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Morphological and Molecular Characterization of *Moniliophthora roreri* Causal Agent of Frosty Pod Rot of Cocoa Tree in Tabasco, Mexico

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Abstract: Cocoa farming is affected by many fungal diseases and frosty pod rot is one of the biggest constraints of cocoa cultivation in Latin America cocoa growing countries. Three *Moniliophthora roreri* strains have been isolated from frosty pod rot diseased cocoa tree (*Theobroma cacao*) in Tabasco, Mexico. The strains were identified by morphological analysis and by sequencing the internal transcribed spacers 1 and 2. The microscopic and macroscopic morphological characteristics observed are similar to those reported in the literature for those species. The phylogenetic analysis of the *M. roreri* sequences formed three groups supported by high bootstrap values and the sequences of the isolates clustered close to the sequences of strains previously isolated from *T. cacao* in Peru and Mexico. The alignment of ITS1-5.8s-ITS2 sequences of *M. roreri* strains revealed for ITS1 and ITS2 four regions with interspecies divergence (80, 84-91, 110 and 130) and six (395, 444, 459, 539, 570 and 585), respectively. In conclusion, the identification of *M. roreri* cocoa strains by ITSs sequences confirmed the identification by morphological methods and provides information on the variability in comparison with *M. roreri* strains of South and Central America. Besides this, the identification could be important for developing strategies for its prevention and control.

Key words: *Theobroma cacao*, isolation, phytopathogenic, internal transcribed spacers, phylogenetic analysis

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is one of the most important crops, providing the food industry with chocolate, cocoa and cocoa butter (Erukainure *et al.*, 2010). Cocoa tree is grown throughout the world in humid tropics. High humidity favors the development of fungal diseases (Heydari and Pessarakli, 2010). Devastating regional problems include *Phytophthora megakarya* in West Africa (Djocgoue *et al.*, 2006), vascular-streak dieback in Papua New Guinea and the co-evolved pathogens, *Crinipellis perniciosa* (witches'broom) and *Moniliophthora roreri* (Moniliasis or frosty pod rot), in South America (Tiburcio *et al.*, 2010). Frosty pod rot is one of the biggest constraints of cocoa cultivation in Latin America cocoa growing countries (Andrade *et al.*, 2009). *M. roreri* cause damages in almonds and is considered to have its origins in western Ecuador, where it was first described (Rorer, 1926) and/or Colombia (Holliday, 1971) and from there it has spread to other cocoa producing countries. *M. roreri* is still in an invasive

phase having reached Nicargua in 1980, Peru in 1988, Honduras in 1997, Guatemala in 2002 and Belize in 2004 (Evans *et al.*, 2003; Phillips-Mora *et al.*, 2006a). In March 2005, Phillips-Mora *et al.* (2006b) identified four strains of *M. roreri* in diseased cocoa trees in the vicinity of Ignacio Zaragoza, Pichucalco in northern Chiapas, Mexico. Wherever, the pathogen has invaded, cocoa production has been severely affected, with, for example, an estimated 40-70% fall in production in Mexico (Ramirez-Gonzalez, 2008). In 2009, Mexico cocoa production was estimated in 24 600 tonnes of which the state of Tabasco contributed 67% production and the municipality of Huimanguillo contributed with 90% of cocoa produced in Tabasco (SAGARPA, 2010). For that reason the identification of phytopathogenic cocoa strains is important for developing strategies for its prevention and control. Analysis of variable nucleic characters currently comes closer than the others to recognizing fungi species (Taylor *et al.*, 2000). In *M. roreri*, this has been corroborated by studies of Kennedy and Aime (2005) who didn't find correlation between phenotype and *M. roreri*

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groups. Genetic variability of *M. roreri* strains has been studied in Colombia (Ortega and Kafurt, 2007) and Ecuador, Venezuela, Peru and Central America (Phillips-Mora, 2003). Alternatively, the increase of *M. roreri* in cocoa crops in Central America and southeast Mexico, in the last 5 years has increased interest in identifying characteristics of strains mainly by molecular methods.

The aim of this study was to isolate, characterize morphological and molecularly *M. roreri* strains isolated from diseased fruits of cocoa tree from three commercial plantations in Huimangüillo, Tabasco, Mexico.

MATERIALS AND METHODS

Collection of infected frosty pod rots: Cocoa frosty pod rots with typical symptoms were collected from three cocoa commercial plantations: El Caobonal, La Hacienda and La Noria, these cocoa plantations belongs to the municipality of Huimanguillo, Tabasco.

Fungal cultures: Fungal strains used in this study were isolated from diseased fruits of cacao trees collected during October 2007 and November 2009. To isolate fungi, fragments of diseased fruits were placed in a moist chamber and incubated at 25°C for 3 days in darkness. After incubation, individual fungi were recovered of the diseased tissue and then were subcultured on potato dextrose agar, PDA (Sigma St. Louis MO.) at room temperature for 5 days. Fungal strains were maintained on PDA at 4°C and the spores suspension at -20°C. The strains were subcultured every month on PDA medium.

Morphological identification: Morphological descriptions are based on comparisons of *M. roreri* (Phillips-Mora *et al.*, 2006a; Phillips-Mora *et al.*, 2006b; Evans *et al.*, 2003; Evans, 1981), augmented by new observations as noted. Potato dextrose agar (BD Bioxon®) was the standard growth medium for spore of *M. roreri* morphology and growth rate. Scanning electron microscope (SEM) picture (JEOL; JSM-5600LV) was taken at Institute of Ecology (INECOL, Xalapa, Veracruz. Mexico).

DNA extraction, PCR and sequencing: *M. roreri* strains were cultivated in potato broth medium (Nanda *et al.*, 2001) at room temperature and 250 rpm. Cells mycelia were grown for three days, recovered and washed twice with TE buffer (Tris HCl 10 mM, EDTA 1 mM). DNA was extracted by the sonication method described by Kurzatkowski *et al.* (1996). A region of nuclear DNA of

M. roreri strains, containing the ITS1-5.8s-ITS2 sequence, were amplified by PCR using the primers LV1 (5-cctgccagtagtcatatgcttgtct-5´) and LV2 (5´-cacctacggaaaccttgttacgact-3´) (Hernandez *et al.*, 2006). PCR amplifications were performed in a total volume of 50 µL reaction, which contained: 10 µL of 5x Colorless buffer, 200 µM dNTPSs, 0.2 µ of each primer, 2.5 units of *Taq* DNA polymerase and 10-50 ng of template DNA. The PCR reactions were placed in a thermal cycler (Bio-Rad, Hercules, CA) under the following parameters: 5 min initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min primer annealing at 57°C, 1 min extension at 72°C and a final extension period of 12 min at 72°C. The resulting products were purified with the Geneclean® II kit (Hercules, CA) according to the manufacturer's protocol. DNA was sequenced by the Biotechnology Institute, Cuernavaca, Mor., Mexico.

DNA sequence analysis: BLAST search (Zhang *et al.*, 2000) was performed to compare the consensus sequences generated with those deposited in GenBank database. The consensus sequences for each species of *M. roreri* were aligned with other through Clustalw (Larkin *et al.*, 2007) to determine variable regions or base sequences unique to each species or isolate. Distance matrices between all pairs of sequence from the multiple alignments were calculated and one tree was generated by neighbor joining method developed by Saitou and Nei (1987). Bootstrap analyses of 1000 interactions were performed by the Consense routine in the ClustalX inference package (Thompson *et al.*, 1997).

RESULTS AND DISCUSSION

Isolation of strains: Five random samples of cocoa fruits with frosty pod rot symptoms of each of the three plantations were collected. One hundred and twenty eight strains were isolated of which 13 were identified by their morphological characteristics as *M. roreri.*

Morphological identification of *Moniliophthora roreri* strains: The cacao tree (*T. cacao*) is susceptible to various fungal pathogens, many of which are poorly studied. In tropical American countries, the most important fungal pathogens which affect the cocoa tree are *M. roreri* and *Phytophthora* spp. (Ploetz, 2007). In this study three strains of *M. roreri* were isolated and identify by morphological means from commercial plantations in Huimanguillo, Tabasco, the most important cocoa producing region of Mexico. *M. roreri* strains grew on PDA medium (25°C, 7d). Colonies showed a diameter of

Fig. 1(a-d): *M. roreri* (a) Colony on PDA medium (7d, 25°C) front plate (b) Electronic microscopy of phialides. Bar L: 5 μm; (c) Optic microscopy of phialides. Bar: 5 μm; (d) Conidia in PD medium. Bar: 10 μm

70-77 mm, with an initial whitish growth, later salmon-cream color and later acquires a dark brown color due to the massive production of spores (Fig. 1a-d). This characteristics are agree with the descriptions of other authors for other strains of *M. roreri* incubated in modified V8 medium (Evans, 1 981; Phillips-Mora *et al.*, 2006a; Phillips-Mora *et al.*, 2006b) and MEA medium (Evans *et al.*, 2003). Spores, size 8-19×5-11 microns, are produced in chains, thick-walled, pale yellow, brown mass heteromorphic, most frequently globose/subglobose (69%) to ellipsoidal (31%). These observations are similar to those described for other strains of *M. roreri* isolated in Mexico (Phillips-Mora *et al.*, 2006a) and Belize (Phillips-Mora *et al.*, 2006b) as well as with those described previously by Evans (1981). Kennedy and Aime (2005) didn´t find differences in growth rate, hyphal diameter or spore size in *M. roreri* strains isolated of cocoa cultures from Central and South America, however the size of the spores globose/subglobose of the strains described in this work are bigger than those reported by Evans *et al.* (2003) for *M. roreri* strains isolated in Ecuador. The phialides are septate, 10-15×2-5 microns, with disperse mycelium in the culture medium. The front of the colony has a cream color and eventually acquires a dark brown color.

M. roreri molecular identification and phylogenetic analysis: Using the primers LV1 and LV2 the ribosomal DNA region containing the ITS1-5.8s-ITS2 sequence was amplified from DNA of 13 *M. roreri* isolates. According to their sequences three strains were considered unique. The amplified regions of the isolates HT-ITV01, HT-ITV20 and HT-ITV27 of *M. roreri* had a size of 975, 820 and 595 bp, respectively and were deposited in the NCBI GenBank (HQ231236, JN241966 and JN241967, respectively). The ribosomal DNA of *M. roreri* strains HT-ITV01 (GenBank HQ231236) and HT-ITV20 (GenBank HQ231237), showed the highest similarity of 99 and 98%, respectively with GenBank DNA sequences of *M. roreri* DQ222923, DQ222925 and DQ222927, the first two isolated from Mexico (Phillips-Mora *et al.*, 2006b) and the third from Belize (Phillips-Mora *et al.*, 2006a). Whereas, the sequence of ITS1-5.8s.ITS2 of *M. roreri* HT-ITV20 showed similarity (98%) with *M. roreri* AY194150 isolated in Ecuador by Griffith *et al.* (2003) and with *Crinipellis roreri* var. *roreri* AY230154 isolated in Peru (Evans *et al.*, 2003). *M. roreri* strain HT-ITV27 showed highest similarity (99%) with *M. roreri* strain DQ222925, isolated by Phillips-Mora *et al.* (2006b) and with the *M. roreri* strains AY916746, GU457437, GU457438, the first isolated in Costa Rica and the others isolated in El Salvador and Guatemala (Phillips-Mora *et al.*, 2006a; Phillips-Mora *et al.*, 2006b).

Although, the identification of fungi by their morphological characteristics does not provide information or discrimination of some fungi, because morphology can be strongly influenced by the environment (Weising *et al.*, 1995). In our study the sequence of the ribosomal DNA confirmed the species *M. roreri*. In addition, according to the Blast search the ITS1-ITS2 sequences of ribosomal DNA of *M. roreri* strains isolated showed a difference from 1 to 2% compared to sequences of *M. roreri* strains previously isolated in Central and South America. Our results
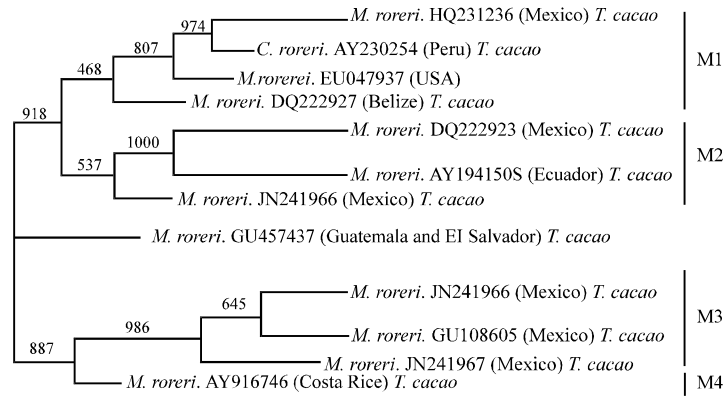
Fig. 2: Relationships of *Moniliophthora* and *Crinipellis* species on cocoa based on neighbour-joining analysis of rDNA-ITS region sequences. Numbers adjacent to branch points are bootstrap values from 1000 interactions

showed that 5' and 3' termini of both ITS1 and ITS2 sequences were highly conserved. These results are similar to those obtained in other fungi (Appiah *et al.*, 2004; Skouboe *et al.*, 1999). ITS sequences of the strains *M. roreri* ITV27 and *M. roreri* ITV20 showed higher homology with ITS sequences of *M. roreri* strains from Central America and southeast of Mexico. These results are consistent with the expansion of *M. roreri* to Mexico from South America through Central America. On the other hand, Phillips-Mora (2003) analyzed 94 isolates of *M. roreri* of tropical America to determine the origin of biogeography and molecular variation using Amplified Fragment Length Polymorphism analysis (AFLP), Inter Simple Sequence Repeat Analysis (ISSR) and Internal Transcribed Spacer (ITS). Five groups were identified. The group named Co-West group encompasses western Colombia, central Ecuador and four countries in Central America (Panama, Costa Rica, Nicaragua and Honduras). Our results are in agreement to locate our isolates *M. roreri* ITV20 and *M. roreri* ITV27 in Co-West group because the DNA ribosomal sequence have the highest homology with *M. roreri* strains isolated from Central America cocoa plantations. Although ITS1 and ITS2 regions exhibited sufficient variability for studying *M. roreri* intraspecific variation, ITS sequences of the strain *M. roreri* ITV01 showed higher homology (99%) with ITS sequences of the *M. roreri* strains isolated from cocoa cultures in Ecuador (AY194150). It is possible that ITS sequences are not enough for identification of *M. roreri* strains. In some fungi species little variation in the ITS1-ITS2 regions between closely related species has been described (Forster *et al.*, 2000). Analysis of DNA sequences of β-tubulin gen and translation elongation factor (EF-1α) could be other options for fast and accurate identification of *M. roreri* strains (Attitalla *et al.*, 2011; Kumar *et al.*, 2011; Peterson, 2008).

Phylogenetic analysis of the 12 representative isolates of *M. roreri* from *T. cacao* formed three major groups (I and II) supported by high bootstrap values (Fig. 2). Group I (91.8% bootstrap value) could be subdivided into two subgroups (M1 and M2). The subgroup M1 includes isolates HQ231236, AY230254, EU047937 and DQ222927. The subgroup M2 includes isolates DQ222923, AY194150 and DQ222925. The group II (88.7% bootstrap value) could be subdivided into two subgroups (M3 and M4). The subgroup M3 includes isolates JN241966, GU108605 and JN241967. The subgroup M4 includes AY916746.

Alignment of ITS1-5.8s-ITS2 sequences of *M. roreri* strains: DNA sequence alignments of the ITS1 and ITS2 regions were conducted to identify areas within each region displayed the greatest diversity and which might best discriminate among the *M. roreri* species examined. The 5.8S subunit sequence was highly conserved within all the *M. roreri* species (161 bp for all the sequences), only a substitution nucleotide was observed in AY230254 sequence (not shown). The ITS1 sequence alignment revealed four regions with interspecies divergence (80, 84-91, 110 and 130) (Fig. 3). These results are opposite to that obtained by Phillips-Mora (2003) who detected mutations (66, 133 and 134) for ITS1 of 95 *M. roreri* strains isolates obtained from five genetic groups of Colombia, Ecuador, Peru, Veneuela and Central America. There was significant variation in terms of deletions, insertions and substitutions between strains within the ITS2 region (395, 444, 459, 539, 570 and 585).

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                        Start                              Start  Start                                      End
                        ITS1                               5.8S   ITS2                                       ITS2
                        1    80   84    91 110  130 217 377 395   444 459   539 570 585666
                        ↓    ↓    ↓     ↓ ↓    ↓   ↓   ↓   ↓     ↓   ↓     ↓   ↓   ↓ ↓   ↓
M.roreri.HQ231236.Mexico    TTG~CTATGATGGGGGCA~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
M.roreri.DQ222923.Mexico    TTG~CTGTGAACTTCTTA~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
M.roreri.DQ222927.Belize    TTG~CTGTGAACTTCTTA~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
M.roreri.DQ222925.Mexico    TTG~CTGTGAACTTCTTA~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
M.roreri.JN241966.Mexico    -TG~CTGTGAACCTTGTC~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
M.roreri.AY194150.Ecuador   TTG~CTGTGAACCTTTTG~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
M.roreri.GU457437.Guatemala TTG~CTGTGAACCTTTTG~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
M.roreri.AY230254.Peru      TTG~CTGTGAACCTTTTG~GGA~CAGT~ATA~AAT~AAAG~T-GAA~GCT~TTG~ACG~G-C~TCA
M.roreri.EU047937.USA       TTG~CTGTGAACCTTTTG~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
M.roreri.AY916746.C.Rica    TTG~CTGTGAACCTTTTG~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
M.roreri.JN241967.Mexico    TTG~CTGTGACCTTTTTG~G-A~CACT~ATA~AAT~AA-G~TTGAG~GTT~TAG~ATG~GGC~TCA
M.roreri.GU108605.Mexico    TTG~CTGTGAACCTTTTG~G-A~CAGT~ATA~AAT~AA-G~TTGAA~GCT~TTG~ATG~GGC~TCA
                            **~** ***              ~* *~** *~***~**~** *~* ***~* *~* *~* *~* *~***
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Fig. 3: Nucleotide sequences of the ribosomal DNA internal transcribed spacer ITS1 and ITS2 of *M. roreri* causing frosty pod rot in *T. cacao*. *M. roreri* strains of this study are in bold. Mark * indicates 100% homology of nucleotides between *M. roreri* strains. Mark ~ indicates that the DNA sequence was cut and the nucleotide not shown have 100% homology

CONCLUSION

In this study three strains of *M. roreri* were isolated from diseased cocoa trees in Tabasco, Mexico and identified by morphological and molecular methods. The strains showed a high homology in their DNA internal transcribed spacers sequences with strains from Central America and Southeast Mexico but analysis of alignment of ITS sequences showed differences in the position of the mutations.

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