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Molecular identification of *Moniliophthora roreri* isolates from cocoa orchards in Norte de Santander, Colombia

Identificación molecular de aislamientos de *Moniliophthora roreri* en huertos de cacao de Norte de Santander, Colombia

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Abstract

The plant pathogenics fungus *Moniliophthora roreri,* causes losses to cocoa farmers and it causes the "Moniliasis", too. The disease destroys the cocoa (*Theobroma cacao* L.) pod and a decreasing crop production, the losses reach up to 60% in the department of Norte de Santander, Colombia. Little is known of the fungus biology, studies are currently identifying macroscopic, microscopic, and *in vitro* behavior with antagonists such as *Trichoderma asperellum, Trichoderma longibrachiatum, Paecilomyces sp.* and *Bacillus brevis,* respectively. Fifty-six isolates of *M. roreri* were obtained from eight municipalities and townships of the department (El Zulia, Cucuta, Tibú, Sardinata, Bucarasica, Teorama, Agua clara, El Tarra) with the aim to establish DNA extraction and PCR analysis. The method of isolation was performed from infected cocoa pods with *M. roreri,* which were washed with distilled water, and cut into 2 x 2 cm, and were disinfected with hypo-chlorite, 2.5% sodium and 60% alcohol. Then sown in Petri dishes containing PDA medium and incubated for 10 days at 28°C. Ribosomal regions "ITS" (internal transcribed spacers) for the 18S rDNA, ITS1, 5.8S, ITS2 and 25S, were amplified and sequenced. Amplicons of 600 bp (8 isolates), 740 bp (12 isolates) and 750 bp for the remaining of 36 isolates. The amplicons sizes corresponded to those expected for *M. roreri* species, and analysis of "BLAST" level confirmed this characterization.

Keywords: *Moniliophthora roreri, Theobroma cacao*, moniliasis, ITS.

Resumen

El hongo fitopatógeno *Moniliophthora roreri* produce la moniliasis, una enfermedad que destruye la mazorca de cacao (*Theobroma cacao* L.) y, por tanto, reduce la producción del cultivo y los ingresos de los agricultores. En el departamento de Norte de Santander, Colombia, las pérdidas por esta enfermedad pueden alcanzar hasta 60% de la producción. El conocimiento sobre la biología del hongo es escaso y actualmente se están realizando estudios de identificación macroscópica, microscópica, y comportamiento *in vitro* frente a antagonistas como: *Trichoderma asperellum*, *T. longibrachiatum*, *Paecilomyces* sp. y *Bacillus brevis*. En ocho municipios y corregimientos del departamento (El Zulia, Cúcuta, Tibú, Sardinata, Bucarasica, Teorama, Agua clara y El Tarra) se obtuvieron 56 aislamientos de *M. roreri* con fines de extracción de ADN y análisis de PCR. El método de aislamiento se realizó a partir de mazorcas infectadas por *M. roreri*, las cuales fueron lavadas con agua destilada, fraccionadas en trozos de 2 x 2 cm y desinfestadas con hipoclorito de sodio (2.5%), y alcohol (60%), antes de la siembra en cajas de Petri con medio PDA e incubación por 10 días a 28°C. Las regiones ribosomales, ITS, (espaciadores internos de transcritos) correspondientes al ADNr 18S, ITS1, 5.8S, ITS2 y 25S fueron amplificadas y secuenciadas. Amplicones de 600 pb (8 aislamientos), 740 pb (12 aislamientos) y de 750 pb, para los 36 aislamientos restantes. Los tamaños de los amplicones correspondieron a los esperados para la especie *M. roreri* y los análisis de BLAST, confirmaron este nivel de caracterización.

Keywords: *Moniliophthora roreri, Theobroma cacao*, moniliasis, ITS.

Introduction

Moniliasis is a cocoa disease (*Theobroma cacao* L.) produced by the fungi *Moniliophthora roreri,* which was presented in 1916 for the first time in virulent form in the republic of Ecuador (Barros, 1977), being currently a common problem for Colombia, Ecuador and Peru (Sánchez & Garcés, 2012). According to Rorer (1918), moniliasis is a pathogenic fungus native from Ecuador, found in species like *T. bicolor* and *T. baloensis* (Barros, 1977). It was also thought that *T. gireli* was the native host of *M. roreri* at north from Colombia in the Antioquia department, where it was observed infecting forest plants to the west of the Andean cordillera of Colombia, a rich area in *Herrania* and *Theobroma species*. Evans (1985), considers that the fungus is native from the Northeast of this part of South America, since is present in these species in Ecuador and Colombia. Is probable that *T. gileri* represents a coevolutionary host of *M. roreri*, which extended from the west of Ecuador to the Northwest of Colombia, in this way, Ecuador is considered the origin center of the pathogen. However, some reports suggest that Colombia could be the first origin place of the pathogen (Phillips, 2003).

In Colombia, there are no accurate date records of the disease emergence. In the literature is reported from Ecuador throughout Colombia and later found in Peru, in plantations in western Venezuela and in southern Panama (Barros, 1977). There are reports from 1969, which state that the first flood produced by Cauca River, affected the 2-year-old plantations in the dike, which caused a generalized attack of cancer or 'chancro' tree stem caused by *P. palmivora.* Later, in 1971, the moniliasis presence was observed for the first time slightly affecting the other region plantations (Barros, 1980). In addition, the first pathogen detection was carried out at the northeastern from Colombia, where the greatest genetic diversity of the causal agent was described and a disease recognition had achieved for approximately 200 years (Sánchez & Garcés, 2012).

In Colombia, this fungal disease is the most limiting factor for the cocoa production (Sánchez & Garcés, 2012), due to a cocoa pod destruction and a decreasing production in Norte de Santander department and the rest of the country, with losses up to 60% of the harvest (Suárez & Rangel, 2013). In this region, there are few studies about this pathogen damage and is necessary to identify not only at morphological but molecular level. Molecular markers exhibits the physicochemical characteristics of DNA and its inheritance, which is explained by the same laws established by genetics. As a consequence, they have been useful for the polymorphisms detection in animals, higher plants, plant pathogens and pests (viruses, fungi, insects, nematodes) (Rocha, 2003). ITS are some of the DNA markers used for the molecular identification of *M. roreri*. The internal transcript spacers are non-coding regions of the DNA sequence that separate the genes coding for the highly RNAs conserved ribosomal throughout taxa, while nucleotides among them may be species-specific. The rRna conservation genes have allowed an easy access to ITS regions with 'versatile' primers to perform the PCR. The variation in spacers is a useful tool in distinguishing among a wide variety of difficult taxa to identify. For the ITS detection, the primers are used in conserved regions of the 28S, 5.8S and 18S genes (White, Bruns, Lee & Taylor, 1990).

Currently, studies of the *M. roreri* genus are carried out, which will allow a more complete image of its evolution and improve the knowledge of its biology, to assist in the development of strategies for a better disease control (Sánchez & Garcés, 2012). Likewise, other investigations are carried out to isolate microorganisms with antagonistic potential to phytopathogen and to observe their *in vitro* antagonistic capacity for biological control in Norte de Santander, Colombia (Suárez & Cabrales, 2008).

Given these concerns, the aim of this research was to standardize the internal transcript spacers (ITS) technique, specifically for application in *M. roreri* isolates and thus, to determine their genetic variability in Norte de Santander, Colombia using molecular markers. This will become in one of the strategies for possible disease control and plant breeding programms, the selection of cacao genotypes resistant to *M. roreri* cultivars and, in the future, to improve the phytosanitary conditions of cocoa cultivation.

Materials and methods

Isolates. The isolates of *M. roreri* were obtained from *T. cacao* taken from farms in different municipalities and townships from the north of Colombia from fruits with disease symptoms as follows: in Zulia (8 isolates), Cúcuta (7), Tibú (5), Sardinata (6), Bucarasica (11), Teorama (5), Agua Clara (6) and El Tarra (8), respectively.

The isolates were extracted from infected cacao pods with *M. roreri,* which were externally washed with distilled water and cut into small cross sections. From this sections,

pieces of 2 x 2cm were taken, which were washed and disinfected with sodium hypochlorite (2.5%) for 2 minutes and alcohol at 60% for 3 minutes. Later, they were planted in petri dishes in potato dextrose agar medium culture (PDA) with an incubation time of 10 days at 28°C (Suárez, 2006).

Maintenance and preservation of the isolations. The isolates of *M. roreri* grew in PDA medium maintained during the study in fresh media. Two replicates of this collection were conserved in tubes and in Petri dishes in dark conditions at room temperature (28°C), while others were stored under refrigeration at 4°C.

Mycelium production. The genomic DNA of *M. roreri* was extracted from the mycelium obtained after petri dish peals to test-tube, where it grew between 11 and 15 days. The isolates were cultured in DPA (Dextrose Potato Agar) at 25°C.

Extraction and purification of genomic DNA.

The DNA was extracted from the mycelium of *M. roreri* (0.5- 0.75 g) that had previously grown in DPA. Each sample was macerated in a 2 ml microtube with 500 µl of extraction buffer, using a motor and pistil. Then the sample was vortexed for one minute and centrifuged at 6000 x g for ten minutes. The supernatant was transferred into a microtube, where it was incubated at 70°C for 15 minutes; followed by an extraction with a volume of phenol-chloroform (1:1) and centrifugation at 10,000 x g for 10 minutes. The aqueous phase was transferred into another microtube, where a volume of isopropanol was added. It was left cold (freezing) for 10 minutes and again centrifuged at 10,000 x g, for 10 minutes. The supernatant was removed and the precipitate was washed in 150 µl of 70% ethanol, dried at 37°C for 3 minutes and resuspended in 50µL of TE 1X pH buffer: 8 with RNAase (Suárez, 2005). This extraction protocol was applied to the 56 strains of *M. roreri*.

DNA quantification. The quantity and quality of the DNA extracted from each sample was estimated on an agarose gel. (0.9% (w/v)) stained with ethidium bromide (0.5 µg /ml) and displayed under UV light using a trans illuminator. The DNA concentrations obtained varied between 1 and 2 µg/µl.

PCR Amplification. The ribosomal ITS regions were amplified using ITS4 (5´-TCCTCCGCTTATTGATATGC-3´), ITS5 (5'GGAAGTAAAAGTCGTAACAAGG-3´) primers (White *et al.*, 1990), which are anchored in rDNAs 25 S and 18 S, respectively, so they also include 5.8 S. PCR amplification was performed in a final volume of 25 µl, consisting of a 1x taq polymerase buffer; MgCl: 1.5 mM, 0.5 µl of each primer, 0.2 mM of DNTPs (Promega®) and 10 (0.4) ng of total genomic DNA from *M. roreri* (Table1).

Table. 1. Reagent mixture used for the PCR-ITS. 2 µl of 5 ng/µl from DNA solution equivalent to 10 ng/ of sample. Taq (Promega®).

	Base concentration	Final Concentration	One sample
Buffer	10x	1x	2,5 µl
MgCl2	50 Mm	1.5 Mm	0.75 µl
ITS4 primer	10 Mµ	0.5 µM	1.25 µl
ITS5 primer	10 Mµ	0.5 µM	1.25 µl
DNTPs	10 Mm	0.2 Mm	0.50 µl
Taq polimerase	5 U/µl	0.02 U/µl	0.10 µl
Nanopure water	—	—	16.65 µl
DNA solution	5 ng/µl	0.4 ng /µl	1.0 µl
Fetal serum	—	—	1 µl
Final volume	—	—	25.0 µl

The PCR reaction was performed in a thermocycler (MJ.Research®) with the following conditions: an initial cycle at 94°C for 1 minute (denaturation); 35 cycles of 94°C for 1 minute (denaturation), 52°C for 45 seconds (hybridization) and 72°C for 1 minute (extension) and a final cycle of 72°C for 7 minutes. The amplifications were checked on a 1.5% w/v agarose gel (1xTAE buffer) stained with ethyl bromide (0.5 µg / ml) and visualized under UV light in a trans illuminator. The fragments were determined by comparison with a 1 Kb marker (Promega®). And the unique bands obtained by PCR, were subsequently sequenced.

Sequentiation. Typical bands obtained by ITS, were sequenced by Macrogen Inc. in South Korea. The chemistry of the ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kit was used using capillary electrophoresis and an ABI PRISM® 3730XL Analyzer sequencer (96 capillary type) following the protocols of the manufacturers. Both strands were sequenced by amplicon. In this research, microbiological and molecular tools that were applied to the phytopathogen were used. The ITS technique was standardized in the way that was mentioned in materials and methods. Only one modification of the technique proposed by Phillips was carried out, in terms of adding bovine fetal serum 1 µl per sample to avoid a PCR inhibition and the number of cycles in the PCR was also varied, increasing 5 cycles from 30 to 35 cycles as follows: an initial cycle at 94 °C for one minute (denaturation); 35 cycles of 94°C for one minute (denaturation), 52°C for 45 seconds (hybridization) and 72°C for one minute (extension) and

a final cycle of 72°C for 7 minutes; and thus good results were shown in the amplifications.

Results and discussion

In total 56 isolations of *M. roreri* were obtained from the 8 sampled municipalities and townships in the department of Norte de Santan der, Colombia (Table 2).

Amplicons

Amplicons of 600 bp were obtained for 8 isolates, 740 bp for 12 isolates and 750 bp for the 36 remaining isolates (Figure 1). The sizes of these isolates corresponded to those expected for the *M. roreri* species and the BLAST analyzes confirmed this level of characterization. Orozco, Osorio, Botero, Rivera and López (2011), they found similar results for *M. roreri* (amplicons of 710 bp), and Phillips, Aime and Wilkinson (2007), they found 741 bp amplicons for this fungus.

The size of the amplicons observed by ITS, did not coincide with all phenotypes of the isolates (Table 3), thus corroborating a difference at the molecular level that cannot be seen in the phenotype of each *M. roreri* isolate. The study of DNA ribosomal in numerous basidiomycetes shows that ITS had achieved a variation that makes them a marker.

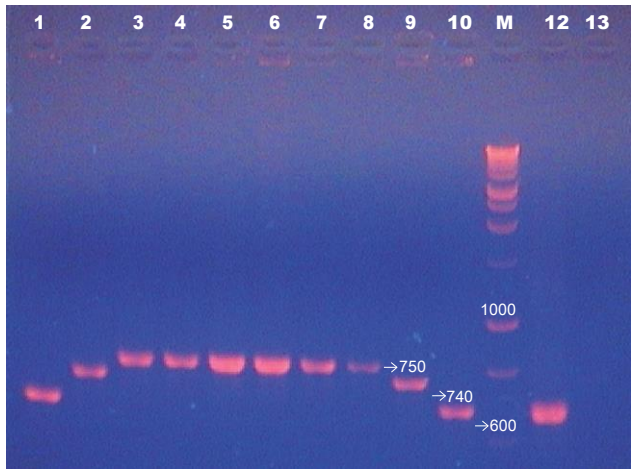

Figure 1. PCR amplification of *Moniliophthora roreri* strains in 1.5% Agarose gels using the primers (ITS4/ITS5). Line 1. M10, Line 2. M11, Line 3. M12, Line 4. M13, Line 5. M14, Line 6. M15, Line 7. M16, Line 8. M17, Line 9. M18, Line 10. M19, Line 11. M: Marker size of 1Kb. Line 12, positive control. Line 13, negative control.

Table. 2. Number of isolates of *Moniliophthora roreri* obtained from *Theobroma cacao* L. in eight municipalities and townships of Norte de Santander, Colombia.

Sample	Location	Name of the farm	Sample	Location	Name of the farm
M 1	El Zulia	Brisas del Zulia A	M 29	El Zulia	Brisas del Zulia D
M 2	Cúcuta	San Isidro A	M 30	Sardinata	Los Cocos C
M 3	Tibú	Buenos Aires A	M 31	Cúcuta	San Isidro F
M 4	El Zulia	Brisas del Zulia B	M 32	EL Tarra	La Constancia B
M 5	Cúcuta	San Isidro B	M 33	Bucarasica	San Isidro G
M 6	Sardinata	La Piñuela	M 34	Teorama	La Fortuna E
M 7	Tibú	Buenos Aires B	M 35	Sardinata	Los Cocos D
M 8	Bucarasica	Miraflores A	M 36	Tibú	Buenos Aires C
M 9	El Zulia	La Perla A	M 37	EL Tarra	El Lucero B
M 10	Bucarasica	San Isidro C	M 38	Bucarasica	El Porvenir B
M 11	Sardinata	Los Cocos A	M 39	El Zulia	Brisas del Zulia G
M 12	Teorama	La Fortuna A	M 40	Agua Clara	La Fortuna E
M 13	Bucarasica	El Porvenir A	M 41	Cúcuta	San Isidro H
M 14	El Zulia	La Perla B	M 42	EL Tarra	La Constancia C
M15	Agua Clara	Jardín Clonal A	M 43	Bucarasica	Miraflores D
M16	Agua Clara	La Fortuna B	M 44	Teorama	San Roque
M 17	Teorama	La Fortuna C	M 45	Agua Clara	Jardín Clonal C
M 18	Sardinata	Los Cocos B	M 46	Cúcuta	San Isidro I
M 19	El Zulia	La Perla C	M 47	EL Tarra	El Lucero C
M 20	Agua Clara	Jardín Clonal B	M 48	Bucarasica	Miraflores E
M 21	Cúcuta	San Isidro D	M 49	Teorama	La Fortuna G
M 22	EL Tarra	El Lucero A	M 50	Sardinata	Los Cocos E
M 23	Bucarasica	Miraflores B	M 51	Tibú	Buenos Aires D
M 24	El Zulia	Brisas del Zulia C	M 52	EL Tarra	El Lucero D
M25	Agua Clara	La Fortuna D	M 53	Bucarasica	El Porvenir C
M 26	Cúcuta	San Isidro E	M 54	Tibú	Buenos Aires E
M 27	EL Tarra	La Constancia A	M 55	EL Tarra	La Constancia D
M 28	Bucarasica	Miraflores C	M 56	Bucarasica	Miraflores F

Table. 3. Amplicons obtained using the ITS technique, applied to 56 isolations of *M. roreri.* Norte de Santander, Colombia.

Amplicon size (ITS fragment).	Sample of *M. roreri*
600 bp	M6, M8, M10, M19, M20, M23, M24, M37.
740 bp	M5, M7, M9, M11, M18, M22, M50, M51, M52, M53, M54, M55.
750 bpv	M1, M2, M3, M4, M12, M13, M14, M15, M16, M17, M21, M25, M26, M27, M28, M29, M30, M31, M32, M33, M34, M35, M36, M38, M39, M40, M41, M42, M43, M44, M45, M46, M47, M48, M49, M56.

taxonomic very useful to distinguish among species (Orozco *et al.*, 2011). The phenotypes of the samples presented three sizes of ITS amplicons that in some cases are common. One group consists of isolates that have an amplicon of 750 bp and had achieved a large phenotypic variation and are characterized by 36 isolates of *M. roreri.* Another group of 12 isolates of *M. roreri* showed amplicons of 740 bp and a last group of 8 isolates exhibited amplicons of 600 bp (Figure 1). The three amplicons observed coincided with the size of 760 bp reported by Phillips (2003).

Isolates

Only two strains of *M. roreri* shared the same morphology or phenotype, group I, and the same amplicon size of 600 bp: the M10 strain from the municipality of Bucarasica and the M19 strain from the municipality of El Zulia; both geographically close and located in the central region of the department. An isolation of *M. roreri*, M54 constitutes the phenotype of group II, with an amplicon size of 740 bp and is found in the municipality of Tibú, northeastern region of the department. Only two isolates of *M. roreri* shared the same morphology or phenotype, group I, and the same amplicon size of 600 bp: the M10 strain from the municipality of Bucarasica and the M19 strain from the municipality of El Zulia; both geographically close and located in the central region of the department. An isolation of *M. roreri*, M54 constitutes the phenotype of group II, with an amplicon size of 740 bp and is found in the municipality of Tibú, northeastern region of the department.

The isolates of the group I (M1, M4, M5, M10, M13, M15, M19, M26, M27, M30, M31, M32, M33, M34, M35, M38, M43, M46, M48, M49, M50, M51, M52, M55), showed a sparse growth of the radial mycelium, with light brown and dark colouration and interspersed with short and interspersed filaments, the obverse color of the colony was light brown and the reverse cream.

In the group II (M14, M16, M17, M20, M21, M25, M28, M41, M54) the isolates showed more abundant mycelium, more compact and with mottled salmon. The obverse color of the colonies was brown and salmon, the reverse was cream. The group III (M2, M3, M6, M7, M8, M9, M11, M12, M18, M22, M23, M24, M29, M36, M37, M39, M40, M42, M44, M45, M47, M53,M56), which was characterized by having abundant mycelium and more compact than for the two previous groups, with an obverse of dark brown and light brown reverse. All groups presented powdery, flat appearance with radial growth, without diffusible pigments in the medium, being similar to those observed by Arbeláez (2010).

ITS regions

A low level of intraspecific variation of the ITS sequence data was observed for band sizes of some samples analyzed for *M. roreri*, which may possibly be due to mutations produced by bases deletion and insertion such as reported by Phillips (2003).

The ITS region consists of the uncoded variables that are located between the rDNA repeats between the highly conserved small subunit, 5.8S, and the genes of the large subunit rRNA, ITS1, and ITS2 that have a functional role, which explains the high level of sequence variation among them. STI is now the most sequential region of the DNA genome in fungi and has been exploited to explain or infer phylogenetic affinities of related groups (Lee and Taylor, 1992). To study the genetic relationship of several species of *Phytophthora* that attack cocoa, the sequences of both ITS1 and ITS2 are also used (Phillips, 2003).

According to Bridge and Arora (1998), the ITS region is used for studies of molecular characterization in fungi due to a relatively short region (500-800 bp) and can be easily amplified by PCR using only pairs of universal primers, which are complementary to conserved regions between the genes of the rRNA subunit. The multicopy nature of the repetitive rDNA makes the ITS region easy to amplify, as was confirmed in this study by sequencing some strains of *M. roreri*, where the ranges are ranged from 762-787-827 bp.

With the PCR technique it was proved that the three ITS bands are in a relatively short range of 600-780 bp. This region can also

be highly variable among morphologically distant species and the generated RFLP-ITS restriction data can be used to estimate genetic distance and provide characteristics for systematic and phylogenetic analysis. Likewise, the PCR-ITS generates species-specific tests which can be reproduced quickly, without the need to produce a chromosomal library, in addition, sequences of the ITS can be selected for species-specific tests, due to sequences that occur in multiple copies and they are similar to each other and are variable among fungal species. The ITS analyzes do not generate enough information in fungi (Bruns, White and Taylor, 1991). The primers designed by White et al. (1990), have allowed the determination of several ITS fungi sequences that serve for taxonomic research and phylogenetic relationship between species of different genus such as *Colletotrichum, Phytophthora* and *Penicillium* (Edel, 1998). In addition, they have allowed the development of rapid and simple procedures for the detection of phytopathogenic fungal species (Bridge and Arora, 1998; Edel, 1998).

ITS sequence analyzes are useful for phylogenetic reconstructions in a wide variety of fungi, algae and plants (Berbee and Taylor, 1999). *Colletotrichum* and *Alternaria* have shown sequence variation at the interspecific level but low levels of intra-specific variation in genus of well-defined species (Cooke et al., 1997; Gardes *et al.*, 1991). On the other hand, variation in ITS sequences between Laccaria species and between *L. bicolor* of 3-5% and 1-2%, respectively, has been estimated. In addition, this variation in intraspecific information has been reported for some fungal species such as *Fusarium oxysporum* (Bridge and Arora, 1998) and *Plerotus tuberregium* (Isikhuemhen, Monalvo, Nerud, and Vilgalys, 2000). It has also been shown by ITS that *M. roreri* is related to *C. perniciosa* (Aime, 2005; Phillips, 2006).

According to Grisales and Afanador (2007) in Antioquia (Colombia) it has been observed that *M. roreri* has a great genetic similarity with low levels of diversity and differentiation, indicating that the introduction of this pathogen in Antioquia is very recent. These studies were performed with the molecular markers AP-PCR and RAPD. Information that has been relevant to continue studies with molecular markers from the samples of *M. roreri* isolated in Norte de Santander and will clarify many doubts regarding the origin of the phytopathogen in this department and in Colombia. According to Arbeláez (2010), who made molecular characterization throughout the activity of isoenzymes, Colombia is one of the countries with the greatest diversity and possible origin of *M. roreri.*

Conclusions

In this research, *Moniliophthora roreri* extracted from *Theobroma cacao* L. in the department of Norte de Santander, Colombia was molecularly isolated and identified using the ITS molecular marker. The development of the ITS technique have allowed to demonstrate its applicability in *M. roreri*, being also a reliable tool for the study of genetic variability between different samples and the relationships among them.

The ITS were useful to define three subspecific groups. A relatively low intraspecific variation was obtained from the ITS analyzed for the 56 isolates of *M. roreri*. The presence of three amplicon sizes of 600 bp, 740 bp, and 750 bp was observed, distributed as follows: 600 bp for eight isolates, 740 bp for twelve isolates and 750 bp for the 36 remaining isolates.

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