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Employing molecular markers to identify *Monilinia fructicola* in Ecuadorian peach orchards

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Abstract ITS and SCAR molecular markers were used to identify *Monilinia* species in 21 fungal isolates from infected peaches in four Ecuadorian provinces. The results confirmed *Monilinia fructicola* as the causal agent of brown rot in the analysed samples. This is the first report that uses molecular methods to identify this pathogen in Ecuadorian peach crops.

Keywords *Monilinia fructicola* · *Prunus persica* · Brown rot · ITS regions · SCAR markers

Monilinia sp. is the causal agent of brown rot disease, which affects peach crops causing in-field losses of over 75 % and up to 100 % in post-harvest stages (Agrios 2005). The first typical lesions of the fungal infection appear as brown spots on the surface of inflorescences and/or fruits. These spots turn greyish as conidia spread, covering the surface of the fruit and surrounding tissues, such as blossoms and branches (Fulton et al. 1999). Brown rot can lead to a condition in which the peach fruit becomes mummified (Fig. 1), acting as a reservoir of spores that are dispersed by environmental factors such as wind and rain and by insects (Gell et al. 2007). High humidity (60-80 %) and temperature (20-25 °C) are environmental conditions that increase the severity of the infection, favoring spore germination (Agrios 2005).

Five species of *Monilinia* that can infect peach plants have been reported worldwide: *M. laxa, M. fructigena*, *M. fructicola* (Gell et al. 2007), *M. mumecola* and *M. yunnanensis* (Hu et al. 2011). The latter two species have only been reported in Asia (Hu et al. 2011), while the remaining three are commonly found in American peach cultivars (Snyder and Jones 1999; Malvárez et al. 2004). Van Leeuwen and Van Kersten (1998) reported that *M. fructigena* causes infection especially on fruits of the Rosaceae family, while *Monilinia laxa* is considered more a pathogen of blossoms and twigs than of fruits. *M. fructicola* affects twigs, blossoms and fruits of stone fruits more than other members of the Rosaceae family. To control brown rot and blossom blight on peach trees, the use of fungicides has been implemented. Due to the prolonged use of these fungicides, there are reports that suggest the appearance of dicarboximide resistance in *M. fructicola* (Lim and Cha 2003).

Pathogen identification at species level is an important tool for epidemiology studies, and for the implementation of preventive practices and control measures. Peach crops in Ecuador are commonly affected by *Monilinia* infections, causing yield reductions of over 50 % (P. Viteri pers. comm. October 19, 2012). Therefore, there is a need for a more detailed characterisation of the fungal species involved in these events. The Ministry of Agriculture and Animal Husbandry of Ecuador published in 1986 an Inventory of Pests, Diseases and Weeds of the country, listing *M. fructicola* as the causal agent of brown rot in peach orchards. However, there is no information about how this phytopathogen species was identified (MAG 1986). Phenotypic identification is inefficient since fungal morphological traits (growth rate, growth pattern in culture media, mycelial coloration and conidial dimensions) tend to overlap between species and some isolates present atypical morphologies (EPPO 2009; Snyder and Jones 1999; Van Leeuwen and Van Kesteren 1998). Molecular methods, on the other hand, are better suited for the identification of the pathogen (Côté et al. 2004) since they rely on the ability to distinguish differences between species at DNA level. Among the DNA-based methods, ITS (Internal Transcribed Spacer of rRNA genes) (Ioos and Frey 2000) and SCARS (Sequence Characterized Amplified

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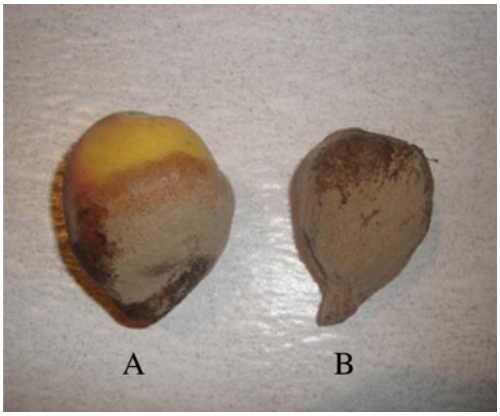

Fig. 1 Development of symptoms and mummification of the fruit affected by brown rot disease. **a** blighted peach fruit. **b** mummified peach fruit

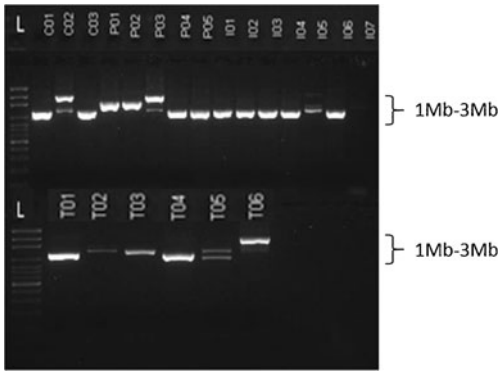

Fig. 2 PCR amplification products using NS5 and ITS4 primers as a fungal DNA positive control. Upper panel: first lane, 1,000 bp Ladder (Axygen, Inc.), followed by 15 samples (C: Carchi; P: Pichincha; I: Imbabura). Lower panel (larger well sizes): first lane, 1,000 bp Ladder (Axygen, Inc.), followed by 6 samples (T: Tungurahua). The expected band sizes for this reaction range from 1.3 Mb to 3 Mb (Perotto et al. 2000).

Regions) (Côté et al. 2004) have been developed for the identification of *Monilinia* species.

To date, there are no reports identifying *Monilinia* species in Ecuadorian peach orchards using molecular techniques. Thus the aim of this report was to implement two DNA – based approaches to adequately identify which *Monilinia* species are present in Ecuador.

Thirty four affected fruits, fresh and mummified, were collected from 34 locations in four different provinces of Ecuador (Carchi 4, Pichincha 7, Imbabura 9, and Tungurahua 14). To isolate the fungus from fresh fruit, infected rectangular pericarp segments were surface sterilised by submerging in sodium hypochlorite 2.5 % for 30 seconds and rinsed with sterile water in a laminar flow hood (Thermo Fisher Scientific, Inc.). After being dried by sterile paper towels, the pericarp segments were transferred to V8-MA culture media (Cañedo and Ames 2004) and incubated in the dark at 25 °C for four days. Colonies presenting typical *Monilinia* mycelial colour were transferred to fresh V8-MA media (EPPO 2009). Mummified fruit were incubated at 25 °C for four to six days in a humid chamber to induce sporulation. Spores were transferred to V8-MA media and incubated for four days at 25 °C in darkness.

Mycelia and conidia from each isolate were examined under an optical microscope using methylthioninium chloride staining to confirm *Monilinia* identity. Isolates that presented typical *Monilinia* morphological traits (conidial shape and septum distribution in mycelia) (EPPO 2009) were transferred to fresh V8-MA media for sample conservation (Van Leeuwen and Van Kesteren 1998).

To confirm Koch's postulates, peach fruits without apparent lesions were surface sterilised, as described above, inoculated with agar plugs containing mycelia of the previously obtained fungal isolates, and maintained for seven days in a humid chamber. A negative control consisting of a peach fruit with an agar plug without fungus mycelia was included. Fungus was re-isolated from peaches that showed apparent *Monilinia* infection following the protocol described above. Twenty one out of 34 fungal isolates subjected to Koch's postulates confirmation developed apparent typical brown rot symptoms in inoculated peaches after seven days, which progressed from superficial soft brown spots to concentric rings of grey conidial pustules. The negative control (peach with agar plug without fungus mycelia) showed no lesions of the infection. The remaining 13 samples were discarded because no typical brown rot symptoms were observed.

For DNA extraction, mycelia were grown in 100 mL of Difco™ PDB (Potato Dextrose Broth) (Becton, Dickinson and Company) supplemented with gentamicin (40 mg/L), and incubated in a MaxQ 4,000 temperature controlled shaker (Thermo Fisher Scientific, Inc.) (0.9 xg) at 27 °C for 24 hours. DNA was extracted from 0.3 g of mycelia of each isolate following the CTAB protocol described by Shagai-Maroof et al. (1984) using Sand White Quartz (Sigma-Aldrich Co.) in a 3:1 proportion.

Universal fungal primers (NS4 primer forward and ITS4 primer reverse) (White et al. 1990) were used to amplify DNA from the isolates to confirm its fungal origin. Reactions were carried out in a Tpersonal thermocycler (Biometra) following the protocol described by White et al. (1990) with an annealing temperature of 54 °C.

For the identification of *Monilinia* species, three independent PCR amplification reactions were performed using three different primer sets that target the ITS region of the most prevalent *Monilinia* species: *M. fructicola*, *M. fructigena* and *M. laxa*. The reactions were carried out following the protocol by Ioos and Frey (2000) using 40 ng of DNA of each isolate as a template, and changing the reported annealing temperature to 57 °C. PCR products were resolved by electrophoresis in a 1.5 % agarose gel. Additionally, multiplex PCR reactions, with 40 ng of DNA of each isolate, were performed using species

specific SCAR primers (MO368- 12 for *M. laxa*, MO368-8R for *M. fructigena* and MO368-0R for *M. fructicola*) and a reverse common primer, MO368-5 (Côté et al. 2004). The annealing temperature was 63 °C. Amplification products were resolved by electrophoresis in a 1.5 % agarose gel.

The fungal origin of the 21 isolates tested was confirmed by PCR using the universal fungal primers (NS4 forward and ITS4 reverse) since all of them showed bands in the range of 1.3-3.0 Mb (Perotto et al. 2000) (Fig. 2). PCR reactions performed with *M. fructicola* specific ITS primers generated the expected size product (350 bp) in 11 out of the 21 samples, while no amplification products were observed for *M. fructigena* and *M. laxa* primer sets. Positive bands were sequenced[1] (Functional Bioscience, Wisconsin U.S.A.) and homology searches using BLAST (NCBI) were performed. High sequence identities (98 – 100 %) were obtained when compared with other reported *M. fructicola* sequences (Myoung et al. 2007; Zhu et al. 2011), confirming the species identity of the fragments.

When multiplex PCR using SCAR primers specific for the three *Monilinia* species was performed, the same 11 samples that yielded positive results with the ITS primers showed an amplification product of about 500 bp in this experiment, which corresponds to the expected size of *M. fructicola* (Côté et al. 2004). Two *M. fructicola* isolates from this report were deposited in the Fungal Taxonomy, Biochemistry and Bioprospection Laboratory at Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) under accession numbers: IOC-4631 and IOC-4629.

The molecular approaches used in this study for the identification of *Monilinia* species proved to be more reliable than the phenotypic method, as twenty one isolates were identified as *Monilinia* using different taxonomic keys (EPPO 2009; Van Leeuwen and Van Kesteren 1998), but only eleven were confirmed by PCR. For the ten remaining isolates, although they displayed apparent typical *Monilinia* conidial shape and mycelium septum, failed to show PCR products for the ITS and SCAR tests. This may indicate researcher misinterpretation of morphological traits (Vienne 2003). Interestingly, one of the isolates from the Imbabura province (I03), which showed a *Monilinia fructicola* specific morphology in V8-MA medium, shifted its appearance when transferred to a PDA culture medium, where it displayed a typical *M. laxa* morphology (EPPO 2009). These observations are consistent with the ones reported by Malvárez et al. (2004), suggesting that growth patterns can be altered during sub cultivation and when cultured in different media (EPPO 2009; Malvárez et al. 2004).

This research identified *Monilinia fructicola* as the causal agent of brown rot in the peach crops sampled from the Ecuadorian highlands, using two different types of molecular markers (ITS and SCARs) and confirmed by sequencing the ITS amplified fragments. Despite the fact that the present study identified only one species of *Monilinia* in Ecuadorian peach crops, the presence of *M. laxa* and *M. fructigena* cannot be ruled out because of the small sample size ($n = 34$). Other studies with limited sample sizes state that the presence of species that occur in less than 1 % of the population could pass undetected (Malvárez et al. 2004). A wider study could confirm or contradict this result.

The molecular identification of the prevalent *Monilinia* species may enable the execution of epidemiology studies and the adoption of efficient prevention and control methods of this phytopathogen, to avoid its expansion to other susceptible peach cultivars and other *Prunus* species in Ecuador.

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[1] Sequences submitted to GenBank under accession numbers JX878906 to JX878916.

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