Mating System in the Brown Rot Pathogens *Monilinia fructicola*, *M. laxa*, and *M. fructigena*

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ABSTRACT

Monilinia fructicola, *M. laxa*, and *M. fructigena* are the most important pathogens responsible for brown rot disease of stone and pome fruits. Information on their mating system and sexual behavior is scant. A mating-type-specific PCR-based assay was developed and applied to 155 *Monilinia* isolates from 10 countries and 10 different host plants. We showed that single isolates carry only one of two opposite idiomorphs at the *MAT1* locus consistent with a heterothallic mating system for all three species. *MAT1-1* and *MAT1-2* mating types were detected in similar proportions in samples of isolates of each species and hence there do not appear to be genetic obstacles to the occurrence of sexual reproduction in their populations. Inter simple sequence repeat markers suggested that asexual reproduction is prevalent, but that sexual recombination occurs in *M. fructicola* populations in Italy. The genetic architectures of the *MAT1* loci of the three pathogens were analyzed.

MAT1-1 and *MAT1-2* idiomorphs are flanked upstream and downstream by the *APN2* and *SLA2* genes and resemble those of *Botrytis cinerea* and other heterothallic fungi in the family *Sclerotiniaceae*. Each idiomorph contains a specific couple of genes, *MAT1-1-1* (with alpha-box domain) and *MAT1-1-5* in *MAT1-1*, and *MAT1-2-1* (with HMG-box domain) and *MAT1-2-10* in *MAT1-2*. Small gene fragments (*dMAT1-1-1* and *dMAT1-2-1*) from the opposite idiomorph were detected close to their flanking regions. Constitutive expression of the four *MAT1* genes during vegetative growth was ascertained by transcriptomic analysis (RNA-Seq). Antisense transcription of the *MAT1-1-1* and *MAT1-2-1* genes and intergenic transcribed regions of the *MAT1* locus were detected. These results represent new insights into the mating systems of these three economically-important pathogens which could contribute to improve the knowledge on their population biology.

Brown rot is an important disease affecting stone- and pome-fruits in the field as well as in postharvest (Byrde and Willetts 1977). The disease is caused by one or more of six closely related species of *Monilinia*: *M. fructicola* (G. Winter) Honey (Mordue 1979a), *M. fructigena* Honey ex Whetzel (Mordue 1979b), *M. laxa* (Aderhold & Ruhland) Honey (Mordue 1979c), *M. polystroma* G.C.M. Leeuwen (van Leeuwen et al. 2002), *M. mumeicola* Y. Harada, Y. Sasaki & T. Sano (Harada et al. 2004), and *M. yunnanensis* M.J. Hu & C.X. Luo (Hu et al. 2011).

M. fructicola, *M. laxa*, and *M. fructigena* are the major causal agents of brown rot. *M. laxa* is the most common brown rot pathogen and occurs in many places worldwide (<https://www.cabi.org/isc/datasheet/34749>). *M. fructigena* is mostly restricted to European countries and parts of Asia, but is absent from South America, Australia, and New Zealand (<https://www.cabi.org/isc/datasheet/34747>). Both species are indigenous to Europe and are associated with European brown rot, being the only species present in Europe before the new millennium. *M. fructicola* is responsible for the American brown rot and originally occurred in North and South America, Japan, Australia, New Caledonia, and New Zealand

but now it occurs worldwide (<https://www.cabi.org/isc/datasheet/34746>). In Europe, it is recommended for regulation as a quarantine pest by the European and Mediterranean Plant Protection Organization and is included in the A2 List (<https://gd.eppo.int/taxon/MONIFC/categorization>). *M. fructicola* was first introduced to Europe in 2001 in France (OEPP/EPPO 2002), and subsequently, the pathogen was reported in restricted areas or with sporadic occurrence in numerous other countries (Riccioni and Valente 2015; <https://gd.eppo.int/taxon/MONIFC/distribution>). In Italy, *M. fructicola* was first detected in Piedmont (North Italy) (Pellegrino et al. 2009), and successively, it was reported in Emilia-Romagna and Lazio (Central Italy) (Martini et al. 2013) and in South Italy (Abate et al. 2018).

In contrast to other fungi belonging to family *Sclerotiniaceae*, such as *Botrytis cinerea* Pers. and *Sclerotinia sclerotiorum* (Lib.) de Bary, knowledge on the sexual behavior and mating system in *Monilinia* species is scant. Apothecia, rarely reported for *M. fructigena* and *M. laxa*, are commonly differentiated by *M. fructicola* and analysis of variation in populations of the fungus through classical and molecular markers suggests that sexual recombination occurs in Europe and the United States (De Cal et al. 2014; Free et al. 1996; Holtz et al. 1998; Jänsch et al. 2012; Michailides et al. 2007; Ogawa and English 1991; Villarino et al. 2012; Willetts and Harada 1984). Apothecia of *M. fructicola* and *M. fructigena* could be obtained under laboratory conditions, allowing genetic analysis of meiotic progenies (Batra 1991; Free et al. 1996).

The *MAT1* locus controls the mating system in Ascomycetes (Debuchy and Turgeon 2006). In heterothallic fungi, cross-compatible mating types carry opposite idiomorphs, i.e., two alternative highly divergent sequences containing unrelated *MAT1* genes at the same chromosomal location. In homothallic fungi, single isolates carry both idiomorphs (Martin et al. 2010). The family *Sclerotiniaceae* include both homothallic and heterothallic species. For instance, *B. cinerea*

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displays a bipolar heterothallism and the *MAT1-1* or the *MAT1-2* idiomorphs are singly carried by self-sterile individuals; alternatively, *S. sclerotiorum* displays homothallism and the corresponding *MAT1-1* and *MAT1-2* idiomorphs are closely linked end-to-end at a single *MAT1* locus in self-fertile individuals (Amselem et al. 2011; De Miccolis Angelini et al. 2016; Faretra et al. 1988).

This work was aimed at (i) developing a molecular assay for mating-type assessment in *Monilinia* isolates; (ii) evaluating the presence and distribution of mating-type-specific genes in *M. laxa*, *M. fruticola*, and *M. fructigena* isolates from different countries and host plants; (iii) investigating the occurrence of sexual recombination in *M. fruticola* populations in Italy; and (iv) characterizing the genetic organization of the *MAT1* loci in the three *Monilinia* species.

MATERIALS AND METHODS

Fungal isolates and DNA extraction. Ninety isolates of *Monilinia* spp. from Italy and 65 isolates from different origins were used in this study (Table 1). The isolates, derived from single germinating conidia, were routinely grown on potato dextrose agar (PDA, ingredients per liter of distilled water: infusion from 200 g of peeled and sliced potatoes kept at 60°C for 1 h, 20 g of dextrose, adjusted at pH 6.5, 20 g of agar Oxoid No. 3) at 24 ± 1°C in the darkness. For long-term storage, suspensions of conidia and

mycelium of each isolate were maintained at –80°C in 10% glycerol and revitalized on PDA just before use.

Genomic DNA was extracted and purified from mycelium grown on cellophane disks overlaid on PDA per 4 days at 24 ± 1°C, according to De Miccolis Angelini et al. (2010). In brief, mycelium collected by scraping the cellophane was powdered under liquid nitrogen and added with 600 µl of CTAB buffer (100 mM Tris-Cl, pH 8.0; 1.4 M NaCl; 20 mM EDTA, pH 8.0; 2% cetyltrimethylammonium bromide [wt/vol]; and 0.2% β-mercaptoethanol [vol/vol]). Samples were then frozen and thawed three times using liquid nitrogen and a water bath at 75°C, and then incubated at 75°C for 1 h. After chloroform extraction, the clear supernatant was transferred to a new tube and precipitated with isopropanol at –80°C per 30 min. After centrifugation (14,000 rpm for 15 min at 4°C), the nucleic acid pellet was washed with cold 70% ethanol, air-dried, and dissolved in TE (10 mM Tris-Cl; 1 mM EDTA, pH 8.0). Samples were then treated with 0.1 µg µl^{–1} of DNase-free pancreatic RNase (Sigma-Aldrich, Milan, Italy) for 2 h at 37°C, precipitated by the addition of 0.6 vol of 5 M ammonium acetate and 2 vol of cold absolute ethanol. The final DNA pellets, washed with 70% ethanol and air-dried, were dissolved in water and stored at –80°C until use.

Molecular assay for mating type. Primer pairs were designed on the conserved regions of the *MAT1* locus in two closely related species, the homothallic *S. sclerotiorum* and the heterothallic

TABLE 1. Origin of *Monilinia* isolates used in this study

Species	Isolate code ^a	Location	Host plant	Year
<i>M. fruticola</i>	Mfrc69, Mfrc77, Mfrc78 , Mfrc83 , Mfrc88, Mfrc93	Italy (Caserta, Campania)	Cherry	2014
	Mfrc106, Mfrc111, Mfrc113, Mfrc114, Mfrc118, Mfrc123 , Mfrc129, Mfrc140, Mfrc144, Mfrc145	Italy (Bisceglie, Apulia)	Cherry	2014
	Mfrc146, Mfrc147, Mfrc148, Mfrc149, Mfrc150, Mfrc151, Mfrc152, Mfrc153, Mfrc154, Mfrc155, Mfrc156, Mfrc157, Mfrc158, Mfrc159 , Mfrc160, Mfrc161, Mfrc163, Mfrc164, Mfrc165, Mfrc168, Mfrc177, Mfrc180, Mfrc186, Mfrc190, Mfrc197, Mfrc210, Mfrc214, Mfrc222, Mfrc266, Mfrc273, Mfrc275	Italy (Tursi, Basilicata)	Plum	2014
	Mfrc228, Mfrc230, Mfrc233		Donut Peach	2014
	Mfrc247, Mfrc261, Mfrc262, Mfrc265	Italy (Policoro, Basilicata)	Donut Peach	2014
	Mfrc301, Mfrc303, Mfrc305, Mfrc312, Mfrc320, Mfrc321, Mfrc322	Italy (Gioia del Colle, Apulia)	Cherry	2014
	Mfrc426	France (Provence-Alpes-Côtes d'Azur)	Apricots	2010
	Mfrc427		Cherry	2008
	Mfrc428, Mfrc429, Mfrc430		Peach	2010
	Mfrc362, Mfrc363	Greece (Imathia, Central Macedonia)	Peach	Unknown
	Mfrc364, Mfrc365	Greece (Pella, Central Macedonia)	Peach	Unknown
	Mfrc407	Serbia (Udovice, Smederevo)	Nectarine	2012
	Mfrc408	Serbia (Osečina, Kolubara)	Plum	2013
	Mfrc412	Serbia (Požarevac, Braničevo)	Cherry	2015
	Mfrc414	Serbia (Golobok, Smederevska Palanka)	Peach	2016
	Mfrc415	Serbia (Šabac, Mačva)	Sour cherry	2016
	Mfrc391, Mfrc392, Mfrc393	Spain (Lleida, Alcarràs)	Unknown	2013
	Mfrc394	Spain (Lleida, Albesa)	Unknown	2009
	Mfrc395		Unknown	2010
	Mfrc396, Mfrc400	Spain (Lleida, Alfarràs)	Unknown	2009
	Mfrc397	Spain	Unknown	2011
	Mfrc398		Unknown	2014
	Mfrc399		Unknown	2015

(Continued on next page)

^a The isolate Mfrc534 was supplied by F. Nigro (DiSSPA – University of Bari, Italy); the isolates from Mfrc347 to Mfrc358 were supplied by G. Schnabel (Clemson University, USA); Mfrc401 to Mfrc405 by G. C. M. Van Leeuwen (National Reference Centre – NPPO, The Netherlands); Mfrc344 to Mfrc345 by M. Mari (University of Bologna, Italy); Mfrc426 to Mfrc430 by C. Guinet (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail – ANSES); Mfrc362 to Mfrc365 by G. Karaoglani (Aristotle University of Thessaloniki, Greece); Mfrc407, Mfrc408, Mfrc412, Mfrc414, Mfrc415 by J. Hrustic (Institute za Pesticide, Serbia); Mfrc391 to Mfrc400 by A. De Cal (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain); Mfrc326 to Mfrc333 and Mfrc335 to Mfrc339 by T. Michailides (University of California, Berkeley, USA); Mfrc416, Mfrc418 to Mfrc420 and Mfrc424 by B. Weir (Landcare Research Manaaki Whenua, New Zealand); and Mfrc435 to Mfrc438 by L.-Y. Guo (China Agricultural University, China). The isolates Mfrc509 and Mfrc510 correspond to CBS 101503 and CBS 101501, respectively. The *Monilinia* isolates used in setting up the PCR assay for mating type are underlined and those used for the molecular characterization of the *MAT1* loci are in bold.

B. cinerea, with the aim to amplify portions of the mating-type genes in *Monilinia* spp. since no information was available at the beginning of the work. In detail, nucleotide *MAT1* sequences from *S. sclerotiorum* (JQ815883.1) and *B. cinerea* (*MAT1-1*, KF944385.1; *MAT1-2*, KF944386.1) were aligned using SeqMan Pro (Lasergene v.14.1, DNASTAR Inc., Madison, WI) and primer pairs, 1A and 1B specific for the *MAT1-1-1* gene and 2A and 2B specific for the *MAT1-2-1* gene, were designed using the Primer3 software (Rozen and Skaletsky 2000) (Table 2). The primer pairs were preliminary tested on seven isolates per species (underlined in Table 1).

PCR amplifications were performed in a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer, Norwalk, CT) using the following conditions: 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 54°C (Table 2), and 1 min at 72°C, followed by a final extension of 7 min at 72°C. Reaction mixtures (50 µl) consisted of 1× LA PCR Buffer II (Mg²⁺ free), 2 mM MgCl₂, 0.2 mM each dNTP, 2 U of TaKaRa LA Taq (all PCR reagents were from Takara Bio Inc., Otsu, Shiga, Japan), 0.1 µM of each primer, and 0.1 to 0.2 µg of DNA template. Negative control, in which water was used instead of the target DNA, was always run to detect potential contaminations. Aliquots (5 µl) of PCR products were size-separated on 1.5% agarose (Bio-Rad Laboratories, Hercules, CA), added with 0.05 µl ml⁻¹ GelRed (Biotium, Hayward CA), in 1× TAE (40 mM Tris; 20 mM acetic acid; and 1 mM Na₂EDTA, pH 8.0) buffer at 110 V for 110 min. Amplicons were purified with Quantum

Prep PCR Kleen Spin columns (Bio-Rad Laboratories) and subjected to direct sequencing in both forward and reverse directions by external service (Genewiz, Takeley, UK) using the same primers as for PCR. New primer pairs (2C-2F, Table 2) were designed on the conserved regions of the obtained *MAT1-2* amplicons in the three *Monilinia* species for improving reaction specificity and sensitivity. All primers were synthesized by external service (Macrogen Inc., Seoul, Republic of Korea). Lasergene was used for sequence analysis and BLASTn and BLASTx were used for similarity search and multiple sequence alignments with sequences available in NCBI GenBank (<https://www.ncbi.nlm.nih.gov>).

For the assessment of mating type of *M. fructicola*, *M. laxa*, and *M. fructigena* isolates, PCR conditions were the same as above but the reaction mixture (final volume of 25 µl) contained 1× Green GoTaq Flexi buffer (Mg²⁺ free), 2 mM MgCl₂, 75 µM each dNTP, 1.25 U of GoTaq DNA Polymerase (all from Promega Co., Madison, WI), 0.5 µM of each primer (pairs 1B and 2C, Table 2), and 0.1 µg of DNA template. PCR products were visualized by electrophoresis as previously described. Frequencies of *MAT1-1* and *MAT1-2* idiomorphs on total count in each species were tested by the χ^2 test to show possible deviations from the 1:1 ratio.

Inter simple sequence repeat (ISSR) profiling and linkage disequilibrium analysis in *M. fructicola*. Nine isolates (Mfrc77, Mfrc78, Mfrc106, Mfrc123, Mfrc148, Mfrc150, Mfrc233, Mfrc320, and Mfrc321) were used in a pilot experiment to screen ISSR

TABLE 1. (Continued from previous page)

Species	Isolate code ^a	Location	Host plant	Year
<i>M. laxa</i>	Mfrc326, Mfrc327	USA (Reedley, California)	Nectarine	1995
	Mfrc335, Mfrc336, Mfrc337		Nectarine	2002
	Mfrc328	USA (Sanger, California)	Peach	1994
	Mfrc329	USA (Merced, California)	Nectarine	2002
	Mfrc330, Mfrc331	USA (Parlier, California)	Peach	2002
	Mfrc338		Nectarine	2002
	Mfrc332	USA (Livingstone, California)	Peach	2002
	Mfrc333		Peach	2002
	Mfrc339	USA (Dinuba, California)	Nectarine	1992
	Mfrc349	USA (Fresno, California)	Peach	2005
	Mfrc347, Mfrc348	USA (Geneva, New York)	Cherry	2007
	Mfrc350, Mfrc351	USA (Oak Grove, Virginia)	Plum	2012
	Mfrc352, Mfrc353	USA (Byron, Georgia)	Peach	2006
	Mfrc354, Mfrc355	USA (Clemson, South Carolina)	Peach	2016
	Mfrc356, Mfrc357, Mfrc358	USA (Sandy Springs, South Carolina)	Peach	2016
	Mfrc401, Mfrc402, Mfrc403	USA	Plum	1995
	<u>Mfrc534</u>		Apricots	Unknown
	Mfrc404	Australia	Peach	1995
	Mfrc405	New Zealand	Plum	1995
	Mfrc416	New Zealand (Central Otago)	Apricots	1969
	Mfrc418	New Zealand (Royal Oak-Auckland)	Peach	Unknown
	Mfrc419	New Zealand (Whenuapai-Auckland)	Peach	1979
	Mfrc420	New Zealand (Hastings-Hawke's Bay)	Peach	1979
	Mfrc424	New Zealand (Christchurch, Mid Canterbury)	Plum	2014
	Mfrc435	China (Linyi City, Shandong Province)	Peach	2005
	Mfrc436	China (Yantai City, Shandong Province)		
	Mfrc437	China (Chaoyang, Pechino, Beijing)		
	Mfrc438	China (Fangshan, Pechino, Beijing)	Plum	
	Mlax18	Italy	Unknown	1993
	Mlax23, Mlax24, Mlax51			1994
	Mlax30, Mlax33, <u>Mlax34</u> , Mlax36	Italy	Almonds	1994
	Mlax56	Italy	Plum	1996
	Mlax61	Italy (Casalnuovo Monterotaro, Apulia)	Apricots	1997
	Mlax66	Italy (Acquaviva delle Fonti, Apulia)	Cherry	2004
	<u>Mlax292</u> , Mlax293, Mlax294, <u>Mlax297</u> , <u>Mlax302</u> , Mlax309, <u>Mlax316</u>	Italy (Gioia del Colle, Apulia)	Cherry	2014
	Mlax370, Mlax371	Italy (Sammichele di Bari, Apulia)	Apricots	2014
	Mlax346	Italy (Gaudiano, Basilicata)	Apricots	2014
	Mlax509	Italy	Peach	1992
<i>M. fructigena</i>	<u>Mfrc269</u> , Mfrc340, Mfrc341	Italy (Tursi, Basilicata)	Plum	2014
	<u>Mfrc342</u> , <u>Mfrc343</u>	Italy (Gaudiano, Basilicata)	Peach	2014
	<u>Mfrc344</u>	Italy (Emilia Romagna)	Pear	Unknown
	Mfrc345		Apple	Unknown
	<u>Mfrc510</u>	The Netherlands (Utrecht)	Peach	Unknown

primers. Fifteen ISSR primers previously reported to generate polymorphic fingerprinting patterns in fungi were screened: Mf-1 (AT)₈, Mf-2 (AC)₈, Mf-3 (AAG)₅, Mf-6 (AAT)₅, Mf-4 (AG)₈, Mf-5 (AAC)₅, Mf-7 (AAG)₈ (Lim et al. 2004), Mf-8 (AG)₈C, Mf-9 (GACA)₄, M-13-Mf (GAGGGTGGCGGCGGTTCT) (Ma et al. 2003), Mf-10 (GTG)₅ (Zhou et al. 2001), Mf-11 (TGTC)₄ (Longato and Bonfante 1997), SSR-2 (ACTG)₄, SSR-5 (GACAC)₄, and SR10 (CAC)₅ (Jana et al. 2005). All primers were synthesized by Macrogen Inc. and PCR amplifications were performed according to Villarino et al. (2012). The PCR mix (final volume 25 µl) consisted of 1× Green GoTaq Flexi Buffer (Mg²⁺ free), 2 mM or 2.5 mM (Mf-7, Mf-8, and Mf-9) MgCl₂, 0.2 mM each dNTP, 1 U (Mf-2), 1.5 U, or 2.25 U (Mf-7) of GoTaq DNA Polymerase (all reagents from Promega Co.), 1 µM or 2 µM (Mf-2 and Mf-7) primer, and 50 ng of DNA template. Standard PCR conditions were as follows: 3 min at 95°C for initial denaturation; 40 cycles of 1 min at 94°C, 1 min at 50°C, 45°C (Mf-8), or 36°C (Mf-9), 1 min at 72°C; ending with 10 min at 72°C and a final hold at 4°C. For the Mf-2 primer, PCR conditions were as follows: 3 min at 95°C for initial denaturation; 35 cycles of 30 s at 94°C, 30 s at 53°C, 1 min at 72°C; ending with 10 min at 72°C and a final hold at 4°C. Each reaction was performed twice, and PCR products separated on 3% agarose gel added with 0.05 µl ml⁻¹ of GelRed (Biotium) in 1× TAE buffer and photographed with a Gel Doc 1000 System (Bio-Rad Laboratories). A 100-bp DNA Ladder (New England Bio-Labs, Beverly, CA) and a EZ Load 500-bp Molecular Ruler (Bio-Rad Laboratories) were used as molecular size markers. The experiments were repeated at least twice. Thirteen primers that generated reproducible and clearly discernible DNA fingerprint patterns were selected and then used to analyze the full set of 61 *M. fructicola* isolates from South Italy (Table 1).

By assuming that *M. fructicola* is haploid, like other Ascomycetes, each reproducible band in the selected ISSR patterns was considered as a putative locus with two alleles and visually scored for the presence (1) or absence (0) of the amplicon to obtain a binary matrix. An equal weight was given to all scored bands. A single multilocus profile was then constructed for each isolate and used to estimate linkage disequilibrium. The MultiLocus program v1.3b (Agapow and Burt 2001) was used to calculate the following parameters (Xu 2006): (i) the number of different genotypes; (ii) the genotypic diversity (GD; Simpson's index after correction for sample size); (iii) the index of association (IA); (iv) the proportion of compatible pairs of loci (PrCP). GD ranges from zero (all individuals with a same genotype) to one (all individuals with different genotypes). IA has an expected value of zero if the alleles at different loci are randomly associated with each other in the population, while significant associations between alleles at different loci would be expected in clonally reproducing populations.

PrCP approaches a value of 1 for no recombination. Briefly, two loci are compatible if it is possible to account for all the observed genotypes by mutations without inferring homoplasmy or recombination; otherwise, the loci are incompatible. For example, for two loci with two alleles each, the loci are compatible if no more than three genotypes are observed; if all four genotypes are observed, these two loci are phylogenetically incompatible. Phylogenetic incompatibility suggests recombination in the population. IA and PrCP were calculated based on the clone-corrected data (McDonald 1997). The statistical significance of GD ≠ 1, and the null hypotheses of full panmixia (IA = 0) and full clonality (PrCP = 1) were evaluated against 1,000 randomized data sets.

Characterization of the *MAT1* loci. Two isolates of opposite mating type per each species (bold in Table 1) were selected and used as references in the identification and characterization of the mating-type loci. The approach for the sequencing of the full-length loci was the primer-walking method (Sterky and Lundberg 2000) that was previously used to sequence the *MAT1* locus in *B. cinerea* (De Miccolis Angelini et al. 2016). Two rough draft genomes of the strains Mlax316 and Mfrg269, obtained by de novo assembly of next generation sequencing (NGS, Illumina) paired-end short reads (2 × 100 bp) and the assembled transcriptomes of *M. fructicola*, *M. laxa*, and *M. fructigena* (De Miccolis Angelini et al. 2018; data not published) were used as a starting point to identify and characterize *MAT1* sequences in the *Monilinia* species.

tBLASTn was used to identify sequences with high similarity to the amino acid sequences encoded by *MAT1-1-1* (AHX22632.1), *MAT1-1-5* (AHX22633.1), *MAT1-2-1* (AHX22634.1), *MAT1-2-4* (AHX22635.1), recently renamed *MAT1-2-10* (Wilken et al. 2017), and the flanking genes *APN2* (EMR81656.1) and *SLA2* (EMR81653.1) in *B. cinerea*, in our *Monilinia* genome and transcriptome databases. Based on the identified genomic and transcriptomic sequences, along with the partial *MAT1-1-1* and *MAT1-2-1* sequences previously obtained, we designed primer pairs to amplify by PCR overlapping genome sequences included between the *APN2* and *SLA2* genes and to sequence the entire *MAT1-1* and *MAT1-2* idiomorphs in each *Monilinia* species. The selected primer pairs, the size of amplified DNA fragments and PCR conditions are reported in Supplementary Table S1. PCR and sequencing were carried out as described above using 0.1 to 0.3 µg of template DNA and cycling conditions as follows: 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 58 to 65°C and 1 to 8 min at 72°C, followed by a final extension of 7 min at 72°C. The PCR products were visualized on gels and sequenced as described above. All partial *MAT1* sequences obtained were aligned in SeqMan Pro and new primers designed for filling the gaps within the two idiomorphs of each *Monilinia* species.

The sequences were analyzed with the 'Start/Stop/ORF' procedure of the gene-finding program GeneQuest (Lasergene) by

TABLE 2. Primer pairs used to determine the mating type of *Monilinia* species^a

Idiomorph	Primer pair	Sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)
<i>MAT1-1</i>	1A	Fw ATATGGTGCCTGGGAGATGC Rev AGCCATCATACCACTGGACC	54	166
	1B	Fw CATTGCTTCCAACCTCAGAATGC Rev TCATACCACTGGACCAAGGAG	54	182
	2A	Fw TCCCACGTCCMGCTAATGAG Rev AGCCTTCTGTGAGCTTCCT	54	246
	2B	Fw CTCAAGCCAAAGATCCCACG Rev RATCTTGACTTCARRCGCC	54	223
<i>MAT1-2</i>	2C	Fw AGGAAAGCTTATCCAGGGTATCA Rev TATTTCAAGCGTCGTTCCGG	60	150
	2D	Fw TCAAGGAAAGCTTATCCAGGGT Rev AGCCTTCTGTGAGCTTCCT	60	204
	2E	Fw AGCTTCAATTATCGCTGGCA Rev AGCCTTCTGTGAGCTTCCT	60	158
	2F	Fw CCCCGCTATGATTTCAATTTTCT Rev TTCCTTCAAACATCGGCGC	60	122

^a Primer pairs used for assessing the mating type of field isolates are in bold.

using the Borodovsky method to predict the open reading frames (ORFs) and putative splicing sites. BLASTx analysis suggested the gene structure and the presence of introns and exons. SeqBuilder (Lasergene) was used to make a graphical representation of the structural organization of the mating genes.

BLASTn was used for the identification of unique idiomorphic sequences in the three *Monilinia* species and the pairwise comparison of the *MAT1* genes and intergenic regions. Multiple sequence alignments of the *MAT1*-encoded proteins in the *Monilinia* spp. and the homologous proteins in fungi belonging to the order *Helotiales* were carried out using ClustalW and phylogenetic trees generated using MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 (Kumar et al. 2016).

Transcript analysis of *MAT1* genes. Transcript identification and quantification of the expression of *MAT1* genes were assessed by high-throughput mRNA sequencing (RNA-Seq) from the isolates used for the molecular characterization of *MAT1* loci (bold in Table 1). Total RNA was extracted from about 100 mg of mycelium or germinating conidia by using RNeasy Plant Mini kit (QIAGEN, Milan, Italy), according to the manufacturer's instructions, and used to prepare cDNA libraries with TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, CA). Sequencing was carried out on an Illumina HiScanSQ platform using TruSeq SBS kit v3 (Illumina, Inc.) to obtain paired-end reads, each 92 nt in length. After removing indexed adapters, reads were filtered for quality score (QS) ≥ 30 using CASAVA v1.8 software (Illumina, Inc.) and aligned on the assembled *MAT1* loci, quantified and visualized using SeqMan NGen (Lasergene) with default parameters for the option "classic assembler". Detection of reads spanning across putative splice junctions was used to assess prediction accuracy for protein coding gene structures and annotate transcribed sequences within the *MAT1* loci in *Monilinia* species. Gene expression was measured as reads per kilobase per million mapped reads (Trapnell et al. 2010).

RESULTS

Molecular assay for mating type. Four primer pairs, designed on conserved regions of the mating-type genes of *B. cinerea* and *S. sclerotiorum*, were preliminary tested on a pool of *Monilinia* isolates. Single isolates produced amplicons with only one of the primer pairs: the pair 1B, designed on the *MAT1-1-1* gene, yielded a single 182-bp amplicon from genomic DNA of the isolates Mfrc123, Mfrc150, Mfrc261, Mfrc159; Mlax34, Mlax316, Mlax509, Mlax292; Mfrg342, Mfrg343, Mfrg269, Mfrg340, Mfrg341, and Mfrg510; the pair 2A, designed on the *MAT1-2-1* gene, yielded a single 246-bp amplicon from genomic DNA of the isolates Mfrc78, Mfrc534; Mlax297, Mlax302, Mlax346, and Mfrg344. PCR products from one isolate per species and mating

type (Mfrc159, Mfrc534, Mlax316, Mlax297, Mfrg340, and Mfrg344) were sequenced and analyzed by BLAST search to confirm their nature. The 182-bp products showed 74 to 76% identity with the *MAT1-1* idiomorph (KF944385.1) and the 246-bp products showed 82 to 86% identity with the *MAT1-2* idiomorph (KF944386.1) of *B. cinerea*. The primer pair 2C designed on the 246-bp sequences of the strains Mlax297 and Mfrc83, yielding a 150-bp amplicon (Table 2), improved the sensitivity and specificity of the assay for the *MAT1-2* idiomorph compared with the pair 2A and was then used for further analysis.

The mating type of 155 isolates (125 of *M. fructicola*, 22 of *M. laxa*, and 8 of *M. fructigena*) was assessed by PCR (Supplementary Fig. S1). Both mating types were detected in each *Monilinia* species and a total of 77 isolates were *MAT1-1* and 78 isolates were *MAT1-2*. In detail, among Italian *M. fructicola* isolates, 34 were identified as *MAT1-1* and 27 as *MAT1-2*. Both mating types were also generally detected among *M. fructicola* isolates from other countries except those represented by only few isolates. All the isolates from Greece (four isolates), China (four), Georgia (two), and New York (two) were *MAT1-2*, while the only one from Australia was *MAT1-1*. *M. laxa* samples included 10 *MAT1-1* and 12 *MAT1-2* isolates. Finally, six *MAT1-1* and two *MAT1-2* isolates constituted the *M. fructigena* samples (Table 3). The two mating types were in ratios statistically close to 1:1 in each of the three species (χ^2 values were 0.04 for *M. fructicola*, 0.09 for *M. laxa*, and 1.0 for *M. fructigena*; $\chi^2 = 3.84$ for $P = 0.05$).

ISSR profiling and linkage disequilibrium analysis in *M. fructicola*. Eight of the 13 ISSR primers (i.e., Mf-2, Mf-3, Mf-5, Mf-8, Mf-11, M-13-Mf, SRR-2, and SRR-5) used on the whole set of 61 isolates produced reproducible polymorphic bands. Each of the selected primers amplified from 5 (Mf-5) to 10 (Mf-2 and SSR-2) bands per isolate which varied in size from 200 to 2,500 bp. Of the 54 amplified bands, 29 were monomorphic across all isolates and 25 were polymorphic with an average of three polymorphic bands per primer (Supplementary Fig. S2). Forty-nine genotypes were identified among the 61 isolates. GD was 0.992, slightly but significantly ($P < 0.001$) lower than the value of one expected for full random mating. The observed IA was 0.474, significantly ($P < 0.001$) higher than the mean IA (0.003) of the random data set, so that the null hypothesis of complete panmixia was rejected. The PrCP value was 0.899 ($P = 0.015$) and hence the null hypothesis of full clonality was also rejected. When the IA and the PrCP results were combined, there was some evidence for recombination (most likely by means of sexual reproduction) against a background of predominantly asexual reproduction.

Characterization of the *MAT1* locus. tBLASTn using amino acid (aa) sequences from *B. cinerea* identified putative *APN2*, *MAT1-1-1*, *MAT1-1-5*, and *SLA2* genes in genomic contigs of the

TABLE 3. Distribution of the *MAT1-1* and *MAT1-2* idiomorphs in *Monilinia* species according to their origin

Origin	Country/state	<i>M. fructicola</i>		<i>M. laxa</i>		<i>M. fructigena</i>	
		<i>MAT1-1</i>	<i>MAT1-2</i>	<i>MAT1-1</i>	<i>MAT1-2</i>	<i>MAT1-1</i>	<i>MAT1-2</i>
Europe	Italy	34	27	10	12	5	2
	France	2	3				
	Greece		4				
	Serbia	3	2				
	Spain	7	3				
	The Netherlands					1	
USA	California	4	10				
	Georgia		2				
	New York		2				
	South Carolina	3	2				
	Virginia	1	1				
	Unknown	2	2				
Oceania	Australia	1					
	New Zealand	4	2				
Asia	China		4				
Total		61	64	10	12	6	2

strains Mlax316 (55,736 bp) and Mfrg269 (36,171 bp). It was not possible to detect the *MAT1-2* genes because both the strains were *MAT1-1*. The percent identity values of amino acid sequences were up to 76% (*M. laxa*) and 75% (*M. fructigena*) for the MAT1-1-1 alpha-box protein, 77% for the MAT1-1-5 putative protein, 74% (*M. laxa*), and 77% (*M. fructigena*) for the APN2 and up to 93% for the SLA2 proteins (E-values $\leq 1e-85$). Similarly, tBLASTn identified in the transcriptomes of each species partial or complete (119 to 2,010 bp) transcript sequences for the *MAT1*-specific genes, with the only exceptions of *MAT1-2-10* in *M. laxa* and *M. fructigena*, and the flanking *APN2* and *SLA2* genes (E-values $\leq 5e-23$).

Using the primer pairs designed on the identified genomic sequences, we obtained 7,041 (*M. laxa*) and 6,933 (*M. fructigena*) nucleotide sequences encompassing the entire *MAT1-1* idiomorphs and adjacent regions. The assembled sequences were 99% identical to those obtained by NGS assembly. We supposed a similar structure of the *MAT1-1* locus also in *M. fructicola*, and hence, we designed primers on the available transcript sequences of the *MAT1-1* and the flanking *APN2* and *SLA2* genes in *M. fructicola* which were successfully used to amplify and sequence overlapping regions of the locus. The assembled *MAT1-1* sequence of *M. fructicola* was 7,979 bp in length.

The entire *MAT1-2* idiomorphs and adjacent regions of the three species were obtained by designing PCR primers on transcript sequences of the *MAT1-2-1* and *MAT1-2-10* genes and the *APN2* and *SLA2* gene sequences from the reconstructed *MAT1-1* idiomorphs. The assembled sequences were 7,135 bp (*M. laxa*), 7,667 bp (*M. fructicola*), and 8,050 bp (*M. fructigena*) in length (Fig. 1).

The *MAT1-1* idiomorphs include two ORFs identified by both GeneQuest and BLASTx analyses. The predicted *MAT1-1-1* genes are 1,137 bp (*M. fructicola*) or 1,132 bp (*M. laxa* and *M. fructigena*) in length, with two exons (259 and 824 bp), separated by an intron of 54 bp (*M. fructicola*) or 49 bp (*M. laxa* and *M. fructigena*). The encoded proteins (360 aa), containing the conserved alpha-box domain (pfam04769), are 96 to 98% identical in the three species and BLASTp search showed 73 to 74% aa identity with the homologous *Botrytis elliptica* protein (AKC57287.1). The predicted *MAT1-1-5* genes have the same structure in the three species. They are 1,304 bp in length, contain four exons (277, 548, 27, and 279 bp) interrupted by three short introns (49, 65, and 59 bp). The encoded proteins (376 aa), with no putative conserved domain, showed 93 to 98% aa identity in the three species and the highest amino acid identity (70%) with the homologous *Sclerotinia minor* protein (AHA49902.1) in BLASTp search.

The *MAT1-2* idiomorphs include two ORFs. The predicted *MAT1-2-1* genes have the same structure in the three species. They are 1,176 bp in length and contain two introns (58 and 53 bp) and three exons (410, 371, and 284 bp). The encoded proteins (354 aa), containing the conserved HMG-box domain (cd00084), are 91 to 95% identical in the three species and BLASTp search showed the highest amino acid identity (70%) with the homologous *Sclerotinia borealis* F-4128 protein (ESZ90540.1). The predicted *MAT1-2-10* genes are 957 bp in length and contain two introns (both of 48 bp) and three exons (434, 239, and 188 bp). The encoded proteins (286 aa), with no putative conserved domain, showed 90 to 92% aa identity in the three species and the highest amino acid identity (56 to 58%) with the homologous *S. sclerotiorum* protein (AGB05588.1).

The sequences of the *MAT1* locus and their respective flanking regions in the *MAT1-1* and the *MAT1-2* strains of *M. laxa*, *M. fructigena*, and *M. fructicola* have been deposited in GenBank database under accession numbers MG182161 (*MAT1-1*, Mlax316), MG182162 (*MAT1-1*, Mfrg269), MG182163 (*MAT1-1*, Mfrg123), MG182164 (*MAT1-2*, Mlax297), MG182165 (*MAT1-2*, Mfrg344), and MG182166 (*MAT1-2*, Mfrg78).

The assembled *MAT1* locus sequences in each species were aligned using BLASTn. In both *M. laxa* and *M. fructigena*, the 5' (1,200 bp) and the 3' (1,600 bp) termini showed 99% identity. In *M. fructicola*, the 5' (1,310 bp) and the 3' (2,121 bp) termini showed

97 and 95% identity, respectively. Highly divergent idiomorphic sequences of different sizes were hence identified. The *MAT1-2-1* genes extend for 47 bp (*M. laxa*) or 54 bp (*M. fructicola*) in the 5' flanking region and then a homologous *dMAT1-2-1* (87 to 94% identity, E-value $\leq 3e-15$) was detected in the opposite idiomorph. In addition, other *dMAT1-2-1* sequences (203 bp, *M. fructicola*; 249 bp, *M. laxa*; 258 bp, *M. fructigena*; 72 to 76% identity, E-values $\leq 1e-33$) were detected in the unique *MAT1-1* sequences. In the three species, two *dMAT1-1-1* sequences (290 to 299 bp, 73 to 77% identity, E-values $\leq 1e-46$; 170 to 225 bp, 79 to 83% identity, E-values $\leq 1e-46$) were detected in the unique *MAT1-2* sequences. In *M. fructicola* the second fragment corresponds to the *MAT1-1-1* gene extension in the 3' flanking region. Furthermore, the internal *dMAT1-1-1* fragments (290 to 299 bp) showed similarity (E-values $\leq 1e-44$) to the direct or inverted repeats (230 to 250 bp) responsible for intramolecular recombination events in homothallic *Sclerotinia* species (Chitrampalam et al. 2013; Xu et al. 2016).

The two idiomorphs showed high sequence identity in the intergenic regions 5' IGR-1 (97 to 99%) and 3' IGR-3 (90 to 97%), while, as expected, it was lower (46 to 47%) in the internal IGR-2. By comparing the three species, IGR-1 and IGR-3 showed a lower sequence identity (69 to 89%; 67 to 91%) than IGR-2 (91 to 94%), while comparing the mating types, the identity of IGR-1 was lower in *MAT1-1* (69 to 72%) than in *MAT1-2* (82 to 89%). In all pairwise comparisons between species, *M. laxa* and *M. fructigena* IGRs showed higher sequence identity (80 to 94%) with each other than with *M. fructicola* (67 to 92%; 68 to 93%, respectively).

Phylogenetic analysis of the four *MAT1*-specific protein sequences of *M. fructicola*, *M. fructigena*, and *M. laxa* with those of fungi belonging to the order *Helotiales* available in databank yielded very similar results (Fig. 2). The three *Monilinia* species grouped together in a clade close to those formed by the other fungi belonging to *Sclerotiniaceae*, *B. cinerea* and *B. elliptica*, and most of the *Sclerotinia* species. The *MAT1-2-10* proteins of *Monilinia* spp. were more similar to those of *Sclerotinia* spp. than *Botrytis* spp. The clade of *Rutstroemiaceae*, which grouped also *Sclerotinia homoeocarpa*, and at a higher degree, the clade of the *Helotiaceae*, including *Glarea lozoyensis* and *Hymenoscyphus* spp., were evolutionary more distant. Low rates of amino acid substitutions were recorded in the *MAT1* genes of *M. fructicola*, *M. laxa*, and *M. fructigena*, with *M. laxa* and *M. fructigena* more like each other than *M. fructicola*.

Expression of *MAT1* genes. We estimated gene expression and assessed prediction accuracy for the *MAT1* genes by mapping RNA-Seq reads (36.4 to 37.3 million for the *MAT1-1* idiomorphs; 32.1 to 36.2 million from the *MAT1-2*) onto the reconstructed genomic sequences of the two idiomorphs in each species. The expression levels of the mating-type genes were generally low and could not be detected for the *MAT1-2-10* gene in *M. fructigena* (Fig. 3). As expected, reads from single isolates mapped exclusively either on the *MAT1-1* or *MAT1-2* idiomorphic sequences, according to the own mating type.

The reads covered 98% of the *MAT1-1-1* transcript sequence in *M. fructicola*, 88.5% in *M. laxa*, and 77.9% in *M. fructigena*. It was not possible to confirm the predicted splicing site because of low read depth. However, two unpredicted splicing events originated by new candidate introns both with antisense orientation were observed at the 3' terminus (50 bp, nucleotide position 5561 to 5610) and downstream (68 bp, nucleotide position 5808 to 5975) the *MAT1-1-1* gene in *M. fructicola* (MG182163). The mapped reads covered the entire length of the *MAT1-1-5* transcript in *M. laxa*, including the predicted start and stop and three splice sites, validating intron predictions. Partial coverages of the transcripts were obtained in *M. fructicola* (72.4%) and *M. fructigena* (30.2%) due to low expression levels, but the three predicted splice sites were confirmed in *M. fructicola*. Expression level of *MAT1-2-1* was higher in *M. fructicola* (full coverage) than in *M. laxa* (95%) and *M. fructigena*

(78%). The two predicted splice sites were confirmed in *M. fructicola* and *M. laxa*; in *M. fructigena* only the position of the 58-bp intron within the HMG-box domain could be confirmed. Novel candidate introns of 119 bp (from 1642 to 1760) and 53 bp (from 2578 to 2630), originating from antisense-oriented alternative transcripts, were observed in *M. laxa* (MG182164). The *MAT1-2-10* transcript sequence was completely covered although at low read depth in *M. fructicola*, partially covered in *M. laxa* (78.5%), and no mapped reads were obtained for *M. fructigena*. Only the splice junctions at the 5' introns (48 bp) in *M. laxa* and *M. fructicola* were confirmed.

Transcription of a region (289 bp) of the *MAT1-2* idiomorph, including the 170-bp *dMAT1-1-1* sequence and near-identical flanking region downstream the *MAT1-2-10* gene, was detected in *M. fructicola*. Moreover, a transcribed region (1,036 bp) of the *MAT1-1* idiomorph, including the 249-bp *dMAT1-2-1* sequence and extending for 278 bp in the 5' flanking region, was detected in *M. laxa* (MG182161). It contained two putative introns of 53 bp (from 1,457 to 1,405) and 64 bp (from 2,103 to 2,040) in the antisense orientation. Only few reads mapped on the corresponding regions in the *MAT1-1* idiomorphs of *M. fructicola* and *M. fructigena*.

Fig. 1. Structures of the mating-type (*MATI*) loci in the *MATI-1* (a) and *MATI-2* (b) idiomorphs of *Monilinia fructicola*, *M. laxa*, and *M. fructigena*. Solid boxes represent the coding region of the predicted genes interrupted by introns. Internal gray box within the *MATI-1-1* and *MATI-2-1* genes are the conserved alpha-box and HMG-box domains, respectively. Arrows indicate orientation of coding sequences. Sizes of the unique sequences of each idiomorph (delimited by dotted lines) are reported.

DISCUSSION

Sexual reproduction, when occurring, is a major source of genetic variation in fungal populations and may create novel recombinant genotypes with altered virulence or increased fitness in new ecological niches (Heitman 2006; Ni et al. 2011). The mating systems are diverse in the family *Sclerotiniaceae*, which include both homothallic and heterothallic species. For instance, *B. cinerea* and *B. elliptica* display a bipolar heterothallism, *S. minor* and *S. sclerotiorum* display homothallism, while *Sclerotinia trifoliorum* and *S. homoeocarpa* include either homothallic or heterothallic isolates. In heterothallic species, the *MAT1-1* or the *MAT1-2* idiomorphs are singly carried by self-sterile individuals, while in homothallic species they are closely linked end-to-end at a single *MAT1* locus in self-fertile individuals (Amselem et al. 2011; Chitrampalam and Pryor 2015; De Miccolis Angelini et al. 2016; Putman et al. 2015; Terhem et al. 2015; Xu et al. 2016). The mating system governing the sexuality in the genus *Monilinia* was largely unexplored and no information was available for *M. laxa* and *M. fructigena*. *M. fructicola* was initially reported to be homothallic (Ezekiel 1924; Harada 1977), but other studies suggested that it is capable of outcrossing (Sharvell and Chen 1943; Sonoda et al. 1982; Thind and Keitt 1949) and this was finally demonstrated by segregation of mycelial incompatibility and fungicide resistance genes in ascospores progeny (Free et al. 1996). A search of conserved sequences by alignments of the *MAT1* loci in *B. cinerea* and *S. sclerotiorum* allowed us to design a PCR assay based on idiomorph-specific primers for rapid mating-type assessment in *Monilinia* isolates. The molecular assay was applied to 61 *M. fructicola*, 22 *M. laxa*, and 8 *M. fructigena* isolates from 9 districts in South Italy and 64 *M. fructicola* isolates from 14 geographic areas, including other European countries, the United States, Australia, New Zealand, and China. Each isolate of the three *Monilinia* species carried only one

of the two alternative idiomorphs at the *MAT1* locus. Hence, we firstly showed at molecular level that *M. fructicola*, *M. laxa*, and *M. fructigena* have a bipolar heterothallic mating system, typical of other self-incompatible Ascomycetes. Both mating types were represented among the isolates from most countries, except those with small-sized fungal samples. Overall, the two mating types were in balanced distribution in the populations of the three *Monilinia* species and this indicates the absence of genetic barriers to the occurrence of sexual reproduction and the potential for outcrossing in most of the geographic areas in which the pathogens are present.

As to *M. fructicola*, a quarantine pathogen first reported in Europe in 2001 (OEPP/EPPO 2002) and in 2009 in North Italy (Pellegrino et al. 2009), the results of the present monitoring

Fig. 3. Expression levels of the *MAT1* genes in *MAT1-1* and *MAT1-2* isolates of *Monilinia fructicola* (MFRC), *M. laxa* (MLAX), and *M. fructigena* (MFRG).

Fig. 2. Evolutionary relationships of the mating-type-specific proteins of *Monilinia fructicola* (MFRC), *M. laxa* (MLAX), and *M. fructigena* (MFRG) and other fungi belonging to *Helotiales*. The tree was inferred using the neighbor-joining method and the evolutionary distances (number of amino acid substitutions) were computed using the Poisson correction method eliminating all positions containing gaps and missing data. Bootstrap test was carried out with 1,000 replicates.

demonstrated that both idiomorphs were introduced and established in the continent as well as in Italy. However, there are no reports of apothecia findings in the field in Europe in contrast to the common occurrence of sexual reproduction and apothecia differentiation in the United States (Michailides et al. 2007). For this reason, the genetic structure of *M. fructicola* population in Italy was investigated by using ISSR markers. Forty-nine genotypes were found in 61 tested isolates.

On the ground of the statistically significant values of GD (0.992), IA (0.474), and PrCP (0.899), there is some evidence for recombination (most likely by means of sexual reproduction) in a prevalently clonal population, like reported in other European countries (De Cal et al. 2014; Jansch et al. 2012; Villarino et al. 2012). Hence, we can suppose that sexual reproduction potentially occurs at low frequency although other genetic sources of variation, such as heterokaryosis and parasexuality, could also underlie recombination. The predominant clonality of the population and the apparently limited occurrence of the sexual reproduction might be due to different reasons: (i) the prodigious conidial production in *M. fructicola* well timed to initiate infection in susceptible plant tissues; (ii) the hot and dry climatic conditions of South Italy, which are generally not inductive to apothecia differentiation; and (iii) crop management factors, such as removal of infected fruits by growers or destruction and interring of mummified fruits during soil tillage (Holtz et al. 1998; Michailides et al. 2007; Villarino et al. 2012).

Studies on genetic variation and IA analyses revealed comparable results for the closely related species *S. sclerotiorum* and *B. cinerea*. *S. sclerotiorum* populations on different crops in the United States showed prevalent clonality and some evidence of recombination (Aldrich-Wolfe et al. 2015; Dunn et al. 2017). A high level of genetic diversity but nonrandom mating was observed in *B. cinerea* populations in California (Ma and Michailides 2005) and South Africa (Wessels et al. 2016), suggesting the absence or infrequent sexual reproduction, that was instead supposed to occur in other areas on various hosts, like Hungarian vineyards (Vácz et al. 2008).

By combining high-throughput sequencing data with targeted PCRs and sequencing using the primer walking approach, the full-length sequences of the *MAT1* loci from the three *Monilinia* species were determined. The genetic organization of the loci was very similar in the three *Monilinia* species, in terms of gene content, synteny and intron number and positions, and appeared highly similar to those of the closely related heterothallic *B. cinerea* and *B. elliptica* (Amselem et al. 2011; De Miccolis Angelini et al. 2016; Terhøgh et al. 2015). The *Monilinia MAT1* loci are flanked by *APN2* and *SLA2* genes and have typical features of the heterothallic *Pezizomycotina*, in which the *MAT1-1* idiomorph contains a gene, namely *MAT1-1-1*, encoding a protein with a conserved alpha-box domain, while the *MAT1-2* contains a gene, the *MAT1-2-1*, encoding a protein with a conserved HMG domain (Debuchy et al. 2010). In addition, the two idiomorphs contain the *MAT1-1-5* or the *MAT1-2-10* genes, both encoding predicted proteins with no conserved functional domains.

Fragments of the *MAT1-1-1* gene (*dMAT1-1-1*) were detected at the 3' end of the *MAT1-2* idiomorphs and the 3' end of the *MAT1-2-1* gene (*dMAT1-2-1*) was detected at the 5' end of the *MAT1-1* idiomorphs. A duplicated short fragment of *dMAT1-2-1* was also detected bordering the near-identical 5' flanking region of *MAT1-1* idiomorphs in *M. fructicola* and *M. laxa*. Homologous sequences of the opposite idiomorph at similar positions have been reported in the heterothallic *B. cinerea*, *B. elliptica*, and *S. homoeocarpa* and are believed nonfunctional truncated *MAT1* genes. The presence of fragments of mating-type genes in the opposite *MAT1* idiomorph suggests that the heterothallic *MAT1-1* and *MAT1-2* loci evolved from an ancestral homothallic locus by multiple recombination and deletion events with loss of *MAT1*-specific sequences (Amselem et al. 2011; Rodenburg et al. 2018). This evolutionary scenario had been proposed for other Ascomycetes showing similar features

at the *MAT1* locus (Paoletti et al. 2005; Tsui et al. 2013). More recently, different hypotheses have been proposed on the possible evolution of homothallic *Sclerotinia* species from heterothallic ancestors that are consistent with the structure of newly characterized *MAT1* loci in *Sclerotiniaceae* and are more parsimonious involving a lower number of evolutionary changes. According to Xu et al. (2016), indeed, a single structural rearrangement, consisting of a simple insertion of the *MAT1-2* idiomorph into the *MAT1-1* idiomorph at the *MAT1* locus in a heterothallic ancestor with a locus like those of *B. cinerea*, *B. elliptica*, and *Monilinia* species, could be the origin of a homothallic locus, like in the Inv+ *MAT* alleles in *S. minor* and *S. sclerotiorum* and in the L allele in *S. trifoliorum*.

A low genetic diversity is expected in chromosomal regions carrying the *MAT* idiomorphs because recombination is supposed to be suppressed and rare at the *MAT1* locus in Ascomycetes (Idnurm 2011). Mating-type gene sequences have been frequently used as markers in phylogenetic studies or to establish species boundaries in different genera of Ascomycetes (Lopes et al. 2017; O'Donnell et al. 2004), although several authors found them of limited utility (Geng et al. 2014; Strandberg et al. 2010). The three *Monilinia* species showed high similarity of the sequences of genes and intergenic regions within the *MAT1-1* and *MAT1-2* idiomorphs while it was lower in the bordering regions. Phylogenetic analysis of the four *MAT1*-specific protein sequences of *M. fructicola*, *M. fructigena*, and *M. laxa* within the order *Helotiales* yielded quite comparable results and the three *Monilinia* species constituted a clade close to those formed by the other fungi belonging to *Sclerotiniaceae*, i.e., *B. cinerea* and *B. elliptica*, and most of the *Sclerotinia* species. The *MAT1-2-10* proteins of *Monilinia* spp., different from the others, were more similar to those of *Sclerotinia* spp. than to *Botrytis* spp. *M. laxa* and *M. fructigena* were more like each other than *M. fructicola*. This is consistent with the evolutionary hypothesis of the *Monilinia* species based on phylogenetic analysis of coding (G3PDH and TUB2) and non-coding rDNA (ITS) sequences. *M. fructicola* may have evolved earlier than other species, which were polyphyletic and shared a single common ancestor with *M. fructicola*. *M. mumeicola* appears to be a direct descendant of *M. laxa* and *M. fructigena* may have evolved from *M. yunnanensis* (Hu et al. 2011; Yin et al. 2015). In our analysis, the clades made up by *Rutstroemiaceae* and *Helotiaceae* were evolutionary more distant. As previously reported, *S. homoeocarpa*, which does not produce a true sclerotium like the other *Sclerotiniaceae* but an indeterminate plate-like substratal stroma, was more closely related to *Rutstroemiaceae* (Putman et al. 2015).

Transcriptome sequencing data from *Monilinia* isolates of opposite mating types confirmed the accuracy of mating-type gene prediction at intron-exon boundaries and demonstrated that the *MAT1* genes are expressed, although at low levels, during vegetative growth (mycelium and germinating conidia). Constitutive expression of the mating-type genes has already been reported in *B. cinerea* (De Miccolis Angelini et al. 2016), *Sclerotinia* spp. (Doughan and Rollins 2016; Xu et al. 2016), and other unrelated fungi (Bidard et al. 2011; Ferreira et al. 1996). Genes at the mating-type locus typically control sexual reproduction and are required during fruiting-body development (Pöggeler et al. 2006; Doughan and Rollins 2016). *MAT1-1-1* and *MAT1-2-1* are master regulatory conserved genes with an essential role in the mating process. They are thought to act as transcriptional regulators of pheromone signaling and are responsible for distinct patterns of gene expression in sexually compatible mating types (Bidard et al. 2011; Debuchy and Turgeon 2006; Debuchy et al. 2010; Pöggeler et al. 2006). Gene deletion of *MAT1-1-5* and *MAT1-2-10* in knockout mutants of *B. cinerea* and *S. sclerotiorum* proved that these two genes play a role in apothecial disk development (Doughan and Rollins 2016; Rodenburg et al. 2018). Nevertheless, the nature and roles of these additional mating-type genes, not conserved in filamentous fungi, in sexual reproduction remain to be

investigated in *Monilinia*. The expression of all the *MAT1* genes in mating noninducing conditions corroborates the hypothesis that they may have other functions besides sexuality with multiple pleiotropic effects on several phenotypic traits, such as vegetative growth, asexual sporulation, and pathogenicity. For instance, loss-of-function *MAT1* gene mutants of *S. sclerotiorum* produced defects in secondary sexual characters including lower numbers of spermatia and altered expression of pheromone precursor and receptor genes (Doughan and Rollins 2016). Lack of the *MAT1-2* genes in self-sterile isolates results in ascospores of a smaller size in *S. trifoliorum* (Xu et al. 2016) or in reduced fitness and pathogenicity in *Ceratocystis albifundus* (Lee et al. 2015). In *Penicillium chrysogenum*, the *MAT1-1-1* transcription factor also participates in key biological processes such as regulation of asexual development and morphogenesis, as well as amino acid, iron, and secondary metabolism (Becker et al. 2015). The mating-type genes control conidial number and size in *Ulocladium botrytis* (Wang et al. 2017). Virulence genes were also included among targets of mating-type transcription factors in *Cryptococcus neoformans* (Mead et al. 2015).

We found evidence of transcriptional activity beyond the annotated genes at the *MAT1* locus. Transcribed regions corresponded to the *dMAT1-1-1* sequence 3' of the *MAT1-2* idiomorph in *M. fruticola* and to the *dMAT1-2-1* sequence 5' of the *MAT1-1* idiomorph in *M. laxa*. This finding agrees with the observed transcription of the *MAT1-1-1* gene fragments derived from inversion or deletion events in homothallic *Sclerotinia* species (Chitrampalam and Pryor 2015; Chitrampalam et al. 2013; Xu et al. 2016). The regions also contained spliced transcripts with an antisense orientation, as could be deduced from the sequence of the splice junctions. This could mean that *dMAT1-1-1* and *dMAT1-2-1* are actively transcribed gene fragments with a potential role in sexual reproduction like in *S. sclerotiorum* (Chitrampalam et al. 2013; Doughan and Rollins 2016). Nevertheless, further investigations are required to establish their function and role in mating. Antisense transcription of the *MAT1-1-1* and the *MAT1-2-1* protein-coding genes were also observed in *M. fruticola* and *M. laxa*, but not in *M. fructigena* probably due to the low read depth at the *MAT1* locus in this species. We previously assessed the antisense transcription of the *MAT1-2-1* gene in *B. cinerea* with isoforms from alternative splicing (De Miccolis Angelini et al. 2016), and transcript isoforms of both the *MAT1-2-1* and *MAT1-1-1* genes have been observed in *S. sclerotiorum* (Chitrampalam et al. 2013). Antisense transcription is currently recognized as an important regulator of gene expression, widespread among all life kingdoms, but poorly understood for its role in regulation of basic biological processes such as sexual reproduction, growth and development (Donaldson and Saville 2012; Brophy and Voigt 2016).

In conclusion, this study has clarified for the first time the bipolar heterothallic mating systems governing the sexual reproduction in the major brown rot pathogens *M. fruticola*, *M. laxa*, and *M. fructigena* through the identification and full characterization of the *MAT1* loci for gene content, transcriptional activities and phylogenetic analysis of the mating-type genes. Monitoring of mating types in fungal samples from Italy and other worldwide areas showed that both two mating types are present at similar frequency in the pathogen's populations and hence there are not genetic obstacles to the occurrence of sexual reproduction. A PCR-based method for assessing mating type was made available and will be helpful for further monitoring program on high numbers of isolates within field populations. As to *M. fruticola*, a quarantine pathogen recently introduced in Europe, the genetic structure of its populations present in South Italy suggests that they are primarily clonal but show a certain extent of recombination deriving from the potential occurrence of sexual reproduction. These results contribute to a deeper knowledge of the role of sexual reproduction in the evolution and population biology of these economically important brown rot pathogens.

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In the article “Mating System in the Brown Rot Pathogens *Monilinia fructicola*, *M. laxa*, and *M. fructigena*” by D. Abate, R. M. De Miccolis Angelini, C. Rotolo, S. Pollastro, and F. Faretra, there were errors in the sequences of primer pairs 1B and 2C in Table 2. The correct sequences are shown below in revised Table 2.

Revised Table 2

TABLE 2. Primer pairs used to determine the mating type of *Monilinia* species^a

Idiomorph	Primer pair		Sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)
<i>MAT1-1</i>	1A	Fw	ATATGGTGCCTGGGAGATGC	54	166
		Rev	AGCCATCATACCACTGGACC		
	1B	Fw	CATTGCTTCCACTCAGAATGC	54	182
		Rev	TCATACCAGTGGACCAAGGAG		
<i>MAT1-2</i>	2A	Fw	TCCCACGTCCMGCTAATGAG	54	246
		Rev	AGCCTTCTTGTGAGCTTCCT		
	2B	Fw	CTCAAGCCAAAGATCCCACG	54	223
		Rev	RATCTTGTAATTCARRCGCC		
	2C	Fw	AGGAAAGCTTATCCAGGGTATCA	60	150
		Rev	TATTTCAAGCGTCGTTCCGG		
	2D	Fw	TCAAGGAAAGCTTATCCAGGGT	60	204
		Rev	AGCCTTCTTGTGAGCTTCCT		
	2E	Fw	AGCTTCAATTATCGCTGGCA	60	158
		Rev	AGCCTTCTTGTGAGCTTCCT		
	2F	Fw	CCCCGCTATGATTTCAATTTTCT	60	122
		Rev	TTCCCTTCAAAACATCGGCGC		

^a Primer pairs used for assessing the mating type of field isolates are in bold.

Previously Published Version

TABLE 2. Primer pairs used to determine the mating type of *Monilinia* species^a

Idiomorph	Primer pair		Sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)
<i>MAT1-1</i>	1A	Fw	ATATGGTGCCTGGGAGATGC	54	166
		Rev	AGCCATCATACCACTGGACC		
	1B	Fw	CATTGCTTCCACTCAGATGC	54	182
		Rev	TCATACCAGTGGACCAAGGAG		
<i>MAT1-2</i>	2A	Fw	TCCCACGTCCMGCTAATGAG	54	246
		Rev	AGCCTTCTTGTGAGCTTCCT		
	2B	Fw	CTCAAGCCAAAGATCCCACG	54	223
		Rev	RATCTTGTAATTCARRCGCC		
	2C	Fw	AAGAAAGCTTATCCAGGTATCA	60	150
		Rev	TATTTCAAGCGTCGTTCCGG		
	2D	Fw	TCAAGGAAAGCTTATCCAGGGT	60	204
		Rev	AGCCTTCTTGTGAGCTTCCT		
	2E	Fw	AGCTTCAATTATCGCTGGCA	60	158
		Rev	AGCCTTCTTGTGAGCTTCCT		
	2F	Fw	CCCCGCTATGATTTCAATTTTCT	60	122
		Rev	TTCCCTTCAAAACATCGGCGC		

^a Primer pairs used for assessing the mating type of field isolates are in bold.