AFLP Analysis of Intraspecific Variation Between *Monilinia laxa* Isolates from Different Hosts

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ABSTRACT

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We analyzed with an amplified fragment length polymorphism (AFLP) marker system the genetic diversity and relationships among 67 *Monilinia laxa* isolates obtained from different host plants. From a total of 1,089 amplified bands scored using 20 primer combinations with two selective nucleotides, 354 were polymorphic and further used in genetic diversity analysis. Genetic relationships among isolates were assessed with different phenetic approaches, including unweighted pair group method with arithmetic mean clustering and principal coordinate analysis; the population's differentiation estimate was analyzed by molecular variance; and model-based clustering was employed to infer population structure. All four analyses clearly showed significant differences between isolates from apple trees and isolates from other host plants. No further grouping according to any other host plant was observed. The results indicate host specialization of apple isolates and support the taxonomic grouping of apple isolates.

Additional keywords: forma specialis, molecular markers

Assessment of the level and nature of genetic variation in plant pathogenic fungi has important implications in disease management and understanding host-pathogen interactions. Brown rot of fruit crops caused by species of the genus *Monilinia* is an extremely destructive disease found in most temperate regions in which apples, pears, and other rosaceous fruit trees are grown (2,3). They cause considerable losses to fruit crops and damage trees (39).

The genus *Monilinia* includes three most widely known and economically important species: *M. fructigena* Honey, *M. fructicola* (G. Winter.) Honey, and *M. laxa* (Aderh. & Ruhl.) Honey. Although the three species are polytrophs and infect a wide range of *Rosaceae* species, a certain level of host specialization exists among them. *M. fructigena* is mainly a fruit rot pathogen, although it is also able to infect and colonize blossoms and it occurs predominantly in pome fruit orchards. *M. fructicola* is mostly confined to stone fruits—peach, nectarine, plum, and apricots—and is mainly a fruit pathogen. *M. laxa* is a blossom and twig pathogen, caus-

ing damage mainly in stone fruits when severe infection of blossoms occurs under warm and humid conditions (2). *M. laxa* is distributed in all major areas of stone and pome fruit production with a humid and moderate warm climate (4). A specialized form of this last species exists, *M. laxa* f. sp. *mali* Harrison{?}, which is thought to be restricted to apple and causes blossom wilt, spur-kill, and canker. Few data on *M. laxa* f. sp. *mali* are available in the literature (2,3). A severe blossom blight of apple was observed in apple orchards in India during 1983. The isolated pathogen was identified as *M. laxa* f. sp. *mali* (30,31). The occurrence of *M. laxa* f. sp. *mali* in Slovenia was first reported in 1997, when an unusual dying of apple shoots was observed. During the blooming season of the next year, apple, apricot, and sour cherry trees were artificially inoculated with these apple isolates, and disease symptoms occurred only on apple trees, supporting the existence of the specialized form (5). In the following years, the disease spread throughout apple production areas in Slovenia.

Another species, *M. mali* (Takah.) Whetzel, autonomous and endemic to Japan and Russia, produces similar symptoms on apples as European *M. laxa* f. sp. *mali* (2,3). In a survey of apple diseases in Korea, the authors found blossom blight caused by *M. mali*, a disease which had not occurred on apple since the mid-1970s (16). The infection mechanisms of *M. mali*

are distinct from those described for *M. laxa* and *M. fructigena*, since the wilt appears before the blossoms open. *M. mali* can also be morphologically distinguished from the other three species based on the presence of disjunctors not present in the other three species from the *Junctioniae* group (2).

With the development of molecular tools, various genetic markers have become basic techniques for detecting, identifying, and classifying plant pathogenic fungi and are also very useful tools for evaluating genetic variability within or between species. Delineation of the three *Monilinia* species has been mainly achieved by analyzing certain parts of the genome, mainly for identification purposes: ITS and IGS regions (15), partial sequences of small DNA (ssrDNA), and large (lsrDNA) subunits of ribosomal DNA (10,13). Information about the genetic structure and genetic diversity of *Monilinia* species is scarce. Based on the ITS nucleotide sequence, no variation was observed in isolates of *M. laxa* from *Malus* and *Prunus* spp. (13). On the basis of random amplified polymorphic DNA (RAPD) markers, variation in *M. laxa* exists but is randomly distributed worldwide (11). The first larger diversity study of *M. laxa* populations from peach, apricot, and almond orchards in Spain using the RAPD technique was recently reported (12).

Amplified fragment length polymorphism (AFLP) is a technique that combines the ease of finding polymorphic loci known for RFLP, with the economic applicability of polymerase chain reaction (PCR) and the possibility of controlling the number of fragments generated by primer extensions (17,27). The efficiency of the AFLP technique for taxonomy, genetic diversity studies, and polymorphism assessment has been used in a number of biological systems (9,25,32,34) to clarify closely related species that could not be resolved with morphological or other molecular systematic characters (18). Majer et al. (17) used AFLP fingerprinting for the first time for the detection of genetic polymorphism of two species of plant pathogenic fungi, *Cladosporium fulvum* and *Pyrenopeziza brassicae*.

The purposes of the current study were (i) to optimize the AFLP procedure for

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Table 1. Sixty-seven *Monilinia laxa* isolates used in the amplified fragment length polymorphism (AFLP) study, their host plants, geographical origin, year, and source of isolation

No.	Isolate ^a	Host plant	Origin ^b	Year	Source
12	MLX0601	Apricot (AP)	Ljubljana	2006	Young fruit
13	MLX0602	Apricot (AP)	Ljubljana	2006	Young fruit
21	MLX0603	Apricot (AP)	Ljubljana	2006	Young fruit
32	MLX0605	Apricot (AP)	Ljubljana	2006	Young fruit
40	MLX0606	Apricot (AP)	Vrtojba	2006	Young fruit
33	MLX0607	Apricot (AP)	Štanjel	2006	Young fruit
14	MLX0610	Sweet cherry (CH)	Brdo pri Lukovici	2006	Young fruit
15	MLX0611	Sweet cherry (CH)	Brdo pri Lukovici	2006	Young fruit
22	MLX0612	Sweet cherry (CH)	Brdo pri Lukovici	2006	Young fruit
23	MLX0614	Sweet cherry (CH)	Brdo pri Lukovici	2006	Young fruit
34	MLX0615	Sweet cherry (CH)	Brdo pri Lukovici	2006	Young fruit
67	MLX0616	Sweet cherry (CH)	Brdo pri Lukovici	2006	Young fruit
42	MLX0618	Sweet cherry (CH)	Brdo pri Lukovici	2006	Mummified fruit
35	MLX0619	Sweet cherry (CH)	Brdo pri Lukovici	2006	Young fruit
66	MLX0621	Sweet cherry (CH)	Brdo pri Lukovici	2006	Young fruit
16	MLX0623	Apricot (AP)	Brdo pri Lukovici	2006	Blossom
17	MLX0624	Apricot (AP)	Brdo pri Lukovici	2006	Young fruit
18	MLX0625	Plum (PL)	Brdo pri Lukovici	2006	Young fruit
19	MLX0628	Peach (PE)	Brje pri Komnu	2006	Young fruit
20	MLX0629	Japanese quince (QUI)	Ljubljana	2006	Shoot
24	MLX0630	Japanese quince (QUI)	Ljubljana	2006	Young fruit
25	MLX0631	Apricot (AP)	Ptujska gora	2006	Young fruit
43	MLX0633	Sweet cherry (CH)	Ptujska gora	2006	Young fruit
44	MLX0634	Sweet cherry (CH)	Ptujska gora	2006	Young fruit
45	MLX0635	Sweet cherry (CH)	Ptujska gora	2006	Young fruit
36	MLX0637	Japanese quince (QUI)	Ptujska gora	2006	Young fruit
47	MLX0638	Peach (PE)	Krško	2006	Young fruit
37	MLX0646	Apricot (AP)	Ljutomer	2006	Shoot
26	MLX0652	Japanese cherry (JCH)	Ljubljana	2006	Blossom peduncle
27	MLX0653	Japanese cherry (JCH)	Šmartno pod Šmarno goro	2006	Blossom peduncle
38	MLX0654	Japanese cherry (JCH)	Ljubljana	2006	Blossom peduncle
46	MLX0655	Japanese quince (QUI)	Kranj	2006	Blossom peduncle
48	MLX0656	Sweet cherry (CH)	Horjul	2006	Blossom peduncle
28	MLX0657	Apple (APP)	Ljubljana	2006	Blossom peduncle
29	MLX0658	Apple (APP)	Ljubljana	2006	Blossom peduncle
30	MLX0659	Apple (APP)	Ljubljana	2006	Blossom peduncle
39	MLX0660	Apple (APP)	Ljubljana	2006	Blossom peduncle
49	MLX0662	Sweet cherry (CH)	Ankaran	2006	Blossom peduncle
41	MLX0663	Sweet cherry (CH)	Ankaran	2006	Blossom peduncle
50	MLX0664	Sweet cherry (CH)	Ankaran	2006	Blossom peduncle
51	MLX0665	Apricot (AP)	Strunjan	2006	Blossom peduncle
52	MLX0666	Apricot (AP)	Strunjan	2006	Blossom peduncle
53	MLX0667	Apricot (AP)	Strunjan	2006	Blossom peduncle
54	MLX0668	Apricot (AP)	Strunjan	2006	Blossom peduncle
55	MLX0670	Sweet cherry (CH)	Ljubljana	2006	Blossom peduncle
31	MLX0675	Apple (APP)	Resje	2006	Blossom peduncle
56	MLX0677	Apple (APP)	Resje	2006	Blossom peduncle
57	MLX0678	Apple (APP)	Resje	2006	Blossom peduncle
58	MLX0679	Apple (APP)	Resje	2006	Blossom peduncle
1	MLX11KB	Apricot (AP)	Čentiba	2004	Twig
4	MLX215P	Apple (APP)	Preddvor	2003	Young fruit
2	MLX218K	Apricot (AP)	Maribor	2003	Twig
7	MLX21K	Sweet cherry (CH)	Pince	2004	Mummified fruit
5	MLX3K	Apricot (AP)	Pijava Gorica	2004	Twig
8	MLX53P	Apple (APP)	Škofja Loka	2004	Twig
9	MLX63P	Apple (APP)	Zgornja Polskava	2004	Twig
10	MLX64P	Apple (APP)	Cerklje ob Krki	2004	Twig
11	MLX65P	Apple (APP)	Sevnica	2004	Twig
3	MLX67P	Apple (APP)	Pečarovci	2004	Twig
6	MLX71K	Plum (PL)	Rodež	2004	Mature fruit
59	2809G	Unknown (UNK)	Japan	n.a.	n.a.
60	7809D	Unknown (UNK)	Japan	n.a.	n.a.
61	1888D	Unknown (UNK)	Japan	n.a.	n.a.
62	1888G	Unknown (UNK)	Japan	n.a.	n.a.
63	CBS298.31	<i>Malus sylvestris</i> (MSYL)	Ireland	1931	Twig
64	CBS101505	<i>Prunus mume</i> (PMU)	Japan	1989	n.a.
65	CBS101507	Apricot (AP)	Australia	1978	n.a.

^a Isolates 1 to 11 were obtained from the Agricultural Institute of Slovenia, Plant Protection Department, Ljubljana; isolates 59 to 62 were a gift from Kazuaki Tanaka, Laboratory of Phytopathology of the Faculty of Agriculture and Life Science, Hirosaki University; and isolates 63 to 65 were obtained from CBS, Utrecht, The Netherlands.

^b Isolates except 59 to 65 are from Slovenia, their collection sites are presented in Figure 1.

M. laxa; (ii) to ascertain the usefulness of a multilocus marker system for studying genetic variability and to capture the population structure present within the species of *M. laxa* isolates from different hosts; and (iii) to study relationship and diversity among fungal isolates from different hosts. Here we report the results of AFLP analysis of 67 *M. laxa* isolates screened with 20 different AFLP primer combinations.

MATERIALS AND METHODS

Fungal isolates and culture conditions. In total, 67 single-spore isolates of *M. laxa* were included in our research (Table 1). The isolates were collected from a wide range of hosts comprising pome and stone fruits from different collection sites in Slovenia (Fig. 1, 60 isolates): 18 isolates from sweet cherry (CH) (*Prunus avium*), 17 from apricot (AP) (*Prunus armeniaca*), 14 from apple (APP) (*Malus domestica*), 4 from Japanese quince (QUI) (*Cydonia oblonga*), 3 from Japanese cherry (JCH) (*Prunus serrulata*), 2 from peach (PE) (*Prunus persica*), and 2 from plum (PL) (*Prunus domestica*). The remaining 7 isolates were acquired abroad: 4 isolates (2809G, 7809D, 1888D, and

1888G) of *M. laxa* isolated from unknown host plant were from Japan (Laboratory of Phytopathology of Faculty of Agriculture and Life Science, Hirosaki University, Kazuaki Tanaka), and 3 reference isolates from Ireland (apple), Japan (Japanese apricot: *Prunus mume*), and Australia (apricot) (CBS 298.31, CBS 101505, and CBS 101507) were obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Utrecht, the Netherlands.

All collected isolates were cultured from sporodochia that had developed on different parts of infected host tissue. They were identified on the basis of morphological traits and also with molecular diagnostic assay using species-specific primers according to the described methods (14,15) (data not shown). Isolates were maintained on potato dextrose agar (PDA) medium (5 g of potato extract, 20 g of glucose, and 14 g of agar per liter) (Biolife, Italiana) in petri dishes in the dark at 18 to 22°C. For long-term storage, fungi were grown on PDA slants at 4°C and fungal plugs from petri dishes were also stored in glycerol at -80°C.

Fungal culture and DNA isolation. Liquid fungal cultures were initiated by

inoculating 150 ml of malt extract broth (17 g/liter) (Merck KGaA, Darmstadt, Germany) in Erlenmeyer flasks with three 4-mm-diameter fungal plugs from PDA medium. They were incubated at 25°C, 100 rpm, for 5 days. Mycelia were harvested by vacuum filtering through Whatman filter paper and washed twice with sterile distilled water.

DNA was extracted from 0.5 g of fungal mycelium wrapped in foil to avoid losing the tissue during grinding with liquid nitrogen in a mortar. One milliliter of warm (68°C) CTAB extraction buffer was added to the ground mycelium (2% [wt/vol] CTAB, 100 mM Tris-HCl, pH 8, 20 mM EDTA, pH 8, 1.4 M NaCl, and 0.2% [vol/vol] β -mercaptoethanol) and incubated at 68°C for 2 h. After incubation, phenol:chloroform:isoamyl alcohol (25:24:1) extraction was performed. The emulsion was centrifuged for 15 min and the DNA-containing aqueous phase was again extracted with chloroform:isoamyl alcohol (24:1). DNA was precipitated with sodium acetate (3 M, pH 5.2) and ice-cold isopropanol. DNA was pelleted by centrifugation, rinsed with 70% ethanol, and resuspended in 30 μ l of TE buffer (10 mM Tris-

Fig. 1. Map of collection sites of 60 Slovenian *Monilia laxa* isolates (marked with asterisks) from different host plants. Names correspond to the names in Table 1. Host plants are marked with abbreviations after the isolate name: AP, apricot; APP, apple; CH, sweet cherry; JCH, Japanese cherry; PE, peach; PL, plum; and QUI, Japanese quince. In addition to Slovenian isolates, 4 Japanese isolates from unknown plants and 3 reference (CBS) *M. laxa* isolates were obtained: 2809G, 7809D, 1888D, 1888G, CBS298.31 (*Malus sylvestris*, Ireland), CBS101505 (*Prunus mume*, Japan), and CBS101507 (apricot, Australia).

HCl, pH 8.0, 1 M EDTA, pH 8.0). The extracted DNA was stored at -20°C until further use. Extracted DNA was electrophoresed on 0.8% agarose gel and stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) for quality control. DNA concentration was quantified by fluorimetry (Amersham Biosciences DynaQuant 200).

AFLP analysis. AFLP reactions were performed as originally described (37) with minor modifications including exchanging *Mse*I AT rich recognizing restriction enzyme for GC rich *Msp*I restriction enzyme (23).

Two hundred and fifty nanograms of fungal genomic DNA was digested with restriction enzymes *Eco*RI and *Msp*I (2.5 U of each enzyme, New England Biolabs) in a reaction volume of 40 μl at 37°C for 3 h. After digestion, 5 pmol of *Eco* and 50 pmol of *Msp* double-stranded adaptors were ligated (1 Weiss U of T4 DNA ligase) to the sticky ends of the genomic fragments in a final volume of 50 μl at 37°C for 3 h. Adaptors were obtained by mixing equimolar amounts of primers *Eco*RI linker1 (5' CTCGTAGACTGCGTACC 3')

and *Eco*RI linker2 (5' AATTGGTACG CAGTCTAC 3') for *Eco* adaptors and *Msp*I linker1 (5' GACGATGAGTCC TGAG 3') and *Msp*I linker2 (5' CGC TCAGGACTCAT 3') for *Msp* adaptors.

Five microliters of ligation reaction served as a template in the PCR preamplification step in a 50- μl volume with the following components: onefold PCR buffer, 1.5 mM MgCl_2 , 0.2 mM of each dNTP, 75 ng of *Eco*RI and *Msp*I preamplification primers without any selective nucleotide (*Eco*RI+0: 5' GACTGCGTAC CAATTC+0 3' and *Msp*I+0: 5' GAT GAGTCCTGAGCGG+0 3'), and 1 U of *Taq* DNA polymerase. Reactions were amplified in a GeneAmp PCR System 9700 (Perkin Elmer) repeating 20 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min.

The PCR products from the preamplification step were diluted 10-fold, and 2 μl of the dilution was used for selective amplification, using the same core primers as in the first amplification with an additional two arbitrary (selective) nucleotides at the 3' end. Selective reactions were performed

in 10 μl of PCR reaction mix with the same reaction components as for the pre-amplification step, except that 0.3 U of *Taq* polymerase was used and 50 ng of each selective primer, whereby *Eco*RI primers were Cy5 labeled at their 5' end to allow automated laser fluorescence analysis. The reaction was amplified with an initial touch down protocol: 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min for 12 cycles, in each cycle the annealing temperature was decreased by 0.7°C , followed by 23 cycles: 94°C 30 s, 56°C 30 s, and 72°C 1 min. Based on initial screening of 40 different primer combinations on 4 different fungal isolates, 20 primer combinations were chosen (combinations of 4 *Eco*RI [E] and 10 *Msp*I [M] primers) based on amplification profile intensity, number of amplified bands, and indicative polymorphism. The combinations of primers used for amplification were as follows: E-AC in combination with M-TA, M-GT, M-TC, M-CT, M-TG, and M-AG; E-GA in combination with M-CG, M-CT, M-AT, M-TA, M-AG, M-TC, and M-GT; E-GT in combination with M-CG, M-GA, and M-GC; and E-TC

Table 2. Characteristics of amplified fragment length polymorphism (AFLP) markers generated by 20 primer combinations in analysis of 67 *Monilinia laxa* isolates

Comb.	Primer pair		No. of all fragments	Fragment length	No. of polymorphic fragments	% Polymorphism	No. of fragments per isolate (min; max)	No. of polymorphic fragments per isolate (min; max)	No. of unique fragments in one isolate	No. of unique fragments in apple isolates	No. of unique fragments in other isolates
1	AC	TA	58	55-695	28	48.28	36;46	6;16	4	4	3
2	AC	GT	57	65-725	24	42.11	33;52	1;9	4	1	2
3	AC	TC	46	59-607	22	47.83	33;39	9;15	2	1	1
4	AC	CT	56	50-568	18	32.14	41;51	3;13	2	0	0
5	AC	TG	59	50-636	22	37.29	39;47	2;10	4	1	0
6	AC	AG	55	50-717	21	38.18	40;49	6;15	2	1	0
7	GA	CG	58	71-525	18	31.03	44;52	4;12	5	1	0
8	GA	CT	49	74-484	27	55.10	21;42	4;20	2	1	0
9	GA	AT	96	50-524	29	30.21	36;94	5;16	5	1	2
10	GA	TA	69	50-340	22	31.88	51;63	6;16	0	0	0
11	GA	AG	70	50-607	18	25.71	58;63	6;11	1	3	1
12	GA	TC	52	60-565	17	32.69	37;49	2;14	0	1	1
13	GA	GT	71	80-745	16	22.54	56;63	1;8	2	0	0
14	GT	CG	33	50-476	4	12.12	29;33	0;4	0	1	0
15	GT	GA	33	69-483	11	33.33	24;29	2;7	0	1	1
16	GT	GC	35	50-534	5	14.29	31;35	1;5	0	0	0
17	TC	TA	45	50-494	15	33.33	35;42	5;12	0	1	0
18	TC	GA	50	50-447	6	12.00	27;49	0;5	0	0	0
19	TC	AG	59	50-557	24	40.68	44;55	9;20	0	5	1
20	TC	CG	38	50-428	7	18.42	33;38	2;7	0	0	0
Sum			1,089		354				33	23	12
Average			54.5		17.7	32.51	37.4;51.8	3.7;11.8			

Table 3. Analysis of molecular variance (AMOVA) of 354 polymorphic amplified fragment length polymorphism (AFLP) loci among 67 isolates of *Monilinia laxa*

Source of variation	df	Variance components	Percentage of total variation	Φ -statistics	P
Among isolates from different hosts A (apple vs. all other)	1	0.08802	47.50	0.47496	<0.0001
Within groups of isolates	65	0.09730	52.50		
Among isolates from different hosts B (apple vs. apricot)	1	0.08550	44.24	0.44238	<0.0001
Within groups of isolates	31	0.10778	55.76		
Among isolates from different hosts C (apple vs. sweet cherry)	1	0.08962	47.03	0.47028	<0.0001
Within groups of isolates	31	0.10094	52.97		
Among isolates from different hosts D (sweet cherry vs. apricot)	1	0.00015	0.17	0.00167	0.3529
Within groups of isolates	34	0.09255	99.83		

in combination with M-TA, M-GA, M-AG, and M-CG (Table 2). The amplification reaction was mixed with an equal volume (10 μ l) of formamide loading buffer (5 mg

dextrane blue in 1 ml of formamide), denatured by heating to 94°C for 4 min, and the samples were immediately transferred onto ice. Five microliters of sample was

loaded onto 5% polyacrylamide denaturing gel (5% acrylamide-bisacrylamide 19:1, 1 $\times$ TBE, 7 M urea) and electrophoresed on an ALFexpressII (Amersham Biosciences) sequencer at limiting power 15W and constant temperature 55°C for 300 min. An external size standard ranging from 50 to 500 bp was run together with samples to allow further sizing of the fragments.

Band scoring and genetic data analysis. Allele Locator 1.03 (Amersham Biosciences) program was used for gel analysis. Fragments were sized against a 50- to 500-bp molecular standard, and fragments of the same size present in all isolates were used to correct for smiling effect of the gels.

Only strong and reliably amplified markers were scored as binary variables, either with presence (1) or absence (0). Monomorphic markers were excluded from further diversity assessment. The genetic similarity among isolates was calculated using Dice's similarity index (21). Cluster analysis was generated from the similarity matrix by the unweighted pair group method using the arithmetic averages (UPGMA) algorithm, with the "FIND" option enabled to detect all possible trees. The goodness of fit of the clustering to the data matrix was estimated by the COPH and MXCOPM subprograms of NTSYS, which calculate cophenetic values and perform matrix comparison analysis, respectively. All calculations were performed by the computer program NTSYSpc 2.02 (26). Branch support was performed by bootstrap analysis of 1,000 bootstrap replicates by the TreeCon program (36). Additionally, a principal coordinate analysis (PCO) based on Dice's similarity data matrix was also carried out, using NTSYS-pc program modules DCENTER, EIGEN, and MXPLOT to generate two-dimensional presentation.

Model-based clustering employing a Bayesian algorithm was applied to infer the genetic structure of the analyzed *M. laxa* isolates using Structure software (22). Ten independent runs of the program were performed by setting the number of clusters (K) from 1 to 10; each run consisted of a burn-in period of 10,000 iterations followed by 10⁵ MCMC (Monte Carlo Markov Chain) iterations, assuming an admixture model and correlated allele frequencies. For other settings, program defaults were used and no prior information was used to define the clusters. The most likely number of clusters (K) was chosen, based on the logarithmized probabilities of data Pr(X|K) for each value of clusters (K) and by ad hoc statistic ΔK (7).

An analysis of molecular variance (AMOVA) analysis was carried out using Arlequin software (29) to statistically test the existence of different groups within the species. Several groups of isolates were defined for testing the probable host spe-

Fig. 2. Unweighted pair group method using arithmetic averages (UPGMA) dendrogram based on 67 *Monilinia laxa* isolates and 354 amplified fragment length polymorphism (AFLP) polymorphic loci used to calculate Dice's coefficient of similarity. Numbers at nodes denote bootstrap values for 1,000 replicates, only values greater than or equal to 50% are shown. Numbers before isolate names are numbers from Table 1, abbreviation after isolate name denotes host plant: AP, apricot; APP, apple; CH, sweet cherry; JCH, Japanese cherry; PE, peach; PL, plum; QUI, Japanese quince; PMU, *Prunus mume*; MSYL, *Malus sylvestris*; and UNK, unknown. Foreign isolates are also marked with country name: AU, Australia; JP, Japan; and IRL, Ireland. In group II, only isolates from apple trees are present, while isolates from all other host plants are placed in group I.

cialization: (i) apple isolates (15 samples) and all other isolates (52 samples), (ii) apple isolates and sweet cherry isolates (18 samples), and (iii) apple isolates and apricot isolates (18 samples) (Table 1); and four different AMOVA tests were conducted (apple versus all other, apple versus apricot, apple versus sweet cherry, and sweet cherry versus apricot). The significance level for AMOVA calculations was 0.01 (Table 3).

RESULTS

AFLP analysis. Four isolates from different host plants (2 from apple, 1 apricot, and 1 sweet cherry) were initially analyzed for amplification pattern and tentative level of polymorphism for all 40 possible primer combinations with four different *EcoRI* primers (E-TC, E-AC, E-GA, and E-GT) and 10 *MspI* primers (M-CT, M-TA, M-GC, M-GA, M-TG, M-AG, M-TC, M-CG, M-AT, and M-GT). The unambiguity of the banding pattern, the total number of amplified markers, and the length range of amplified fragments were the main criteria for choosing particular primer combinations. Finally, 20 primer combinations (Table 2) were selected to screen genetic diversity in the whole sample of 67 isolates.

AFLP analysis of 67 different *M. laxa* isolates yielded a total of 1,089 well-spaced fragments from 20 chosen primer combinations. All bands were reproducible when fingerprints were repeated. The highest number of fragments (96) was amplified with primer combination E-GA+M-AT, while the fewest bands (33) were scored in two E-GT primer combinations with different M-selective nucleotides (M-CG, M-GA). On average, there were 54.5 AFLP markers per primer combination in the range from 50 to 745 bp. The highest number of polymorphic fragments (29) was detected by primer combination E-GA+M-AT, while primer pair E-GT+M-CG produced the fewest polymorphic bands (4) in the analyzed group of 67 isolates. The average percentage of polymorphism ranged from 12% for the primer combination E-TC+M-GA to 55.1% for the E-GA+M-CT combination. The main AFLP characteristics for 20 primer combinations are presented in Table 2.

Polymorphisms are noticed between isolates with an average of 17.7 polymorphic bands per primer pair. Consequently, we considered 354 polymorphic bands (32.5% of the total bands) for further analysis. On average, between 37.4 and 51.8 AFLP fragments per isolate were obtained (min, max), and between 3.7 and 11.8 polymorphic AFLP markers per isolate were scored (min, max) (Table 2).

Specific markers present only in one isolate, or within the fingerprints of the isolates grouped according to host, were discovered (apple versus all other isolates). For example, 23 fragments in size from 62

to 583 bp were detected exclusively in fingerprints of isolates from apple; the highest number of unique fragments, 5, was detected in primer combination E-TC+M-AG. Six primer combinations did not amplify any of the specific markers in apple isolates. Fewer, 12 unique markers, were also observed in the group of isolates from other host plants in length between 55 and 576 bp. The highest number, 6, was amplified with E-AC primers (3 with M-TA, 2 with M-GT, and 1 with M-TC). Only 4 fragments with E-GA (2 with M-AT, 1 with M-AG, 1 with M-TC) and 1 specific marker were amplified in two combinations (E-GT+M-GA and E-TC+M-AG).

Genetic distance analysis. Dice's similarity coefficient (21) averaged among all cultivars was 0.743. The highest similarity coefficient, 0.99, was between Japanese isolates 2809G (#59) and 7809D (#60), and the lowest, 0.434, was between an apricot (MLX11KB, #1) and an apple (MLX0660, #39) isolate.

Clustering analysis based on Dice's similarity coefficient and UPGMA clustering revealed two well-supported groups of isolates (Fig. 2). The larger group (group I) consisted of 54 isolates from stone fruits, quince, and 2 apple isolates (MLX64P, #10; and MLX0675, #31), and they clustered together at a bootstrap value of 86%. A second group (group II) contained 13 out of 15 isolates obtained from infected apple trees. High variability of isolates can be seen within the two clusters, but isolates were not further clustered based on host species or geographical origin, with the exception of clustering of four Japanese

isolates (2809G, #59; 7809D, #60; 1888D, #61; and CBS101505, #64). Clustering results were confirmed with a high co-phenetic correlation ($r = 0.94$) and by bootstrap values of some clusters.

Principal coordinate analysis (PCO) of the AFLP-based distance data was performed to provide additional differentiation. The first and second principal coordinates explained 29.6% and 11.1% of the total variation, respectively (Fig. 3). Similar to the UPGMA cluster analysis, the two-dimensional plot resulting from PCO separated the studied isolates into two main groups according to the origin of host plants, group I (right side of the figure, all nonapple isolates) and group II (left side of the figure, apple isolates). Two apple isolates (MLX0675, #31; and MLX65P, #11) did not cluster to either of the main groups. The first isolate was placed in the UPGMA dendrogram in group I, while isolate MLX65P (#11) still belonged to group II. The PCO figure placed apple isolate MLX64P (#10) in group I, as in the UPGMA dendrogram. Two apple isolates, one from Ireland (CBS298.31, #63) and one from Slovenia (MLX0660, #39) were placed slightly away from the primary apple isolates distribution. No other grouping related to the geographic or host origin was observed in the part of the PCO figure in which group I isolates were placed.

An alternative model-based method for inferring the number of different clusters of individuals without predefined structure within 67 isolates was also employed (22). The method also confirmed a clear separation of the two groups of isolates and sup-

Fig. 3. Principal coordinate analysis (PCO) among 67 *Monilinia laxa* isolates based on their Dice's genetic distances.

ported the existence of two clusters (group I and II) in the analyzed *M. laxa* isolates, in terms of host specific origin. The highest value of the estimated log probability of data $\Pr(X|K)$ did not indicate the number of clusters (Fig. 4A), but the results of ad hoc statistic ΔK (7) clearly arranged the

isolates into two groups (group I and group II) (Fig. 4B) composed of 65 isolates, as observed in UPGMA and PCO analysis. Two isolates from apple (MLX64P, #10; and MLX0675, #31) were not assigned as group II isolates, supporting UPGMA and the PCO analysis.

AMOVA analysis (Table 3) was performed separately for four different groups of isolates grouped according to host origin. In three cases, AMOVA resulted in highly significant ($P < 0.0001$) differences between apple isolates and groups of all other isolates, apricot isolates, and sweet cherry isolates, which was not the case for the AMOVA results between sweet cherry and apricot isolates (Table 3). The variance among isolates for the first three groups (A, B, and C; Table 3) accounted for a slightly higher percentage of the total variance (52.5 to 55.7%) than the variance among the groups (44 to 47%). For the last group compared (sweet cherry and apricot isolates, D; Table 3), the variance component among the groups contributed only 0.17% of the total variance, indicating the absence of genetic differentiation at this level and no indicative host specialization.

DISCUSSION

This is the first report of the use of AFLP techniques in diversity analysis of *M. laxa* isolates from different hosts. The AFLP technique can be regarded as a marker system of choice for revealing genetic diversity in closely related and morphologically similar plant pathogenic fungi and has been successfully applied as an effective taxonomy tool among different *Fusarium* (19,28), *Verticillium* (8), and *Botrytis* (33) species.

Twenty different AFLP primer combinations (Table 2) amplified on an average 54.5 fragments per combination, which is similar to reports on *Fusarium* sp. (6) and *V. albo-atrum* (24) isolates (51 and 42.8 fragments, respectively) but distinct from *B. tulipae* isolates (14.6 fragments) (33).

UPGMA and PCO clustering, AMOVA analysis, and a model-based clustering method clearly differentiated 13 out of 15 apple isolates from all other isolates (groups I and II), while isolates from other host plants did not show differentiation. This is the first report that *M. laxa* isolates from infected apple trees are distinguished from other host isolates by genetic polymorphisms and supports the biological specialization of apple isolates and formae specialis *mali* taxonomic classification. Classical plant pathological procedures for host specificity of different *Monilinia* species recognized three levels of specificity, described as polytropy, oligotrophy, and monotrophy. Most of the *Disjunctoriae* species are monotrophic pathogens, although some are oligotrophic. *Junctoriae* species are polytrophic in domesticated and wild *Rosaceae* and may also be found as saprophytes on other hosts, but there are reports on some level of host preference in *M. fructigena* and *M. laxa* (2). *M. fructicola* has a fairly similar host range to *M. laxa*, but they did not originally coexist in the same regions of the world. Biological

Fig. 4. The choice of the most likely number of clusters (K): **A**, plot of mean $\ln P(X|K)$ values ($\pm$ SD) over 10 independent runs for each K (K = 1 to 10); **B**, plot of ΔK values for each K based on the second order rate of change of the likelihood function with respect to K reveals two groups of isolates.

specialization, the ability of a particular fungal isolate to infect and reproduce only on a particular host or group of hosts is very well documented and studied in *F. oxysporum* causing wilt diseases. More than 150 host-specific formae speciales have been described for this species, with nuclear and mitochondrial gene genealogies for eight of them. AFLP polymorphism based phylogenies have also been constructed (1).

Eight formae speciales are found in grass powdery mildew (*Blumeria graminis*). Detailed study of ITS regions, β -tubulin gene, and AFLP analysis showed isolates grouping according to their principal host genus and not according to geographic origin. For the AFLP marker system, authors have reported on such high variation in banding pattern that it was difficult to identify common bands (38). The same observation was noted in our analysis, in which *Monilinia* isolates were not grouped according to geographic origin, with the exception of four Japanese isolates which clustered together in the dendrogram. However, clear separation of apple isolates presenting forma specialis *mali* from all other isolates was clearly seen and supported by the high bootstrap values of the UPGMA dendrogram, PCO analysis, AMOVA statistics, and Structure clustering.

AFLP seems to be the molecular tool of choice for fungal genetic variability analysis. The results of Vakalounakis et al. (35) also showed strong correlation between AFLP haplotypes and formae speciales. The mechanisms responsible for apple specialization in *M. laxa* are not known, but they probably occur at the site of infections – stigmas, since *M. laxa* f. sp. *mali* cannot infect other hosts (5), but other host isolates can easily be grown and maintained on apple fruits (20).

Gell et al. (12) assessed genetic diversity among diverse populations of *M. laxa* isolates from different fruit production areas of peach, almond, and apricot and two isolates of *M. fructigena* and *M. fructicola* in Spain, using RAPD markers. The examined isolates were grouped in three clusters as expected: *M. fructigena*, *M. fructicola*, and *M. laxa*; but a further grouping of *M. laxa* isolates according to sampling place, year, or host was not observed. Our results showed host restricted specialization of isolates, since apple *M. laxa* isolates are clearly distinguished.

This is the first report that molecular marker data support the existence of biological specialization of *M. laxa* isolates for apple trees and forma specialis *mali* taxonomic classification. These data, combined with additional analysis, will contribute to understanding the underlying mechanisms of *M. laxa* host specificity.

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LITERATURE CITED

- Baayen, R. P., O'Donnell, K., Bonants, P. J. M., Cigelnik, E., Kroon, L. P. N. M., Roebroeck, E. J. A., and Waalwijk, C. 2000. Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot disease. *Phytopathology* 90:891-900.
- Batra, L. R. 1991. World Species of *Monilinia* (fungi): Their Ecology, Biosystematics and Control. New York Botanical Garden, Mycological Society of America, Berlin, Stuttgart.
- Byrde, R. J. W., and Willetts, H. J. 1977. The brown rot fungi of fruit: Their Biology and Control. Pergamon Press, Oxford, UK.
- CABI/EPPO. 1991. Distribution maps of plant diseases. CAB International, Wallingford, GB.
- Celar, F., and Valič, N. 1999. The occurrence of fungus *Monilinia laxa* f. sp. *mali* in Slovenia. Page 514 in: 4th Slovenian Conference on Plant Protection. J. Maček, ed. Društvo za varstvo rastlin, Portorož, Slovenia.
- Chulze, S. N., Ramirez, M. L., Torres, A., and Leslie, J. F. 2000. Genetic variation in *Fusarium* section *Liseola* from no-till maize in Argentina. *Appl. Environ. Microbiol.* 66:5312-5315.
- Evanno, G., Regnaut, S., and Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* 14:2611-2620.
- Fahleson, J., Lagercrantz, U., Hu, Q., Steven-ton, L. A., and Dixelius, C. 2003. Estimation of genetic variation among *Verticillium* isolates using AFLP analysis. *Eur. J. Plant Pathol.* 109:361-371.
- Folkertsma, R. T., van der Voort, J. N., de Groot, K. E., van Zandvoort, P. M., Schots, A., Gommers, F. J., Helder, J., and Bakker, J. 1996. Gene pool similarities of potato cyst nematode populations assessed by AFLP analysis. *Mol. Plant-Microbe Interact.* 9:47-54.
- Fulton, C. E., and Brown, A. E. 1997. Use of SSU rDNA group-I intron to distinguish *Monilinia fructicola* from *M. laxa* and *M. fructigena*. *FEMS Microbiol. Lett.* 157:307-312.
- Fulton, C. E., van Leeuwen, G. C. M., and Brown, A. E. 1999. Genetic variation among and within *Monilinia* species causing brown rot of stone and pome fruits. *Eur. J. Plant Pathol.* 105:495-500.
- Gell, I., Larena, I., and Melgarejo, P. 2007. Genetic diversity in *Monilinia laxa* populations in peach orchards in Spain. *J. Phytopathol.* 155:549-556.
- Holst-Jensen, A., Kohn, L. M., Jakobsen, K. S., and Schumacher, T. 1997. Molecular phylogeny and evolution of *Monilinia* (Sclerotiniaceae) based on coding and noncoding rDNA sequences. *Am. J. Bot.* 84:686-701.
- Hughes, K. J. D., Fulton, C. E., McReynolds, D., and Lane, C. R. 2000. Development of new PCR primers for identification of *Monilinia* species. *EPPO Bull.* 30:507-511.
- Ioos, R., and Frey, P. 2000. Genomic variation within *Monilinia laxa*, *M. fructigena* and *M. fructicola*, and application to species identification by PCR. *Eur. J. Plant Pathol.* 106:373-378.
- Lee, D. H., Lee, S. W., Choi, K. H., Kim, D. A., and Uhm, J. Y. 2006. Survey on the occurrence of apple diseases in Korea from 1992 to 2000. *Plant Pathol. J.* 22:375-380.
- Majer, D., Mithen, R., Lewis, B. G., Vos, P., and Oliver, R. P. 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycol. Res.* 100:1107-1111.
- Mueller, U. G., and Wolfenbarger, L. L. 1999. AFLP genotyping and fingerprinting. *Trends Ecol. Evol.* 14:389-394.
- Mule, G., Gonzalez-Jaen, M. T., Hornok, L., Nicholson, P., and Waalwijk, C. 2005. Advances in molecular diagnosis of toxigenic *Fusarium* species: A review. *Food Addit. Contam.* 22:316-323.
- Munoz, Z., Moret, A., and Bech, J. 2008. Morphological and molecular characterization of *Monilinia* sp. isolates and pathogenicity on apple. *Agrociencia* 42:119-128.
- Nei, M., and Li, W. H. 1979. Mathematical model for studying genetic variation in terms of restriction endonuclease. *Proc. Natl. Acad. Sci. USA* 76:5269-5273.
- Pritchard, J. K., Stephens, M., and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155:945-959.
- Radišek, S., Jakše, J., and Javornik, B. 2001. Optimisation of amplified fragment length polymorphism (AFLP) analysis of hop wilt (*Verticillium albo-atrum* and *Verticillium dahliae*). *Acta Agric. Slov.* 77:139-146.
- Radišek, S., Jakše, J., and Javornik, B. 2006. Genetic variability and virulence among *Verticillium albo-atrum* isolates from hop. *Eur. J. Plant Pathol.* 116:301-314.
- Restrepo, S., Duque, M., Tohme, J., and Verdier, V. 1999. AFLP fingerprinting: An efficient technique for detecting genetic variation of *Xanthomonas axonopodis* pv. *manihotis*. *Microbiol. U.K.* 145:107-114.
- Rohlf, J. F. 1998. NTSYS: Numerical taxonomy and multivariate analysis system, version 2.02. Exeter Software, Setauket, NY.
- Rosendahl, S., and Taylor, J. W. 1997. Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP™. *Mol. Ecol.* 6:821-829.
- Schmidt, H., Niessen, L., and Vogel, R. F. 2004. AFLP analysis of *Fusarium* species in the section *Sporotrichiella* - evidence for *Fusarium langsethiae* as a new species. *Int. J. Food Microbiol.* 95:297-304.
- Schneider, S., Roessli, D., and Excoffier, L. 2000. Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.
- Sharma, R. L., and Kaul, J. L. 1987. Blossom blight and fruit rot of apple. *Indian J. Plant Pathol.* 5:205-206.
- Sharma, R. L., and Kaul, J. L. 1988. Susceptibility of apples to brown rot in relation to quantitative characters. *Indian Phytopathol.* 41:410-415.
- Sisto, A., Cipriani, M. G., Tegli, S., Cerbone-schi, M., Stea, G., and Santilli, E. 2007. Genetic characterization by fluorescent AFLP of *Pseudomonas savastanoi* pv. *savastanoi* strains isolated from different host species. *Plant Pathol.* 56:366-372.
- Staats, M., van Baaren, P., and van Kan, J. A. L. 2007. AFLP analysis of genetic diversity in populations of *Botrytis elliptica* and *Botrytis tulipae* from the Netherlands. *Eur. J. Plant Pathol.* 117:219-235.
- Travis, S. E., Maschinski, J., and Keim, P. 1996. An analysis of genetic variation in *Astragalus crennophyllax* var. *crenophyllax* a critically endangered plant, using AFLP markers. *Mol. Ecol.* 5:735-745.
- Vakalounakis, D. J., Doulis, A. G., and Klineromou, E. 2005. Characterization of *Fusarium oxysporum* f. sp. *radicis-cucumerinum* attacking melon under natural conditions in Greece. *Plant Pathol.* 54:339-346.
- Van de Peer, Y., and De Wachter, R. 1994. TREECON for Windows: A software package for the construction and drawing of evo-

- lutionary trees for the Microsoft Windows environment. *Comput. Appl. Biosci.* 10:569-570.
37. Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP - A new technique for DNA-fingerprinting. *Nucleic Acids Res.* 23:4407-4414.
 38. Wyand, R. A., and Brown, J. K. M. 2003. Genetic and forma specialis diversity in *Blumeria graminis* of cereals and its implications for host-pathogen co-evolution. *Mol. Plant Pathol.* 4:187-198.
 39. Zhang, Y., Bodmer, M., and Ruegg, J. 1991. Variation in sensitivity to sterol biosynthesis inhibitors (SBI) of different isolates of *Monilinia* species. *J. Plant Dis. Prot.* 98:317-322.