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SHORT COMMUNICATION

Detection of *Monilinia* spp. by a multiplex real-time PCR assay and first report of *Monilinia fructicola* in South Tyrol (northern Italy)

Urban Spitaler[1] · Anna Pfeifer[1] · Evi Deltedesco[1] · Sabine Hauptkorn[1] · Sabine Oettl[1]

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Abstract

Brown rot decay of stone and pome fruit caused by *Monilinia* spp. is an economically important disease. The main pathogens in Italy are the indigenous species *Monilinia laxa* and *M. fructigena,* and the invasive species *M. fructicola*. The presence of *M. fructicola* in Italy was reported for the first time in 2008 in Cuneo, Piedmont. Further records showed that *M. fructicola* spread and established rapidly in other regions of Italy. In South Tyrol, Europe's largest contiguous apple-growing area, the cultivation of stone fruit is an increasingly important agricultural branch for small-holder farmers of the mountainous areas. Up to now, it was unknown if *M. fructicola* is present and how *M. laxa* and *M. fructigena* are distributed. Therefore, plant protection strategies were applied without knowledge of the species present in the orchards. To detect the three *Monilinia* spp. on stone fruit trees and to determine their distribution, samples of fruit mummies were taken from scattered stone fruit trees and from sweet cherry orchards. A multiplex real-time PCR was applied to detect *Monilinia* spp. in the fruit mummies. The results showed the presence of *M. fructicola* for the first time in South Tyrol on three locations and that *M. laxa* was the predominant species, followed by *M. fructigena*.

Keywords Ascomycota · Molecular analysis · Occurrence of species · *Prunus avium*

Introduction

The fungi *Monilinia laxa* (Aderhold and Ruhland) Honey as well as the two closely related species, *M. fructigena* (Pers.) Honey, and *M. fructicola* (G. Winter) Honey, are the most important fungi responsible for blossom, twig and branch blight, and brown rot in genus *Malus*, *Pyrus*, and *Prunus* (Batra 1991; Petróczy et al. 2012). The genera *Malus* and *Pyrus* are members of pome fruit, while the genus *Prunus* belongs to stone fruit. *Monilinia laxa* is economically important on stone fruits, causing mainly blossom and twig blight, but it has also been detected on pome fruits. *Monilinia fructigena* causes primarily fruit rot, before and after storage and marketing on pome fruit but was also detected on stone fruit. The invasive species *M. fructicola* is mainly a blossom, twig, and fruit pathogen of stone fruits (Di Francesco and Mari 2018; EFSA 2011; Hrustić et al. 2012). All species overwinter in mummified fruits or in canker lesions in wood segments. Under favorable weather conditions, the disease develops rapidly, and several generations of spores can be formed during one vegetation period. Overall, rain falls in the period of blooming are ideal conditions for pathogen spreading and cause blossom, twig, and branch infections, making these pathogens one of the most important diseases on stone fruit. Furthermore, fruit rot caused by *Monilinia* spp., before or during storage, is an important factor for crop losses on stone fruit as well as on pome fruits, especially in the post-harvest (Hrustić et al. 2012).

While *M. laxa* and *M. fructigena* are considered native to Europe, *M. fructicola* was identified for the first time in Europe in 2001 in France (EPPO 2020) and has spread across Europe (EFSA 2011). In Italy, the occurrence of *M. fructicola* was reported first in Cuneo province in 2008 (Pellegrino et al. 2009); subsequently, the pathogen was detected in Emilia-Romagna region in 2010 (Martini 2012; Montuschi et al. 2016), in Veneto and Sardinia region as well as in the Rome province in 2011 (Martinelli et al. 2013; Martini et al. 2016), in the Latium region in 2012 (Martinelli et al. 2013), in the Marche region in 2013 (Landi et al. 2016), in the Pugli region, Basilicata region and Caserta province in 2014 (Abate et al. 2018), and in Campania region in 2016

✉ Sabine Oettl
sabine.oettl@laimburg.it

[1] Institute for Plant Health, Laimburg Research Centre, Laimburg 6, 39040 Auer, South Tyrol, Italy

and 2017 (Regione Campania 2018). Since records are available only for certain regions or provinces but not on an area-wide basis, for many fruit-growing areas of Italy it is not clear if *M. fructicola* is present.

South Tyrol is known as Europe's largest apple-growing area, with over 18,500 ha of apple orchards. In recent years, the area of stone fruit orchards increased notably and reached 108 ha of sweet cherries (*Prunus avium* L.), 81 ha of apricots (*Prunus armeniaca* L.) and 6 ha of plums (*Prunus domestica* L.) in 2019 (Autonome Provinz Bozen 2019). Other stone fruits such as almonds (*Prunus dulcis* (MILL) D.A. Webb) and peaches (*Prunus persica* (L.) Batsch) can be found as scattered trees in domestic gardens, on the edges of apple orchards, vineyards, grasslands, and rural roads. In addition, also forests contain a notable number of wild stone fruit trees, especially cherries. Up to now, due to lack of information about the distribution of the three *Monilinia* spp. in South Tyrol and since the species cannot be distinguished in the field (Michailides et al. 2007), treatments against brown rot were performed without knowledge about the species present in the orchards. *Monilinia* spp. differ in growth rate and virulence (Villarino et al. 2016), host plant preference (Di Francesco and Mari 2018; Vasić et al. 2018), responses to fungicides, and occurrence of resistances (Abate et al. 2018; Chen et al. 2013; Miessner and Stammler 2010). Additional knowledge about the distribution of the various species could improve plant protection strategies.

The present study investigated the presence of *M. fructicola* in South Tyrol, an important fruit-growing region in Europe. Furthermore, the occurrence of *M. laxa* and *M. fructigena* in fruit mummies from stone fruit was observed. Finally, we present a sequence modification within the *M. fructigena* qPCR probe originally described by Guinet et al. 2016.

Materials and methods

Sampling

Fruit mummies present on stone fruit trees were collected during January until March 2020 in South Tyrol, Italy. In total, 154 samples were collected from 20 sweet cherry commercial orchards cultivated according to the guidelines for integrated fruit production and from 57 scattered stone fruit trees located in gardens or on rural roads: 11 almonds, 5 apricots, 10 sweet cherries, 13 peach, and 18 plums. The geographic coordinates are listed in Supplementary 1. Four to five samples were taken per cherry orchard from evenly distributed trees and one sample for the single scattered stone fruit trees. Each sample consisted of five mummies and all samples were stored at − 30 °C until DNA extraction. The reference samples for *M. laxa* and *M. fructigena* were isolates grown in Petri dishes on potato dextrose agar (4 g/L potato starch (from infusion), 20 g/L dextrose, 15 g/L agar; Difco™ Becton–Dickinson, France). For *M. fructicola*, the reference sample was genomic DNA. Reference samples were provided by the Center for Agricultural Technology Augustenberg, the Austrian Agency for Health and Food Safety, and the University of Natural Resources and Life Sciences, Vienna.

DNA extraction

Genomic DNA was extracted with the DNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with the following modifications: In total, 25 mg of fruit flesh with visible *Monilinia* symptoms was cut off with a sterile scalpel from five fruit mummies that belong to one sample. The plant material was disrupted by adding 400 µL of AP1 lysis buffer and three 3 mm Tungsten carbide beads (Qiagen) in a mixer mill (MM 400; Retsch GmbH, Haan, Germany) for 3 min at 30 beats per s. No RNase was added. The DNA was eluted two times with 50 µL nuclease-free water. The DNA concentration of the samples was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Germany) and stored at − 20 °C until use.

PCR and sequence analysis

The MO368 sequenced characterized amplified region (SCAR) markers first described by Côté et al. (2004) for the respective *Monilinia* spp. as well as the 18S rDNA region as universal internal control (based on the DNA of the *M. fructigena* reference sample) were amplified (Table 1). Reaction mixtures (30 µL) contained 17.25 µL nuclease-free water, 6.0 µL 10×DreamTaq™ buffer, 0.6 µL dNTPs (10 mM each), 1.5 µL of each primer (10 µM each), 0.15 µL DreamTaq™ DNA Polymerase (5 U/µL) (all PCR reagents were from Thermo Fisher Scientific Inc., Waltham, USA) and 3.0 µL of DNA template each, obtained from the reference samples. The following cycling conditions were used: initial denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s; and a final 5 min extension step at 72 °C. The amplicons were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and subcloned into pJET1.2 plasmids (ThermoFisher Scientific Inc.) according to the manufacturer's instructions. Sequencing was performed by LGC Genomics GmbH (Berlin, Germany); for manual quality control and analysis of sequencing data, the Geneious v.11.1.4 software (Biomatters Ltd. Auckland, New Zealand) was applied.

Table 1 Original primers and probes for *Monilinia* spp. reported by Guinet et al. (2016) and for 18S rDNA reported by Ioos et al. (2009)

Target	Name	Sequence (5′–3′)
M. laxa	Mlx368-F	CCAAGGGCTCCGTAGGTAA
	Mlx368-R	TCCACACCGTCGAACAATAA
	Mlx368-P	**ROX**-CAGATCGTGAAGGGCGTGAGGT-**BHQ2**
M. fructigena	Mfgn368-F	AGCACAGCGAGTACAATAAGC
	Mfgn368-R	TACCCAGACACCACCTCCTC
	Mfgn368-P	**Cy5**-TGCTCCGTAGGCAATCGGTAAAGA-**BHQ2**
	Mfgn368-P_VZL (modified)	**Cy5**-TGCTCCGTAGGCAATCGGTAATAG-**BHQ2**
M. fructicola	Mfcl368-F	ACTAAACGACGCGGTAATGG
	Mfcl368-R	CTTTTAACTTCTTAGCCGCTCCA
	Mfcl368-P	**FAM**-CACGAATGTCGTGAAAGGATAATGGA-**BHQ1**
18 S	18S Uni-F	GCAAGGCTGAAACTTAAAGGAA
	18S Uni-R	CCACCACCCATAGAATCAAGA
	18S Uni-P	**JOE**-ACGGAAGGGCACCACCAGGAGT-**BHQ1**

The *M. fructigena* probe was modified for the present study. Text in bold corresponds to the dye and quencher of the probes, the underlined text indicates the probe modification

Multiplex real-time PCR

Multiplex real-time PCR for the simultaneous identification of *M. laxa*, *M. fructigena*, and *M. fructicola* was performed using the primers according to Guinet et al. (2016) (Table 1). For *M. fructigena* identification, the probe based on the SCAR region was modified as follows: Cy5-TGCTCC GTAGGCAATCGGTAATAG-BHQ2 and denominated as Mfgn368-P_VZL (Table 1). For the internal control based on the 18S r DNA, the primers described by Ioos et al. (2009) were applied (Table 1). The PCR reactions were carried out in a total volume of 20 µL, consisting of 9.4 µL nuclease-free water, 0.4 µL of each primer of the four pairs (10 µM each), 0.1 µL each of the four probes (10 µM each), 4 µL 5 × PerfeCTa® Multiplex qPCR ToughMix® reaction buffer (Quantabio, Beverly, USA), and 3 µL of template DNA (2 ng). Amplification was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc, Hercules, USA) with an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s denaturation and 60 °C for 60 s primer-annealing. PCR efficacy (95–110%) for each primer and probe combination and a coefficient of determination (R^2; > 0.998) was checked by a four-point serial dilution (3.0×10^6–3.0×10^3 copies per sample) of the pJET1.2 plasmids containing the subcloned MO368 SCAR amplicons for the respective *Monilinia* reference samples as well as the 18S rDNA fragment as universal internal control. All samples and controls were run in duplicates and each run included a non-template control (NTC, nuclease-free water), and a positive control for *M. laxa*, *M. fructigena*, and *M. fructicola*. Positive controls consisted of diluted pJET1.2 plasmids from the serial dilution. Threshold calculation and data analysis were performed by using CFX Manager 3.1 (Bio-Rad Laboratories Inc.). Only real-time PCR runs respecting the following criteria were considered for data analysis: (1) amplification of 18S rDNA fragment as endogenous control; (2) production of an exponential amplification curve with the respective primer/probe combination and mean quantification cycle (C_q) value of the positive control below 30; (3) C_q values for NTC for the *M. laxa, M. fructigena* and *M. fructicola* primer/probe combinations above 32. The C_q values reported by Guinet et al. (2016) for *Monilinia* spp. positive samples originating from naturally infected stone fruit and mummified stone fruit ranged from 16 to 25. Therefore, samples that yielded C_q values < 30 in both runs were considered positive in the present study.

M. fructicola-specific PCR

To confirm the presence of *M. fructicola*, two samples from the fruit mummies, which were tested positive for *M. fructicola* in the multiplex real-time PCR, were analyzed by a *M. fructicola*-specific PCR as described by Côté et al. (2004). The amplicons based on the SCAR marker region were characterized by sequencing (LGC Genomics GmbH, Germany) and a Mega Basic Local Alignment Search Tool (MegaBLAST) analysis against the NCBI GenBank database was performed.

Results

Modification of *M. fructigena* probe

During the implementation of the multiplex real-time PCR, sequencing of each reference isolates' target region was

performed and revealed a 3 bp mismatch between the *M. fructigena* probe (Mfgn368-P) (Guinet et al. 2016) and the *M. fructigena* MO368 SCAR marker region. Alignment of the *M. fructigena* MO368 SCAR sequence obtained in this study (deposited in NCBI GenBank under accession number OK635612) with the sequences deposited by Guinet et al. (2016) in the NCBI GenBank database under the accession numbers KU343261-KU343270 showed 100% identity. Thus, confirmed the mismatch at the 3'-end of the probe. Subsequently, a modified probe named Mfgn368-P_VZL (Table 1) was designed and used for the multiplex real-time PCR assays. A comparison of the Mfgn368-P probe published by Guinet et al. (2016) and the newly designed Mfgn368-P_VZL resulted in lower C_q values (data not shown).

Distribution of *Monilinia* spp.

All three species, *M. laxa, M. fructigena and M. fructicola* were found in cherry orchards as well as on scattered stone fruit trees spread throughout the South Tyrol province (Fig. 1a).

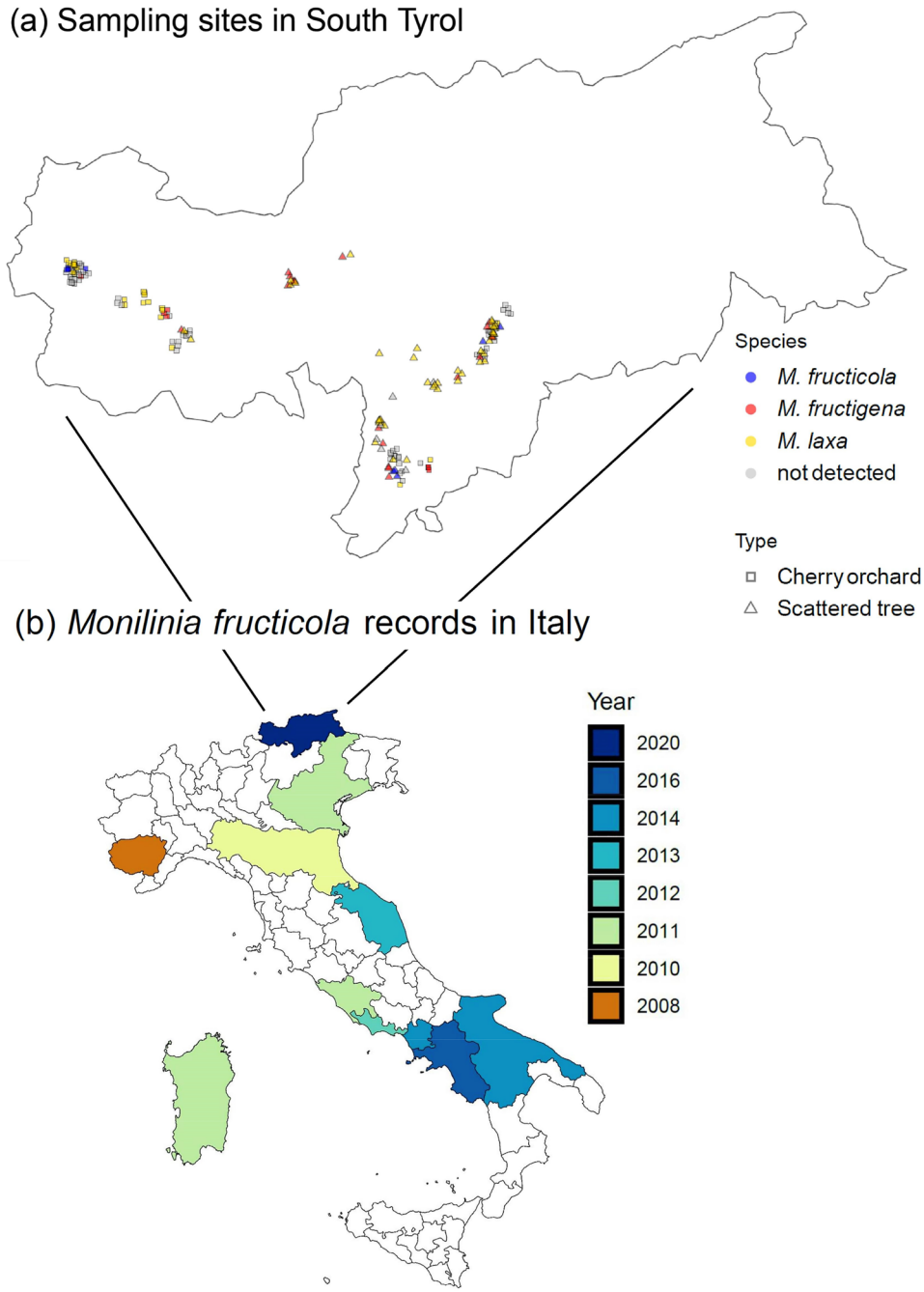

Fig. 1 **a** Sampling sites during the *Monilinia* spp. monitoring from January until March 2020 in South Tyrol. Colored symbols correspond to the detection of *M. fructicola*, *M. fructigena,* or *M. laxa*; grey-colored symbols represent samples without *Monilinia* spp. (not detected). Samples originated from cherry orchards or scattered stone fruit trees. **b** Detection map of *M. fructicola* in Italy based on published records on province or regional level are represented by different colors on the maps according to the year of the first finding (dark blue = new record in 2020)

The presence of *Monilinia* spp. in the fruit mummies varied between cherry orchards and scattered trees. While on scattered trees 89.47% of the samples contained DNA of at least one of the three *Monilinia* spp., in cherry orchards only 32.99% of the samples showed the presence of *Monilinia* spp. (Table 2). The predominant species in cherry orchards was *M. laxa*, which was found in 27.83% overall samples, followed by *M. fructigena* (7.21%) and *M. fructicola* (4.12%). On scattered trees, most samples contained *M. laxa* (71.93%), followed by *M. fructigena* (29.83%) and *M. fructicola* (8.76%). Whereby, 21.69% of the samples contained more than one *Monilinia* spp.

First report of *M. fructicola* in South Tyrol

The invasive fungus *M. fructicola* was found in Italy by previous studies and for the first time on three noncontiguous locations in South Tyrol (Fig. 1b). In total 9 samples belonging to almond, cherry, and peach were tested positive for *M. fructicola* (Supplementary 1).

The first location where *M. fructicola* was detected was in southern South Tyrol where the fungus was detected on three almond trees (46°21′35.0"N 11°17′05.4"E). Interestingly, all three samples also contained *M. fructigena*.

The second location was in central South Tyrol, in a sample from a scattered cherry tree (46°34′14.2"N 11°30′16.8"E) and in a sample from a peach tree (46°35′23.7"N 11°31′29.4"E).). The scattered cherry and peach tree were located at 2.60 km from each other.

The third location was in the west where *M. fructicola* was found in combination with *M. laxa* in three samples originating from two bordering cherry orchards (46°41′04.5"N 10°32′04.2"E) and one sample from a third cherry orchard (46°40′52.8"N 10°33′54.2"E), which was located at a distance of 2.30 km to the other orchards.

The *M. fructicola*-specific PCR for sample number 1 and 2 (Supplementary 1) confirmed the presence of *M. fructicola*. Sequencing of both amplicons revealed 471 bp sequences (deposited in the NCBI GenBank with the accession numbers OM802532 and OM802533) and subsequent MegaBLAST analysis showed 100% similarity to the SCAR marker genomic sequences of *M. fructicola* voucher 10–202 and 10–235 deposited in NCBI GenBank (KU343279 and KU343280).

Discussion

The South Tyrol province is characterized by the economically important cultivation of apples and a recent increase in the cultivation of stone fruits (Autonome Provinz Bozen 2019). Production of sweet cherries and apricots may present an interesting niche for small farmers in the mountainous areas. Thus, considering the particularly difficult production conditions, targeted plant protection measurements are crucial for economically and environmentally sustainable cultivation. One of the most important diseases of stone fruits is *Monilinia* spp., whose control requires several fungicide treatments during the year (EPPO 2004). The predominant species in Italy are *M. laxa*, *M. fructigena,* and *M. fructicola* (Montuschi et al. 2016). *Monilinia laxa* is the dominant species on stone fruit and branches, while *M. fructigena* infests overall pome fruit (Batra 1991; Gril et al. 2008). The invasive fungus *M. fructicola* is present overall on stone fruit, but can also be found on pome fruit and can be detected overall on fruits, while the infection of branches is described as less common (Batra 1991). Furthermore, *Monilinia polystroma* (G. Leeuwen) L. M. Kohn (Johnston et al. 2014), an anamorph species closely related to *M. fructigena* (EPPO 2020; Vasić et al. 2016), was also found in Emilia Romagna region on peaches (Martini et al. 2014) and pears (Martini

Table 2 Occurrence of *Monilinia* spp. in fruit mummies sampled in South Tyrol in 2020

	N samples	*Monilinia* spp.	*M. laxa*	*M. fructigena*	*M. fructicola*	*M. laxa* + *M. fructigena*	*M. laxa* + *M. fructicola*	*M. fructigena* + *M. fructicola*	*M. laxa* + *M. fructigena* + *M. fructicola*
		[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Cherry orchards	97	32.99	21.65	4.12	1.03	3.09	3.09	n.d	n.d
Scattered trees	57	89.47	56.14	10.53	1.75	14.04	1.75	5.26	n.d
Almond	11	63.64	36.36	n.d	n.d	n.d	n.d	27.27	n.d
Apricot	5	100.00	80.00	n.d	n.d	20.00	n.d	n.d	n.d
Cherry	10	90.00	60.00	n.d	10.00	20.00	n.d	n.d	n.d
Peach	13	92.31	38.46	23.08	n.d	23.08	7.69	n.d	n.d
Plum	18	100.00	72.22	16.67	n.d	11.11	n.d	n.d	n.d

Number of collected samples and percentage of samples with at least one of the three species detected and itemized according to the single species as well as the mixed infections. n.d. = not detected

et al. 2015) and in Basilicata region on plums (Abate et al. 2018). *Monilinia polystroma* was not included in the present study, since this species is primarily present in pome fruit (Poniatowska et al. 2016; Vasić et al. 2018).

The aim of the present study was to take advantage of the multiplex format of a real-time PCR described by Guinet et al. (2016) for a fast detection of *M. fructicola* in an important fruit-growing area and simultaneous search for *M. fructigena* and *M. laxa*. The results of the present study showed that on scattered trees, *Monilinia* spp. were found in 89% of the fruit mummy samples, while in integrated managed cherry orchards *Monilinia* spp. were detected only in 33% of the samples. Overall samples, *M. laxa* was the predominant *Monilinia* spp. These observations are consistent with previous studies, which showed that in stone fruits *M. laxa* is the most common *Monilinia* spp. in fruit mummies, followed by *M. fructigena* (Larena et al. 2005). The invasive species *M. fructicola* was found in 9 of 154 samples. Since the records of *M. fructicola* are few, it is likely that *M. fructicola* was recently introduced into South Tyrol. Other studies conducted in Spain showed that after the introduction of *M. fructicola* to a new area, a change in the species spectra occurred very fast and overall *M. fructigena* was displaced by the invasive species *M. fructicola*, while *M. laxa* and *M. fructicola* coexisted with a similar frequency (Villarino et al. 2013). The reduction in the relative frequency of occurrence of *M. laxa* and *M. fructigena* recorded in Spain could be explained by faster growth rates and the higher virulence of *M. fructicola* on stone fruits (Villarino et al. 2016). After the first record of *M. fructicola* for Italy in 2008, for the Emilia-Romagna region, it was shown in 2010 that this species appears almost with the same frequency as *M. laxa* (Montuschi et al. 2016). In contrast to that, in the regions Basilicata, Campania, and Puglia, *M. fructicola* was already the predominant species as it was found in 81% of the sampled orchards and represented 75% of the sampled isolates on stone fruit (Abate et al. 2018). In the alpine region South Tyrol, in contrast to the above-mentioned regions, stone fruit cultivation is mainly located in cool mountain areas. This could promote brown rot caused by *M. laxa* and inhibit *M. fructicola*. Angeli et al. (2017) showed that the optimum temperature and the maximum temperature for brown rot development are higher with *M. fructicola* as the causal agent compared to the disease with *M. laxa* as causal pathogen. Nevertheless, the rise of mean and maximum temperatures in South Tyrol observed in recent years could favor the spread of *M. fructicola* (Schlögel et al. 2020). Since *M. fructicola* grows faster, sporulates more abundantly (Abate et al. 2018), and has a higher aggressiveness (Villarino et al. 2016) than *M. laxa* and *M. fructigena*, a further distribution of *M. fructicola* could require additional plant protection strategies on stone fruit and could lead to increased brown rot post-harvest losses.

Up to now, plant protection measurements against brown rot in South Tyrol were applied without explicitly targeting *M. fructicola* and therefore, this species was unknowingly controlled by treatments against *M. laxa* and *M. fructigena*. *Monilinia* spp. differ in sensitivity and known resistances against fungicides (Abate et al. 2018; Haramoto et al. 2006) and cannot be reliable distinguished in the field (Michailides et al. 2007). Therefore, only the identification of the species in the laboratory followed by fungicide screening can provide information on which fungicides should be applied without selecting resistant strains or species. The main host of *M. fructicola* is stone fruit and, to a lesser extent, pome fruits such as apples and pears (EPPO 2020). The fact that *M. fructicola* can infect apples (Martini et al. 2013; Vasić et al. 2018) makes the 18,300 ha of apple orchards in South Tyrol (Autonome Provinz Bozen 2019) an important spreading source for the disease. Further studies should focus on the presence of the fungus in the apple orchards.

The European Commission lifted the status of *M. fructicola* as a quarantine pest in 2014 but the European and Mediterranean Plant Protection Organization (EPPO) maintained its status on the EPPO A2 List (version 2020–09) of pests recommended for regulation as quarantine pests due to the low number of reports. The detection of *M. fructicola* in South Tyrol contributes to the knowledge of the distribution in Italy. Further studies on a regional basis in Europe are urgently needed for a realistic estimation of the spread of the pathogen and for the performance of targeted plant protection strategies based on the biology, known resistances, and host plant preference of *M. fructicola*.

The revealed 3 bp mismatch between the *M. fructigena* probe (Mfgn368-P) (Guinet et al. 2016) and the *M. fructigena* MO368 SCAR marker region improved the detection of *M. fructigena*.

Generally, mismatches, both in primers and probes, are worrying in terms of quantification accuracy of real-time PCR runs (Süß et al. 2009). Probes whose sequence binds exactly to the target DNA provide more precise fluorescence signals than those with single-nucleotide polymorphism in the sequences (Fish et al. 2007). Since mismatches can lead to a possible attenuation of the signal, the *M. fructigena* exact-binding Mfgn368-P_VZL probe published in the present work should be considered for further experiments.

In conclusion, the results determined *M. laxa* as the predominant species in mummified stone fruits, whereas *M. fructigena* is less abundant. This is the first report of the invasive fungi *M. fructiola* in South Tyrol. The results of the study show that *M. fructicola* is scattered in South Tyrol, which indicates a recent introduction.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s41348-022-00614-7.

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Code availability Not applicable.

Declarations

Conflicts of interest The authors declare that they have no conflict of interest.

Consent to participate Not applicable.

Consent for publication Not applicable.

Ethics approval Not applicable.

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