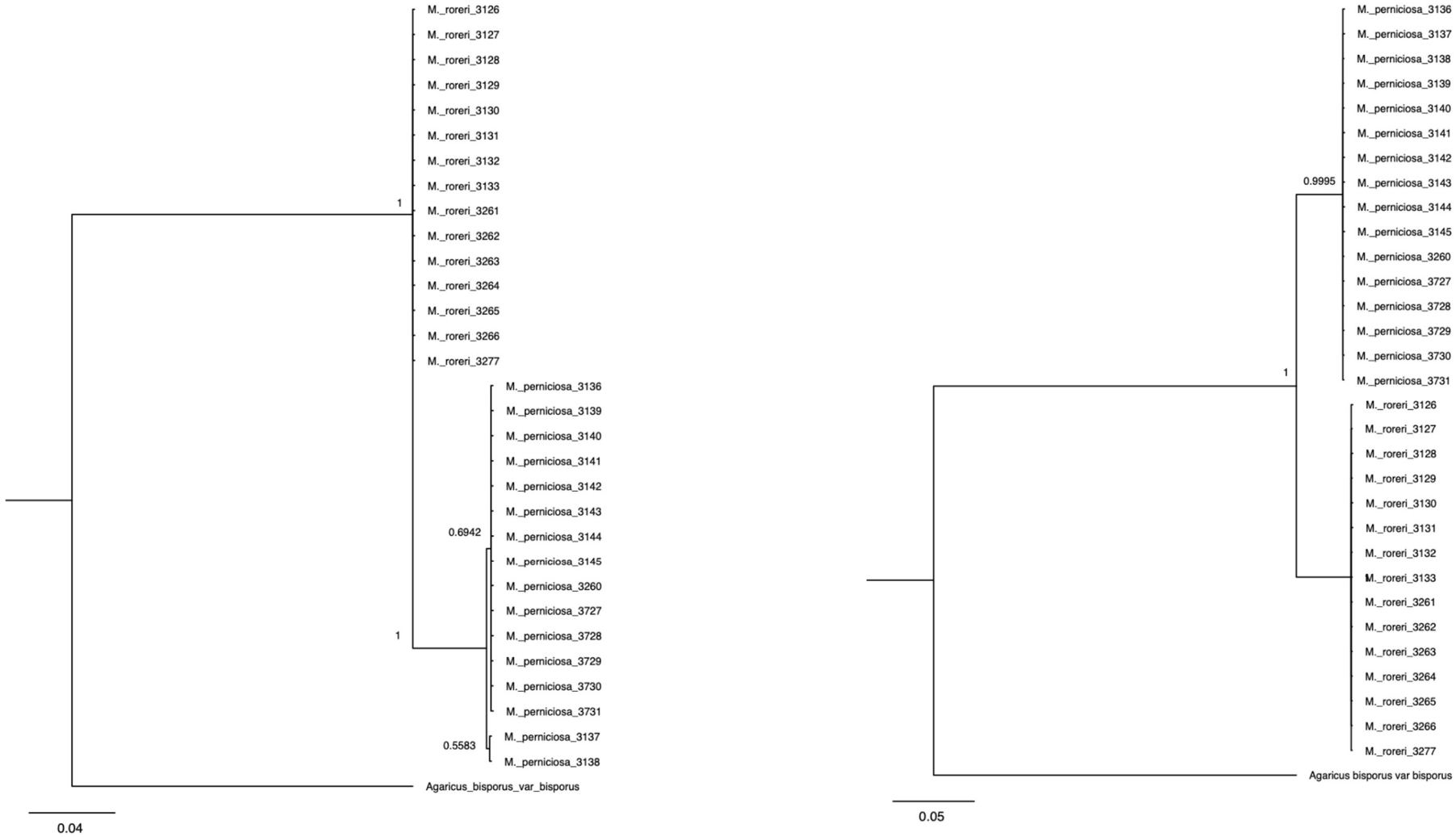

Fig 6. Phylogenetic trees inferred from Bayesian analysis of RPB1 (left) and RPB2 (right) sequences of *Moniliophthora* species. Bayesian posterior probabilities are indicated next to the nodes. Specimen numbers are indicated after species names and locality in parentheses. The scale bar represents the number of expected changes per site.

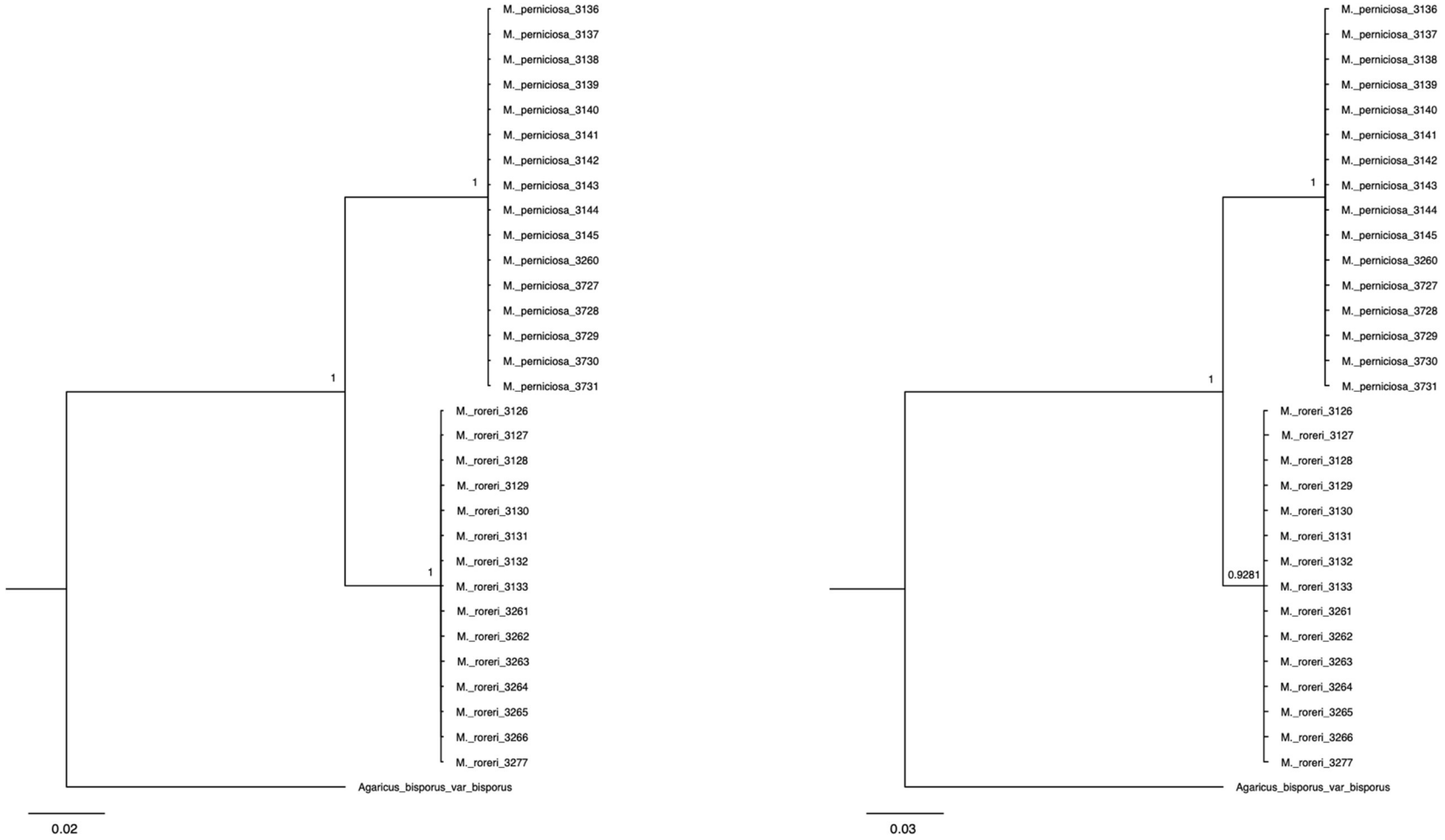

Fig. 7. Phylogenetic trees inferred from Bayesian analysis of TEF1-α (left) and TEF3 (right) sequences of *Moniliophthora* species. Bayesian posterior probabilities are indicated next to the nodes. Specimen numbers are indicated after species names and locality in parentheses. The scale bar represents the number of expected changes per site.

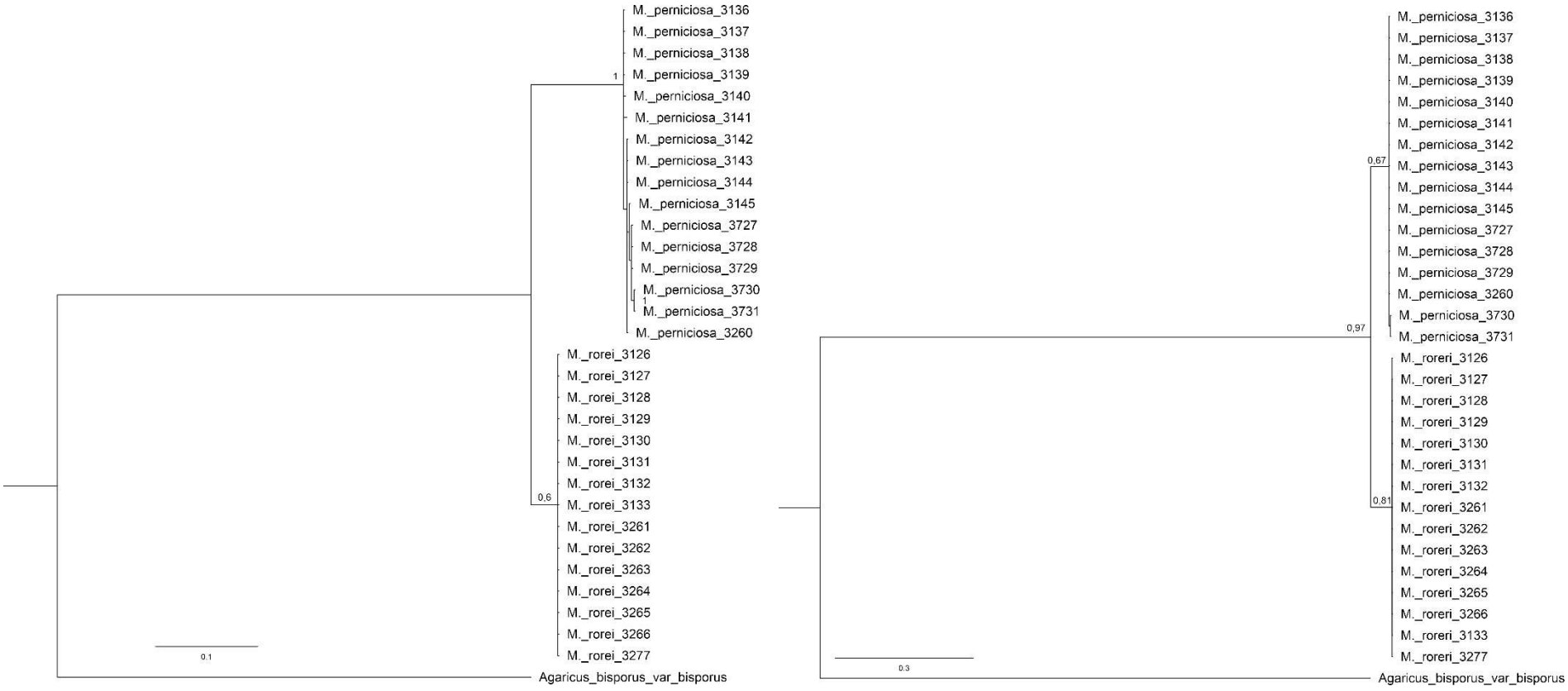

Fig. 8. Phylogenetic trees inferred from Bayesian analysis of TOPI (left) and MCM7 (right) sequences of *Moniliophthora* species. Bayesian posterior probabilities are indicated next to the nodes. Specimen numbers are indicated after species names and locality in parentheses. The scale bar represents the number of expected changes per site.

DISCUSSION

PCR and Phylogenetic Analyses

Few molecular studies investigated the phylogenetic signal for different genomic regions of *M. perniciosa and M. roreri* (Aime and Phillips-Mora 2005; Lisboa et al. 2020). Among the eight genomic regions PCR-amplified and Sanger-sequenced (ITS, LNS2, TEF1-α, TEF3, MCM7, RPB1, RPB2 and TOPI) only ITS, RPB1 and TEF1-α were used in previous studies of *Moniliophthora* species (Aime and Phillips-Mora 2005; Lisboa et al. 2020; Niveiro et al. 2020).

Studies already showed protein-coding genes offering high-resolution and support to fungal taxonomy (Aime and Phillips-Mora, 2005; Schmitt et al., 2009; Feau et al., 2011; Vialle et al., 2013; Stielow et al., 2015; Lisboa et al., 2020). MCM7, RPB1, and RPB2 presented a high-resolution power in Agaricales, within Basidiomycota (Brandon Matheny et al. 2002; Matheny et al. 2006; Stefani et al. 2014). However, PCR and sequencing failures have discouraged their use as potential genetic markers for taxonomic studies in *Moniliophthora* spp.

The new primer sets for MCM7 (n=4), RPB1 (n=2) and RPB2 (n=3) generated individual amplicons and sequences for the corresponding genomic regions. The primers MCM7-Moni_937F/MCM7-Moni_2R, RPB1-Moni_167F/RPB1-Moni_1026R and RPB2-Moni_5F/RPB2-Moni_7cR are highly recommended for PCR-amplification and Sanger-sequencing of MCM7, RPB1 and RPB2, respectively, due to the larger size of the obtained amplicons.

This study revealed a high-interspecific polymorphism and the absence of intraspecific polymorphism among *Moniliophthora* sequences for the genomic regions ITS, LNS2, MCM7, RPB1, RPB2, TEF1-α, TEF3 and TOPI. The *Moniliophthora* isolates were grouped into two distinct phylogenetic clades (Figures 5 to 8), corresponding to *M. perniciosa* and *M. roreri*.

Moreover, thirty-one sequences for each genomic region have been generated and are available for further comparisons. It will increase the usefulness of the regions for comparing and developing new tools for detection of *M. perniciosa* and *M. roreri*.

An extensive taxon sampling of *M. perniciosa* and *M. roreri* populations from all geographical regions and hosts, combined with PCR amplification and sequencing ITS, LNS2, MCM7, RPB1, RPB2, TEF1-α, TEF3 and TOPI genes, would allow reconstructing fully resolved and robust phylogenies for this genus.

Primer design and testing for *Moniliophthora* spp. detection

The sequences obtained from ITS and LNS2 genomic regions showed the required characteristics for developing the detection primers (Ye et al. 2012). The species-specific primers developed in this study are accurate and sensitive to detect only *M. perniciosa* or *M. roreri.* allowing a quicker and less labor-intensive method compared to the current protocol applied by the official laboratories.

The current protocol for *Moniliophthora* detection is based on ITS region sequencing. The sequencing results are inserted on the Basic Local Alignment Search Tool (BLAST) for finding sequence similarity in the NCBI database. This protocol is time-consuming and its use is restricted to few laboratories which possess the necessary equipment for sequencing.

The *Moniliophthora roreri* is considered one of the twenty quarantine pests of most importance for the Brazilian agriculture (MAPA 2020). The likely pathway for *Moniliophthora roreri* entrances in Brazil is associated with infested plant products, especially cocoa pods and seedlings (Fidelis et al. 2018). Refusing the entry of cocoa pods or even limiting their movement within the country is only possible after conclusive identification of the quarantine pest present on a plant material (Luchi et al. 2020). Uncertain identification may lead to introduction of infested plant materials that would expose the Brazilian cocoa production to an unacceptable

risk. At the same time, the phytosanitary control assays must be promptly carried out, using a minimum of equipment.

The witches' broom and frosty pod rot caused by *M. perniciosa* and *M. roreri* are the main diseases of cacao in tropical America (Aime and Phillips-Mora 2005) and their hypothetical introduction in West Africa, Southeast Asia and the Pacific would cause a global crisis in chocolate production (Marelli et al. 2019).

The main pathogens that affect cocoa production in West Africa, Southeast Asia and the Pacific are efficiently monitored by molecular protocols (Muller et al., 2001; Samuels et al., 2012; Ali et al., 2016), while no fast and accessible detection method has been developed for *M. perniciosa* and *M. roreri* yet.

The development of species-specific primers for fast and efficient detection of cacao-witches' broom and frosty pod rot should limit the spread of these destructive diseases and prevent them from entering disease-free areas.

Therefore, these new species-specific primers can be included in international surveillance programs for plant products, and in contingency plans for monitoring plant health of *M. perniciosa and M. roreri* hosts.

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