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Research

Genome-Wide Analysis of *Moniliophthora roreri* Facilitates the Development of Species-Specific Primers for Biomonitoring Frosty Pod Rot of Cacao

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Abstract

Detecting pathogens in asymptomatic plants allows for a rapid response to emerging threats, such as the introduction of new pathogens or the emergence of new resistant strains. Frosty pod rot (*Moniliophthora roreri*) significantly jeopardizes cacao production by impairing pod quality and yield. However, early diagnosis is challenging due to its prolonged incubation period, compounded by symptoms resembling those of *M. perniciosa*, the causative agent of witches' broom disease. To address this, a specific and sensitive quantitative real-time PCR (qPCR) assay was developed for *M. roreri* detection. This involved testing diagnostic primers against a panel of 252 DNA samples collected from target and nontarget species of over 13 fungal species commonly found in cacao plantations from various regions. Species-specific primer pairs were obtained through genomic comparison of *Moniliophthora* spp. genomes. Two primer sets, Mr77F/R and Mr78F/R, were validated using a set of *M. roreri* DNA samples representative of different genetic groups (Ecuador, Peru, and Mexico) and *M. perniciosa*, from which no amplification was detected. The limit of detection (LOD) was determined to be 0.9 ng/µl for both primers Mr77F/R and Mr78F/R. Using this set of primers, the amplification of *M. roreri* DNA from naturally diseased cocoa pods at various stages of infection was also successful. At the practical level, the data provided here confirm the value of molecular diagnostic testing for the early detection of *M. roreri*. These features are desirable for improving fungal diagnostic capacity and assisting in devising strategies to avoid pathogen dispersion within cacao-growing regions.

Keywords: biomonitoring, cacao pathogens, comparative genomics, frosty pod rot, molecular diagnosis, witches' broom

Cacao (*Theobroma cacao* L.), known as the chocolate tree, is a tropical agricultural commodity with increasing demand in the global market (Bennett et al. 2022). Fungal diseases are one of the main problems in cacao plantations, causing losses as high as 40% in global cacao production (Nair 2021). Among those, the diseases caused by *Moniliophthora roreri* (Mr) and *M. perniciosa* (Mp), the causal agent of frosty pod rot (FPR) and witches' broom disease (WBD) of cacao, respectively, have emerged as major threats to cacao plantations (Aime and Phillips-Mora 2005; McElroy et al. 2018), with losses that can reach 90% if proper management is not adopted (Ploetz 2007). Although both fungi are hemibiotrophic and phylogenetically related, they have

different environmental adaptations and pathogenicity strategies (Aime and Phillips-Mora 2005; Meinhardt et al. 2014). Mr has specificity for cacao pods, long biotrophic phase (45 to 90 days), with sporulation after initiating the necrotrophic phase, and the similarities of its symptoms to those caused by Mp, when cacao pods are asymptomatic and progressing to the formation of brown lesions or pod swellings (Bailey et al. 2018; Phillips-Mora et al. 2007). The similarities between these two cacao pathogens pose a challenge when it comes to their early detection and differentiation.

Colombia and Ecuador are the centers of diversity of Mr (Díaz-Valderrama et al. 2022; Phillips-Mora et al. 2007). Until the 1970s, the pathogen was considered geographically isolated, reaching Colombia, Ecuador, Peru, western Venezuela, and eastern Panama (Aime and Phillips-Mora 2005; Ali et al. 2015; Phillips-Mora et al. 2007). However, over the past 60 years, FPR of cacao has spread from Panama throughout Central America to Mexico (Aime and Phillips-Mora 2005) and Jamaica (Johnson et al. 2017). Another expansion occurred through Ecuador, Peru, and Bolivia () toward the north of Brazil (Acre), reaching the cities of Cruzeiro do Sul and Mancio Lima in Acre state (MAPA 2021), and the Brazilian Amazon region neighboring Colombia and Peru. FPR poses a potential threat not only to Brazilian plantations but also to any cacao production areas free of this disease. The primary threat persists in Africa, a major cacao-producing hub, and southern Bahia, the primary cacao producer in Brazil, where the repercussions of the introduction of WBD more than three decades ago are still being addressed.

Considering the long incubation period, the viability and adaptability of Mr (Jaimes et al. 2016), and the scale of the historical outbreaks of FPR in the past 60 years, it is believed that the pathogen will continue to spread and become established in new areas. In addition, symptoms of FPR can be easily confounded with WBD and other cacao pod diseases, i.e., black pod disease of cacao, caused by *Phytophthora* spp. Diseased but asymptomatic pods are the prevalent form of disease dissemination over long distances, which is problematic because it masks the spread of FPR. After all, the classical disease diagnosis takes longer and is based on a visual examination of the spores when pods are at the sporulation stage. The spores have the potential to survive over 3 months and are spread by wind, rain, and human activity. Thereby, FPR threatens the chocolate industry trade and the biodiversity of the agroforestry system in which the cacao trees grow (Evans et al. 2013; McElroy et al. 2018).

FPR and WBD are managed by integrated control practices, including the removal of diseased plant material, timely application of pesticides associated with biological products, and the planting of resistant varieties (Ali et al. 2015). However, pathogen avoidance and exclusion are the most effective strategies for plant disease prevention. The combination of the molecular methods for diagnosing offers a faster and more reliable diagnosis to differentiate these two pathogens and, together with the existing disease management framework, has the potential to improve disease control through biomonitoring, therefore contributing to preventing the pathogens' introduction, establishment, and spread (Adriko et al. 2012). It can help the sustainable integrated management practices required in modern cacao cultivation by increasing productivity and reducing costs (Aslam et al. 2017; McCartney et al. 2003).

Diagnosing the pathogens mentioned above requires a set of information, including history, symptoms examination, isolation, cultural characterization, and genotyping by sequencing. Nevertheless, symptom-based diagnostic methods are laborious, time-consuming, and may not detect the pathogen in the asymptomatic phase of the disease. Molecular diagnosis based on PCR methods provides tools to identify plant-pathogenic fungi faster and more accurately (McCartney et al. 2003). These methods are particularly promising for their speed, specificity, and sensitivity (Aslam et al. 2017; McCartney et al. 2003). Generating a large amount of genomic data enables whole-genome mining to identify regions with better pathogen discriminatory capacity (Feau et al. 2019). Another advantage of molecular detection tools is the possibility of using minimal amounts of DNA, such as from a single fungal spore (McCartney et al. 2003). Specific amplification products that distinguish different fungal species and the early detection of the disease before the presence of visible symptoms are essential for the diagnosis by PCR (Aslam et al. 2017).

Conventional PCR coupled with quantitative real-time PCR (qPCR) methods are valuable for their speed and sensitivity in detecting and diagnosing plant pathogens. In this study, PCR- and qPCR-based disease diagnostics methods were developed to diagnose Mr. These results should be helpful for the surveillance of diseased pods, with the aim of early diagnosis of FPR to achieve early intervention.

Materials and Methods

Fungal collection and DNA extraction

A collection of 252 DNA samples from the target Mr and DNA from various nontarget fungal species known to cause cacao diseases (Mp, *Ceratocystis cacaofunesta*, *Lasiodiplodia* spp., and *Phytophthora* spp.), along with other fungal species commonly found in cacao plantations (*Fusarium* spp., *Trichoderma* spp., and *Clonostachys rosea*; Supplementary Table S1) were included. Except for Mr, all fungal isolates were retrieved from the Brazilian Fungal Collections of the Cacao Research Center (CEPEC-CEPLAC, Bahia, Brazil).

For the development of Mr-specific primers, DNA from two target strains of Mr (MrTcPM37 and MrTcPUNAS3) and two nontarget strains of Mp (MpTcBr1441 and MpTcBr4145) were utilized as positive and negative controls, respectively. DNA samples of Mr were obtained through a technical agreement between CEPLAC and the National Institute of Agricultural Research (INIAP)/Pichilingue Experimental Station in Quevedo, Ecuador; the Universidad Nacional Agraria de la Selva (UNAS) and the Instituto de Cultivos Tropicales (ICT), Peru; and the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Mexico. This ensured a diverse range of samples representing different regions, including Ecuador, which is recognized as one of the centers of Mr diversity (Díaz-Valderrama et al. 2022), therefore guaranteeing the representation of the genetic variability existing in different populations of Mr and Mp (Patrocínio et al. 2017)

To obtain genomic DNA from pure culture, the fungal isolates were cultivated in a liquid mineral medium (10 g of glucose, 1 g of $NH_4H_2PO_4$, 0.2 g of KCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 5 g of yeast extract, 0.05 g of $CuSO_4 \cdot 5\ H_2O$, and 0.05 g of $ZnSO_4 \cdot 7H_2O$ in 1,000 ml of distilled water) (da Silva et al. 2022), except for *Phytophthora* species, which were grown in carrot juice medium (20 g of carrot and 1,000 ml of distilled water), and Mr that was grown on potato dextrose agar. The mycelium was filtered, washed with ice-cold distilled water, and stored at −80°C until extraction. Pure-culture DNA was extracted using the modified SDS protocol (da Silva et al. 2022). All DNA was quantified by Picodrop Spectrophotometer (Picodrop Ltd) and used in PCR reactions at 30 ng/µl.

DNA from naturally diseased pods were obtained without the need to perform pure culture from approximately 100 mg of necrotic internal pod tissue, which was ground into a fine powder using liquid nitrogen. Pods were collected and pro-

cessed in INIAP. In this case, extractions were performed using the cetyltrimethylammonium bromide (CTAB) protocol (Faleiro et al. 2002) or the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions.

Identification, design, and in silico evaluation of species-specific primers

Potential species-specific sequences of Mr were obtained using an in silico comparative pipeline adapted from da Silva et al. (2022). Using a Perl script, the Mr genome MrNCBI of 57 Mb (Ecuador Mr-NCBI, ID 10809) (Meinhardt et al. 2014) were fragmented into sequences of 500 bp with a 250-bp overlap (Pieck et al. 2017). To obtain specific sequences for the target Mr, the fragmented genome Mr-NCBI ID 10809 was compared with the nontarget Mp genomes MpTcBr4145, MpTcBr1441, and MpSlBr178 from CEPLAC/CEPEC/Bahia, Brazil, and MpTcE404, INIAP/Ecuador (Barbosa et al. 2018), using a basic local alignment search tool nucleotide (BLASTn) search (Altschul et al. 1990). This genome was chosen because of their depth coverage sequencing and assembly qualities (Barbosa et al. 2018; Meinhardt et al. 2014). The Mr sequence fragments without significant similarity to the nontarget Mp genomes were used for a BLASTn search against the Mr genome from Peru (Barbosa et al. 2018). This second BLAST was performed as a filter to find sequences shared between different isolates of the same species. This step was carried out to avoid genomic fragments that might be unstable in diverse populations of the target species. After excluding nonsimilar sequences of Mr, the remaining sequences were considered species-specific and, therefore, rated as possible candidates for the design of specific primers (Fig. 1).

A subset of 12 primer pairs (Supplementary Table S2) was designed using the Primer3 program (https://primer3.ut.ee/). Conventional PCR primer pairs were designed to amplify PCR products of approximately 250 to 300 bp. First, primer specificity was analyzed in silico using the Primer-BLAST tools (Ye et al. 2012) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al. 2012) and MFEprimer3.1 (Wang et al. 2019; https://mfeprimer3.igenetech.com/spec). All primer pairs were designed to generate a single peak on the melting curve, indicating a single amplification product. The prediction of the melting curve was performed using the uMELT Quartz software (https://www.dna-utah.org/umelt/quartz/um.php) (Dwight et al. 2011). The formation of secondary structures was analyzed for all primers using the OligoAnalyzer Tool (https://eu.idtdna.com/pages/tools/oligoanalyzer) (Fig. 1).

Evaluation of primers specificity

DNA quality of all samples was validated by PCR with the nuclear ribosomal DNA internal transcribed spacer (ITS) (White et al. 1990) using the universal primers ITS1F (5′-CTTG GTCATTTAGAGGAAGT-3′) and ITS4 (5′-TCCTCCGCTTAT TCATATGC-3′); except for *Phythophthora* spp., DNA samples were amplified using the combination of ITS1 (5′-TCCGTA GGTGAACCTGCGG-3′) and ITS5 (5′-GGAAGTAAAAGT CGTAACAAGG-3′) primers. This test was performed to avoid possible false-negative results caused by the presence of inhibitors in the reaction or by degraded DNA samples.

The in silico specificity of the diagnostic primer pairs was done as described above. To further validate their specificity and applicability, they were tested against a DNA panel of 252 isolates of over 13 fungal species as the Mr (target) samples, the related (nontarget) Mp, and other nontarget species pathogens: *C. cacaofunesta*, *Phytophthora* spp., *Trichoderma* spp., *Fusarium* spp., *Lasiodiplodia* spp., and *C. rosea*, commonly associated with *T. cacao*. Primer pairs that amplified the DNA of nontarget samples were eliminated. Lastly, the selected primer pairs were used to amplify DNA samples of Mr from different locations and of Mp from an array of locations and hosts. Mp DNA samples were named after the host Latin name: *Theobroma cacao*, Tc (MpTcBr4145, MpTcBr1441, MpTcBr4018, MpTcBr4764, and MpTcBr429); *T. grandiflorum*, Tg (MpTgBr4346); *Solanum lycocarpum*, wolf apple-Sl (MpSlBr178); *S. erianthum*, Se (MpSeBr4071); *Capsicum annum*, Ca (MpCaBr4123); and *S. aethiopicum*, Sa (MpSaBr2496), as well as country.

Conventional PCR reactions were carried out in a 20-µl mixture containing 90 ng of DNA, 1× $NHSO_4$ PCR buffer, 1.8 mM $MgCl_2$, 0.20 mM dNTP, 200 nM of each forward and reverse primers, 1 unit of DNA polymerase (Invitrogen), and 0.01% bovine serum albumin (BSA). Amplifications were performed in a Veriti Thermal Cycle (Applied Biosystems) programmed for an initial denaturation at 95°C for 8 min, followed by 35 cycles of denaturation at 95°C for 15 s, the specific annealing temperature of each primer for 40 s, and a 1-min extension at 72°C. The annealing temperatures used in this study were 54°C for primers Mr69F/R and Mr82F/R; 57°C for Mr75F/R; 60°C for Mr72F/R, Mr73F/R, Mr74F/R, Mr79F/R, Mr80F/R, Mr81F/R, and Mr84F/R; and 62°C for Mr77F/R and Mr78F/R. The best PCR performance was achieved with the primers Mr77F/R and Mr78F/R, which were selected as Mr-specific primer pairs.

Amplification products were separated by electrophoresis on a 2% agarose gel stained with GelRed Nucleic Acid Gel Stain, 1× TBE buffer (0.89 M Tris-HCl, pH 8.3; 0.89 M boric acid; and 0.02 M EDTA) at 110 volts for approximately 3 h. Afterwards, the gels were photographed under UV light using the gel documentation system Biolocus L PIX (Loccus Biotecnologia). PCR product sizes were determined using an Invitrogen 100-bp DNA Ladder or GeneRuler 1-kb DNA ladder (Fermentas).

SYBR green qPCR

The candidate diagnostic primer pairs Mr77F/R and Mr78F/R considered specific to Mr were carried forward for the SYBR green qPCR detection assay. The specificity and sensitivity of the specific primer pairs Mr77F/R and Mr78F/R for Mr were investigated and subsequently utilized in the SYBR Green qPCR detection assay. The qPCR protocol was run separately for both primer pairs in a final volume of 10 µl, containing 2× using the Applied Biosystems Power SYBR Green qPCR Master Mix protocol, (Mr77F/R 100nM and Mr78F/R 200nM) of each forward and reverse primers, 0.2 µl of Rox, and 2 µl of 30 ng/µl of DNA template. The reaction conditions were set as follows: initial denaturation cycle at 50°C for 20 s, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 40 s at the specific annealing temperature of each primer (Mr77F/R and Mr78F/R at 63 and 62°C, respectively), and extension at 72°C for 1 min. Subsequently, melting curve analysis was performed by raising the reaction temperature from 60 to 95°C at a rate in intervals of 0.5°C. Data analysis for qPCR was conducted using a 7500 Fast thermal cycler (Applied Biosystems). The reactions in each trial were performed in triplicate. Average threshold cycle values (Ct) and melting temperature (Tm) with their standard deviations were obtained. Nontemplate control (NTC) was used in all assays.

A sample was considered positive for the presence of the target pathogen (in this case, Mr) when it exhibited a single amplification peak, a unique Tm in the amplification, and Ct values of the samples associated with the reference positive control.

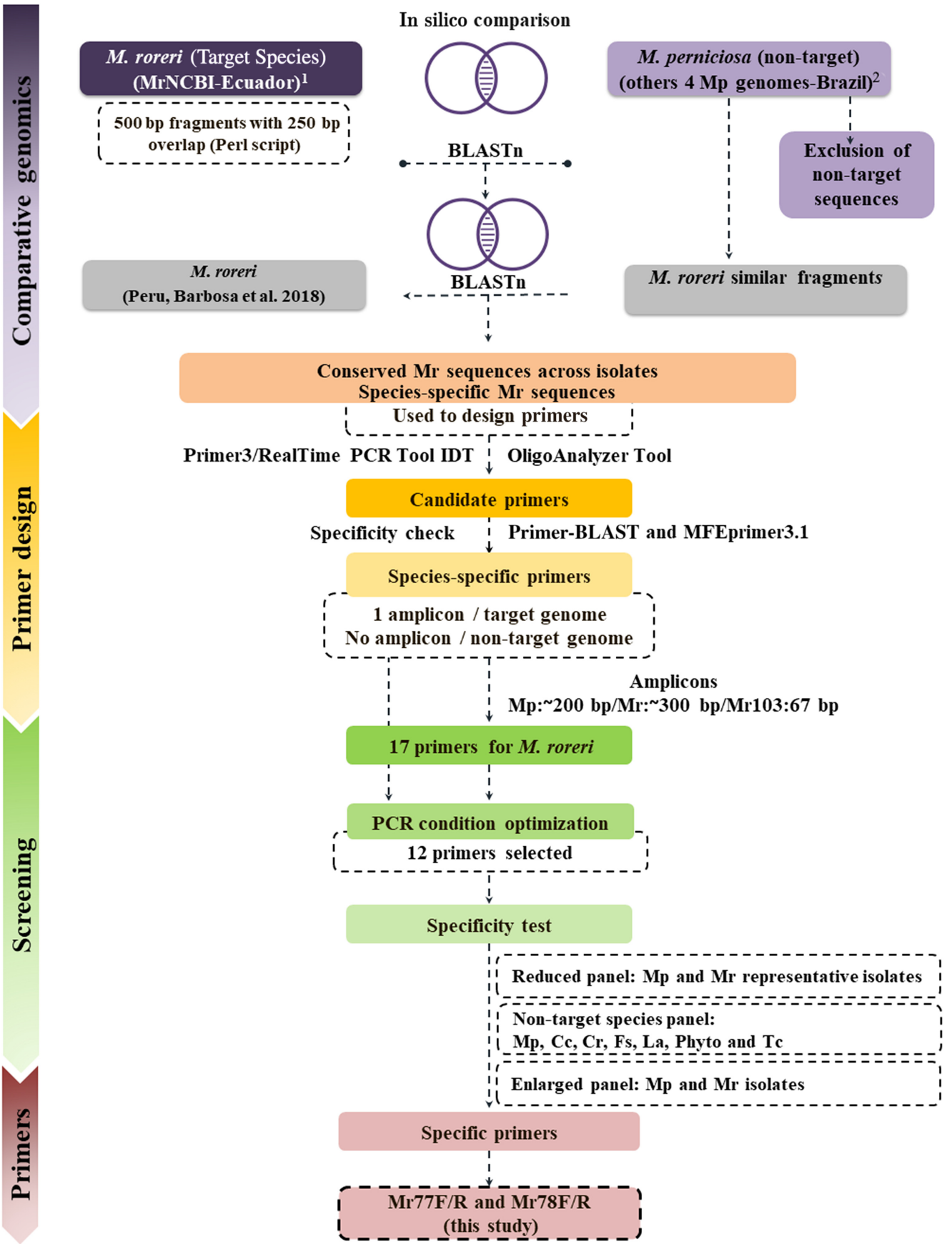


FIGURE 1

Pipeline for obtaining species-specific sequences to *Moniliophthora roreri* (Mr). [1]Genome MrNCBI of 57 Mb (Ecuador Mr-NCBI, ID 10809). [2] Other *M. perniciosa* (Mp) genomes: MpTc1441, MpSI178 (from CEPLAC/CEPEC-Bahia, Brazil), MpTc4145, and MpTcE404, INIAP/Ecuador. Cc, *Ceratocystis cacaofunesta*; Cr, *Clonostachys rosea*; Fs, *Fusarium* spp.; La, *Lasiodiplodia* spp.; Phyto, *Phytophthora* spp.; and Tc, *Trichoderma* spp.

Sensitivity and efficiency of qPCR

The assay's limit of detection (LOD) was determined by analyzing seven two-fold serial dilutions of MrTcPM37 DNA, ranging from 30 to 0.23 ng/µl, using three copies per PCR. DNA concentration was measured with a Picodrop Spectrophotometer (Picodrop Ltd). The assays' overall efficiency (E) was estimated using the slope of the standard curve: $E = ([10 - 1/\text{slope}] - 1) \times 100$, in which E (%) indicates the fraction of target molecules copied in one PCR cycle. An E between 90 and 110% is acceptable (Al-Kahtani et al. 2017).

The coefficient of determination (R^2) is the square of Pearson's correlation coefficient (r) that refers to how well the Ct values correlate with the dilution series. An $R^2 > 0.97$ is acceptable and indicates the consistency of the serial dilutions and possible pipetting errors.

Validation of primers for detecting Mr from diseased cacao pod samples

For the clinical validation, DNA samples were obtained from eight naturally diseased cacao pods showing brown lesions in Ecuador and one from Peru. These pods were collected directly from the field, including those with and without white stroma. DNA extractions were done locally at INIAP in Ecuador and UNAS in Peru. There, pods were disinfected and processed for DNA extraction without pure-culture isolation. The reaction conditions for the qPCR were performed as described above. The negative controls for the detection of Mr in infected pods included DNA from uninfected pods, pods infected with Mp, and Mp mycelia. A set of *T. cacao* chloroplast-specific primers (TcChl-NC_014676.2; F:GCCCAGGTATTAATTACACGAC/R: GCC GATTGATCTTCCAATATGC) was selected as the internal control to amplify only host tissues. The specificity of the primers set for detecting Mr in infected pods was validated using 55 DNA samples from uninfected/infected cacao pods with Mp.

Data analysis by melting curve

The fluorescence data from qPCR with primers Mr77F/R and Mr78F/R were subjected to in-house scripts in Python and R for formatting and analysis of Ct and melting curves. In R, the melt curve function from the {qpcR} package was utilized with parameters norm = FALSE and span. smooth = 0.11, and the negative first derivative of fluorescence during melting was used for subsequent temperature shifts (75 to 88°C and 75 to 85°C for the primers Mr77F/R and Mr78F/R, respectively) and maximum and minimum normalization (R Core Team 2016; Spiess 2018). The analysis of melting curves was conducted for each dilution. Both maximum and minimum normalization were applied for the Ct data from different primers applied to serial dilutions. Graph plotting was accomplished using the plot_ly function within the R environment, providing visual representations of the analyzed data (R Core Team 2016).

Results

Diagnosis of Mr by conventional PCR

Forty-three fragments with 500 bp in length common to Mr were obtained following the pipeline described in Figure 1. The results of Primer-BLAST and the MFEprimer software correctly indicated that the corresponding predicted amplicons of approximately 300 bp in length were only generated in the target species Mr. No amplicons in the nontarget species were obtained (Supplementary Table S2).

The specificity of the primer pairs was tested for conventional PCR in an enlarged panel, with the target Mr and the nontarget species: Mp, *C. cacaofunesta*, *C. rosea*, *Fusarium* spp., *Lasiodiplodia* spp., *Phytophthora* spp., and *Trichoderma* spp. (Table 1). The primer pairs that (i) did not generate amplification products in any sample (primers Mr73F/R and Mr79F/R); (ii) generated poor or not stable PCR product (primers Mr69F/R, Mr72F/R, Mr80F/R, and Mr84F/R); and (iii) generated amplicon in nontarget samples (Mr74F/R, Mr75F/R, Mr81F/R, and Mr82F/R) were considered unsuitable for diagnosis of Mr. DNA viability of the samples was attested by amplifying a PCR product of approximately 700 bp using the universal primers ITS1F and ITS4R (White et al. 1990).

Based on this analysis, the primer sets Mr77F/R and Mr78F/R were selected for further evaluation in a larger panel with DNA of the target (Mr) and nontarget (Mp) species (Fig. 2). The re-

TABLE 1

Specificity test by conventional PCR using reference strains of *Moniliophthora roreri*, *M. perniciosa*, and other main fungal species commonly present in *Theobroma cacao* plantations

	M. roreri			*M. perniciosa*			Other fungi					
Primers	Peru	México	Ecuador	Brazil	Peru	Ecuador	*Cc*[a]	*Phyto*[b]	*Cr*[c]	*Fs*[d]	*La*[e]	*Tc*[f]
ITS[g]	+(41)[h]	+(1)	+(37)	+(65)	+(2)	+(2)	+(22)	+(23)	+(2)	+(2)	+(3)	+(21)
Mr69F/R	+	–	–	–	–	–	–	–	–	–	–	–
Mr72F/R	+	–	–	–	–	–	–	–	–	–	–	–
Mr73F/R	–	–	–	–	–	–	–	–	–	–	–	–
Mr74F/R	+	–	+	–	–	–	–	+(3)	–	–	–	–
Mr75F/R	+	–	+	–	Np	–	+(2)	Np	Np	Np	Np	+(1)
Mr77F/R	+	–	+	–	–	–	–	–	–	–	–	–
Mr78F/R	+	+	+	–	–	–	–	–	–	–	–	–
Mr79F/R	–	–	–	–	–	–	–	–	–	–	–	–
Mr80F/R	+	–	–	–	–	–	–	–	–	–	–	–
Mr81F/R	+	–	+	+(1)	–	–	–	Np	Np	Np	Np	–
Mr82F/R	+	+	+	+(30)	–	+(3)	–	+(17)	Np	Np	Np	–
Mr84F/R	–	–	–	–	–	–	–	–	–	–	–	–

[a] *Cc*, *Ceratocystis cacaofunesta*.
[b] *Phyto*, *Phytophthora capsici*, *P. palmivora*, *P. citrophthora*, and *P. heveae*.
[c] *Cr*, *Clonostachys rosea*.
[d] *Fs*, *Fusarium* spp.
[e] *La*, *Lasiodiplodia* spp.
[f] *Tc*, *Trichoderma longibrachiatum*, *T. stromaticum*, *T. viride*, and *T. harzianum*.
[g] ITS1/4 for all samples except *Phytophthora* spp. ITS1/5.
[h] The number of samples is shown in parentheses. (+), positive PCR amplification; (–), no PCR amplification; and Np, not performed.

sults indicated that well-defined electrophoresis bands could be observed for the Mr DNA samples collected from different countries and locations, and there was no amplification of nontarget species (Fig. 2). These results fully confirmed the applicability of the primer pairs Mr77F/R (Fig. 2A) and Mr78F/R (Fig. 2B) for the molecular diagnosis of Mr and laid a good foundation for the establishment of the qPCR method (Fig. 2C and D).

Diagnosis of Mr by qPCR

The primers Mr77F/R (Fig. 2C) and Mr78F/R (Fig. 2D) were further tested by qPCR in some DNA samples of the target (Mr) and the nontarget species (Mp). Samples with a melting curve profile showing a single amplification peak for Mr77F/R (T_m = 81°C ± 0.6) or Mr78F/R (T_m = 80°C ± 0.4) and Ct values ≤35 were considered positive for the target species. Validation experiments determined that the amplification efficiency of each target was approximately equal to that of the positive control to the target species Mr. In this context, even when different individuals from different Mr groups (Ecuador, Peru, and Mexico) were used, the primers maintained the same detection pattern for Mr. No nontarget DNA (Mp) amplification was detected with these primers. This corroborates their specificity to Mr. Comparing the melting curve's fluorescence values between Mr77F/R (Fig. 2C) and Mr78F/R (Fig. 2D) showed that the amplification curves with primers Mr77F/R had the highest fluorescence values.

LOD and sensitivity of qPCR assays

The analytical sensitivity and LOD of qPCR assays using the primers Mr77F/R and Mr78F/R were tested using a two-fold serial dilution with Mr genomic DNA ranging from 30 to 0.2 ng as a template (Fig. 3A to H). Both Mr77F/R (Fig. 3A and B) and Mr78F/R (Fig. 3E and F) exhibited the same Ct curves for qPCR, melting curves with single amplicon regardless of DNA concentrations, and LOD down to 0.2 ng of DNA. Both Mr77F/R (Fig. 3B) and Mr78F/R (Fig. 3F) exhibited efficiency values within accepted limits (100 ± 10%) and R^2 values of 0.98 and 0.97 after eliminating the lowest concentrated points. In both cases, the qPCR assays exhibited LODs down to 0.9 ng (Fig. 3B to F).

The detection sensitivity of qPCR was explored and compared with that of conventional PCR. For conventional PCR, the amplification products were successful, with LODs down to 15 ng (Mr77F/R) and 3.7 ng (Mr78F/R) (Fig. 4).

Detection of Mr in diseased pods

The primer sets Mr77F/R and Mr78F/R were used in a qPCR assay to detect Mr in DNA extracted from naturally diseased pods from Ecuador and Peru (Fig. 5), which were possibly related to FPR. All pods mainly presented brownish lesions (Fig. 5A) and, in some cases, signs of the pathogen. Amplification analysis successfully demonstrated a single peak for the fungus Mr in all DNA samples extracted directly from the cacao pods

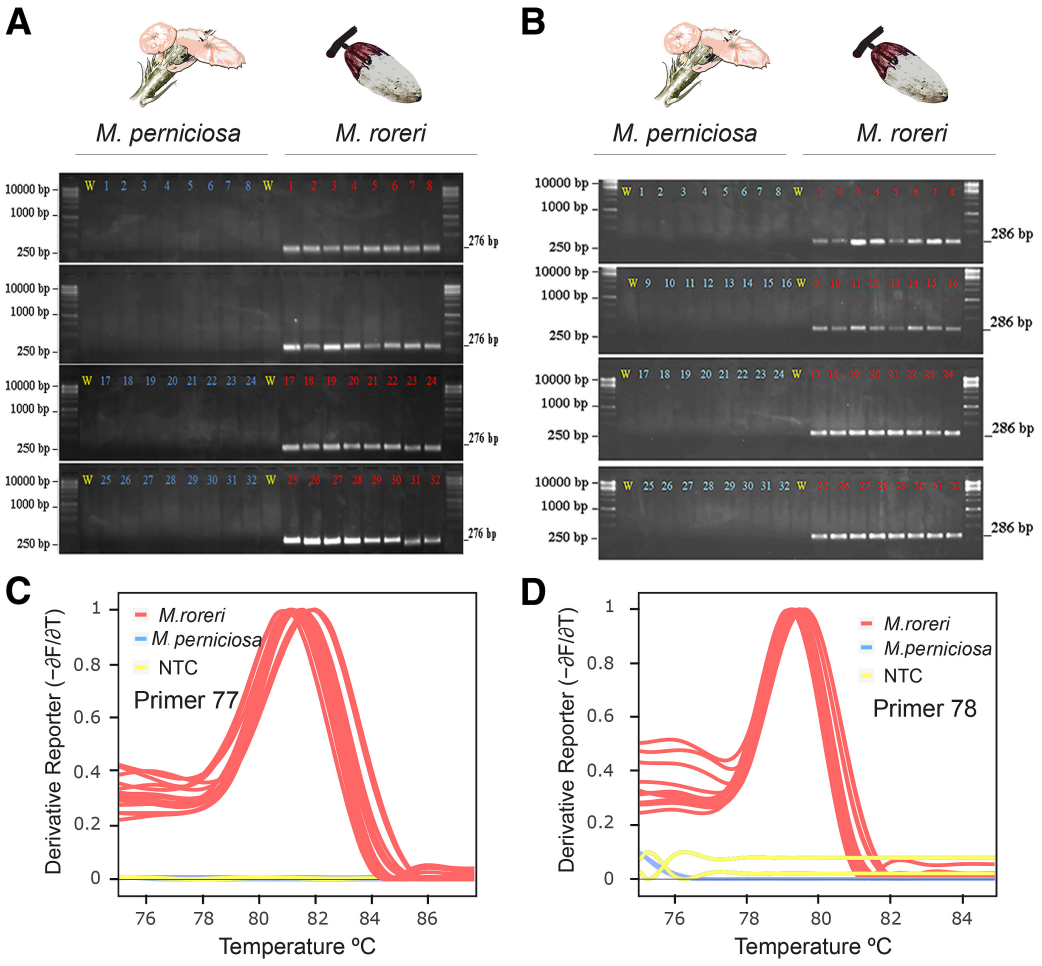


FIGURE 2
Specificity of the primers **A and C,** Mr77F/R and **B and D,** Mr78F/R. In all the images, the blue color represents the results of PCRs conducted with DNA of the nontarget species *Moniliophthora perniciosa*, while the red color corresponds to the results of PCRs using *M. roreri* DNA. A and B, Results of conventional PCR electrophoresis; C and D, negative first derivative of fluorescence during melting, normalized (0-1), obtained after real-time PCR. NTC, nontemplate control.

(Fig. 5) with and without signs of the pathogen. The more evident the disease symptoms on pods, the greater the amount of pathogen DNA detected (lower Ct values) (Fig. 5B). The typical characteristic profile of the Tm was between 80 to 81°C when amplification was performed with primers Mr77F/R and Mr78F/R, corresponding to the Tm from the Mr-positive control obtained from pure-culture Mr DNA (Fig. 5C). All samples from uninfected pods and/or pods infected with Mp were negative.

Discussion

The ongoing geographic spread of FPR into previously unaffected cacao regions is a major concern for growers. Therefore, developing a reliable conventional and real-time PCR method for diagnosing Mr would aid in early detection and assist in devising strategies to prevent pathogen dispersion within cacao-growing regions.

There is significant challenge in developing diagnostic primers for Mr due to its close phylogenetic relationship with Mp (Aime and Phillips-Mora 2005). This challenge is particularly pronounced when there is a scarcity of genomic data available for comparison (Ling et al. 2016). Genome comparison-based diagnosis offers a powerful and efficient approach for identifying specific regions in an organism's genome without relying on single nucleotide polymorphisms (SNPs) (Feau et al. 2018) in conserved regions. SNPs are variations at the level of individual nucleotides in the DNA sequence, and they can be used for genotyping and identifying genetic variation. By avoiding SNPs of conserved regions, genome comparison can focus on specific regions in the target organism's genome using a tiny fraction of the genome, which can be especially useful when SNPs are not informative or when dealing with highly diverse organisms. Another benefit is the possibility of assessing specificity directly in other available genomes (van Dam et al. 2018). However, this approach might encounter limitations due to insufficient genetic variation, which compromises its ability to effectively distinguish between closely related species (Badotti et al. 2017; Zhang et al. 2005).

On the other hand, unique sequences obtained from comparative genomics can be located anywhere in the genome, such as being part of transposable elements or retrotransposons. That might become a problem for the diagnosis if these regions accumulate variations based on less selective pressure

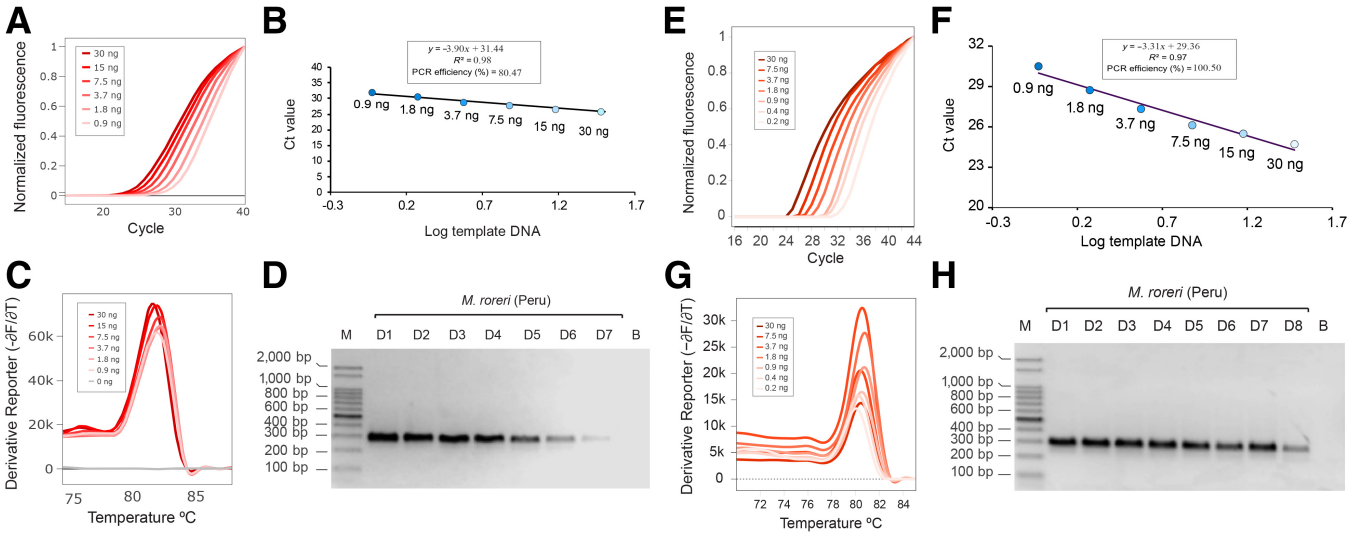
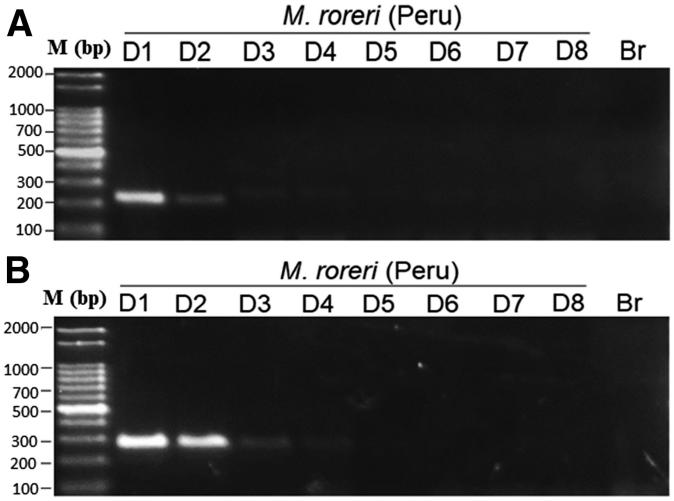


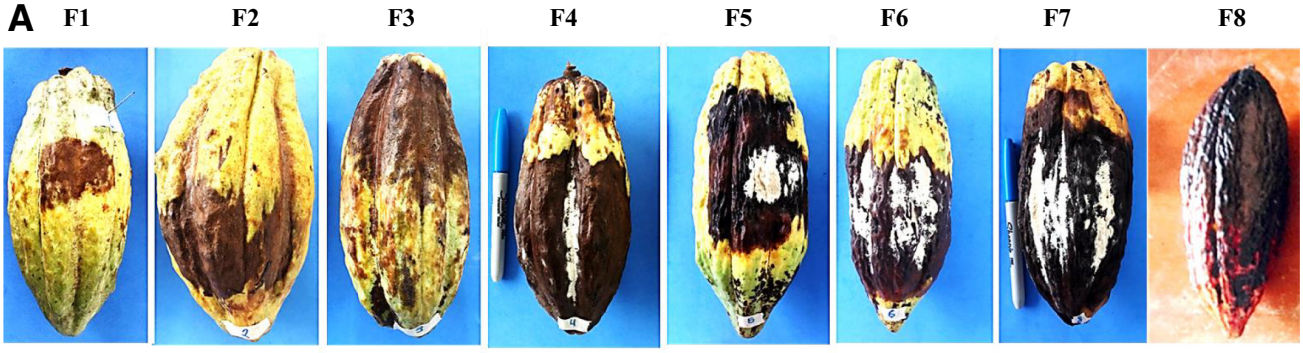
FIGURE 3

Sensitivity evaluation of primers **A, B, C, and D,** Mr77F/R and **E, F, G, and H,** Mr78F/R. A and E, Cycle threshold (Ct) curves for qPCR using different DNA concentrations; C and G, respective linear regression analyses. B and F, Melting curves that indicate a single amplicon, regardless of the DNA concentration used. D and H, Amplification of a single-band pattern and the detection level even at the lowest DNA quantities (D7 and D8).

FIGURE 4

Sensitivity evaluation of **A,** Mr77F/R and **B,** Mr78F/R primers by conventional PCR for *Moniliophthora roreri*. D1 to D8 demonstrate the amplification of a single-band pattern and the detection level using two-fold dilution. D1 = 30 ng; D2 = 15 ng, D3 = 7.5 ng, D4 = 3.7 ng, D5 = 1.8 ng; D6 = 0.9 ng, D7 = 0.4 ng, and D8 = 0.2 ng.





B

	Primers[a]	Mr77F/R		Mr78F/R	
Id	Country[b]	Ct	Tm	Ct	Tm
F1	Ec	28.80±0.23	80.20±0.08	31.88±0.38	79.85±0.08
F2	Ec	26.03±0.03	80.08±0.22	34.05±0.66	79.97±0.17
F3	Ec	26.03±0.03	80.06±0.20	31.00±2.40	79.85±0.10
F4	Ec	23.90±0.04	81.00±0.10	30.60±0.10	79.91±0.10
F5	Ec	27.18±0.52	80.50±0.14	34.60±0.48	79.97±0.08
F6	Ec	30.00±0.67	80.38±0.08	28.00±007	79.99±0.10
F7	Ec	26.05±0.12	80.20±0.08	31.40±0.00	79.91±0.15
F8	Pe	24.43±0.29	80.38±0.08	29.75±0.65	79.91±0.15

C

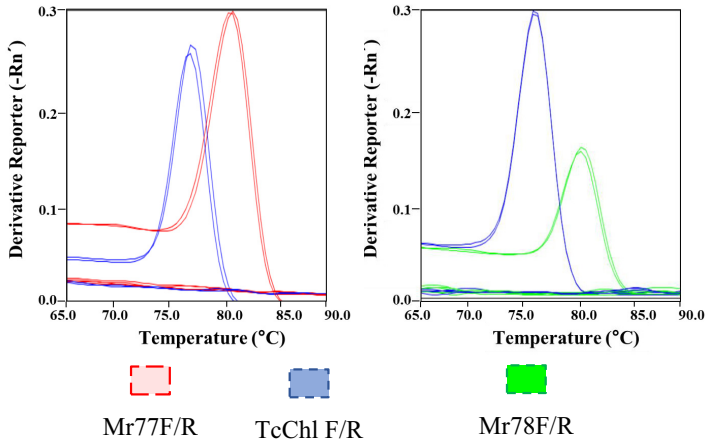


FIGURE 5

Detection by qPCR of *Moniliophthora roreri* (Mr) from diseased pods of *Theobroma cacao*. **A,** Cacao pods showing brown spots and signs of a pathogen. **B,** Amplification profile by qPCR with the primers Mr77F/R and Mr78F/R and the internal control primer set TcChl. **C,** Melting curve profiles of qPCR amplification with the primers Mr77F/R and Mr78F/R using SYBR green. Id, diseased cacao pods; Pe, Peru; Ec, Ecuador; Ct, cycle threshold ± standard error; and Tm, melting temperature.

(Vázquez-Rosas-Landa et al. 2021). Since they are unique regions, there is little or no sequence data available in public databases to compare with other sequences, making it challenging to predict robustness in in silico analysis (van Dam et al. 2018). Analogous comparative genomics provided unique random sequences for rapidly detecting *Fusarium oxysporum* f. sp. *conglutinans* (Ling et al. 2016). The same approach has been successfully applied to obtain species-specific markers to distinguish *Madurella mycetomatis* from the nontarget species *M. pseudomycetomatis* (Lim et al. 2020). Other studies used species-specific primers to distinguish phylogenetically related phytopathogens (Feau et al. 2018).

This article has generated several important findings. Recent genome sequencing efforts of Mr (NCBI's genome resources; Hamilton et al. 2011; Meinhardt et al. 2014) and Mp (Barbosa et al. 2018) supported the genomic comparison approach employed in this study. The bioinformatics analysis of the designed primer pairs, aided by comparative genomics, provided theoretical support for developing species-specific primers to diagnose Mr in cacao pods (Fig. 1). The in silico screening filtered the prior removal of primers with nonspecific amplification in other regions of the proper target organism. In other words, priority was given to primers predicted to yield a single amplicon in the target species, and that was validated using the MFEprimer tool (Qu and Zhang 2015; Wang et al. 2019). All primers were also checked for nonspecificity in nontarget species by the MFEprimer program and Primer-BLAST (Ye et al. 2012). Except for Mr83F/R, amplification with all other primers generated products with the expected size of approximately 300 bp. The specificity of the candidate primer pairs was tested in a total of 252 samples, including the target species and an array of cacao pathogens and other fungi commonly found in cacao plantations. Most primer pairs (approximately 83%) were specific to the target species. The three primers Mr74F/R, Mr75 F/R, and Mr83F/R were excluded not due to nonspecific amplification in Mp but because they were not specific to the target species and resulted in amplification in other cacao pathogens such as *Phytophthora* spp., whose genomes did not enter in our pipeline. The other primers eliminated in the screening steps for specificity by conventional PCR were not excluded due to nonspecific amplification but due to their low detection efficiencies, such as the primers Mr69F/R and Mr72F/R primers. Together, these data support the use of the pipeline in detecting unique regions in the genome of Mr compared with the genome of Mp (da Silva et al. 2022). Routine pipelines are likely to evolve as long as new genomic data from other cacao pathogens become available. This will enhance the in silico screening for distinct sequences of the target organism.

Given the genetic similarity between Mr and Mp (Meinhardt et al. 2014), it is important to use appropriate sampling that accurately represents the biodiversity and ecological variations of these fungal species in the diagnostic process, particularly from regions recognized as centers of diversity for Mr and Mp. A good example is the primer set Mr82F/R that yielded the expected amplicon in Mp isolates from Ecuador. Additionally, the effectiveness of the primers Mr77F/R and Mr78F/R was validated by testing them with several DNA samples, including those from Mp in Brazil and Ecuador and Mr from Peru, Ecuador, and Mexico. The study revealed no visible amplification for Mp. In contrast,

distinct electrophoretic bands were observed in the DNA of Mr from different provinces and in diseased pods at different disease severity stages, enhancing the study's robustness. These results indicate the possibility of diagnosing the pathogen without the need for isolation on the growth medium and enable the differentiation between the FPR and the WBD pathogen.

Sensitivity is an important index for detecting plant pathogens, especially for quarantine and inspection measures, where detecting small amounts of pathogens matters (Ling et al. 2016). In this study, qPCR assays using the primers Mr77F/R and Mr78F/R, initially designed for conventional PCR, showed efficiencies between 90 and 100.94%, which falls within the optimal qPCR efficiency range of 90 to 110% (Kralik and Ricchi 2017). An analysis of the LOD showed that the lowest amount of Mr DNA detected by a qPCR assay was 1 ng for both primers. The sensitivity of the primers can be enhanced by employing alternative methods, such as the TaqMan probe (Garces et al. 2014), which is known for improving assay sensitivity and reproducibility, particularly in the low detection range (Yip et al. 2005). Our group is currently implementing this approach, which is still in progress. Exploring these primers to loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) would also offer rapid and efficient diagnostics suitable for field conditions. These methods provide high sensitivity, specificity, and resilience against sample inhibitors, which are crucial for reliable pathogen detection. Exploring this adaptation aims to expand the practical applications of these primers, thereby improving their effectiveness in early detection of Mr in diverse environmental conditions.

The correlation between fungal DNA levels detected by specific primers in infected pods and disease development stages provides insights into the infection process and its interaction with pod development. Categorizing Ct values into ranges (below 25, >25 to 30, and >30) simplifies the assessment of disease severity. Lower Ct values indicate higher fungal DNA levels, suggesting early disease development and potentially more severe infections. Conversely, higher Ct values suggest lower fungal DNA levels, indicating later disease stages or lower infection severity. However, DNA samples from infected pods contain both fungal and plant DNA. This can influence the Ct value, as the fungal DNA is diluted in the DNA from the plant extract. Consequently, the LOD of fungal DNA might be affected, leading to potential inaccuracies in assessing early infection stages.

Even though molecular diagnostic techniques have increased extensively in recent decades, there is still a long way to go before plant pathologists and technicians develop and apply molecular diagnosis to FPR and WBD. The techniques available for routine detection of Mp and Mr are based, so far, on symptomatology and morphological identification of infected materials that require isolation and cultivation of pathogens in vitro in a culture medium. Identifying pathogens through symptoms and morphology often fails to accurately assess infection severity, leading to potential misdiagnosis. This is especially true for biotrophic fungi with long latent periods that do not fully reveal the extent of fungal presence, which highlights the need for more sensitive techniques like qPCR. Our study compared visual assessments with qPCR results. For example, pods with less visible (F4) necrosis had a Ct value of 23.9, indicating higher fungal DNA, while those with more necrosis (F3) had a Ct value of 26.03, indicating lower fungal DNA. This shows that qPCR offers a more precise way to detect pathogens and diagnose diseases, underscoring the importance of using advanced molecular tools in routine disease assessments for reliable results. Sequencing methods to confirm pathogen species are laborious and are mainly applied in phylogenetic studies (Evans et al. 2003, 2013). Molecular diagnostic techniques are fast and sensitive compared with traditional ones based on morphology (Bilodeau et al. 2007; Boutigny et al. 2013). Nonetheless, the two approaches can be used in tandem, as in regulatory diagnostic protocols, where the confirmation of the isolated properties must include molecular data to avoid misidentification (Bilodeau et al. 2007). Or even in large-scale screening, pod samples could be subjected to molecular testing, and the presence of pathogens is confirmed by morphology in positive samples.

Considering the history of WBD and FPR, delimiting the dissemination and minimizing the impacts of these diseases on cacao production areas is a challenge and a priority. This international collaborative study represents the first crucial step towards international validation of PCR procedures for detecting Mr infection and establishing standard protocols. Implementing these standardized procedures will significantly enhance our ability to detect Mr early in asymptomatic/symptomatic pods. Thus, effective preventive measures to mitigate the disease's impact can be established to safeguard cacao production in affected areas.

Conclusions

Comparative genomics between Mp and Mr enabled the identification of candidate sequences for species-specific diagnosis by conventional and real-time SYBR green PCR. This pipeline can be a model for selecting primer pairs to diagnose other pathogens. Detecting pathogens at the very early stage of the disease allows rapid response to emerging threats. The most important result of this study was that real-time SYBR green PCR is a sensitive and reliable method for detecting Mr in infected cacao pods. Timely and accurately identifying the causative pathogen is crucial for implementing effective disease management strategies, preventing further spread, and protecting cacao crops, which are economically significant for regions and industries relying on cacao production.

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