Journal of Plant Diseases and Protection, **117** (4), 168–172, 2010, ISSN 1861-3829.

Fluorescent AFLP fingerprinting of *Monilinia fructicola*

Fluoreszenz-AFLP-Fingerprinting von *Monilinia fructicola*

T. Gril[1], F. Celar[2], B. Javornik[1] & J. Jakse[1,*]

[1] Chair of Genetics, Biotechnology, Statistics and Plant Breeding, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

[2] Chair of Phytomedicine, Agricultural Engineering, Crop Production, Grassland and Pasture Management, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

[*] Corresponding author, e-mail jernej.jakse@bf.uni-lj.si

Received 19 February 2010; accepted 4 May 2010

Abstract

The fluorescent amplified fragment length polymorphism method (AFLP) has been successfully applied to one of the brown rot fungi species – *Monilinia fructicola*, which causes severe losses in stone fruit production. This is the first report on the use of AFLP methodology for studying genetic variability among different *M. fructicola* isolates. A total of 1256 scorable, well defined AFLP fragments were obtained with 20 primer pairs, of which 462 were polymorphic. An unweighted pair group (UPGMA) dendrogram was constructed based on Jaccard's similarity coefficient. A high similarity of the two *Malus* isolates from the USA was observed, while the other three *Prunus* isolates, from Spain, Japan and New Zealand, formed a second, more variable group. Comparison of AFLP fingerprinting data between *M. fructicola* and *M. laxa* revealed completely different banding patterns and demonstrated the suitability of the AFLP marker system for cross species differentiation of brown rot fungi.

Key words: brown rot fungi, molecular markers, quarantine organism, variability

Zusammenfassung

Die Amplifikationsfragment-Längenpolymorphismen-Methode (AFLP) wurde erfolgreich am Braunfäuleerreger *Monilinia fructicola* angewandt, der hohe Verluste im Steinobstbau verursacht. Dies ist der erste Bericht über die Verwendung der AFLP zur Charakterisierung der genetischen Variabilität unterschiedlicher *M.-fructicola*-Isolate. Insgesamt 1256 differenzierbare, definierte AFLP-Fragmente, von denen 462 polymorph waren, konnten mit 20 Primerpaaren gewonnen werden. Ein nicht gewichtetes Paargruppen-Dendrogramm wurde mit Hilfe des Jaccard-Ähnlichkeitskoeffizienten konstruiert. Eine hohe Ähnlichkeit der beiden *Malus*-Isolate aus den USA wurde beobachtet, während die drei *Prunus*-Isolate aus Spanien, Japan und Neuseeland eine zweite, variablere Gruppe bildeten. Ein Vergleich der AFLP-Fingerprints von *M. fructicola* und *M. laxa* ergab vollkommen unterschiedliche Banden und zeigte die Eignung des AFLP-Markersystems zur Artdiagnose von Braunfäuleerregern.

Stichwörter: Braunfäuleerreger, molekulare Marker, Quarantäneerreger, Variabilität

1 Introduction

The brown rot fungus *Monilinia fructicola* is polytrophic. Its main host range covers Rosaceae fruit trees but principally peaches and other *Prunus* spp, on which it occurs on twigs, blossoms and fruits (van Leeuwen 2000). It is occasionally found on sweet cherry and apple, as well as on ornamental crops. It is more frequently found on the Prunoideae subfamily than on Pomoideae (Batra 1991). Recent reports from Canada (Sholberg et al. 2003) mention incidences of *M. fructicola* also causing brown rot of wine grapes (*Vitis vinifera*).

M. fructicola is known to cause severe losses in stone fruits (*Prunus* spp.), particularly peaches, before and after harvest in North America (California), Australia (Murrumbidgee area) and Canada (Byrde and Willetts 1977). Post-harvest rots result in the most severe losses of yield, in excess of 30% (Boehm et al. 2001). In Europe, disease was not present before 2006, after that several reports of the first occurrence of *M. fructicola* appeared e.g. in Switzerland (Bosshard et al. 2006), Hungary (Petroczy and Palkovics 2006), the Czech Republic (Duchoslavova et al. 2007), Italy (Pellegrino et al. 2009), and Spain (De Cal et al. 2009) showing that it is sporadically spreading. Despite its frequent appearance, *Monilinia fructicola* is regulated as a quarantine organism in the European Union (Council Directive 2000/29/EC) and it is classified to A2 quarantine list proposed by EPPO.

Not only climatic conditions and the presence of a wide host range in Europe but also the fact that it seems to be more variable and adaptable than other European brown species favour the further spread of *M. fructicola*, which could seriously threaten peach, nectarine and apricot orchards in the EU. The spread of the disease to the western part of Europe will have serious consequences for trade, since Italy and Spain have the highest production of peaches and nectarines within the EU (van Leeuwen 2000).

Characterization of *M. fructicola* by classical plant pathology tools requires time, trained personnel and does not directly quantify the pathogen. Alternative approaches to characterizing the genotype are offered by a variety of molecular methods. There have been several reports on using only one part of the fungal genome (ribosomal genes) to distinguish, identify and ascertain the level of genetic variability of *M. fructicola*. Fungal isolates have been distinguished from two other closely related *Monilinia* species on the basis of the presence of a group-I-intron within 18 S rRNA genes (Fulton and Brown 1997). Differentiation between *M. laxa* and *M. fructicola* is possible by restriction digest of the amplified ITS1 region (Snyder and Jones 1999). Based on the variability of the same region, species-specific primers have also been constructed (Ioos and Frey 2000). Genetic variability studies have been concentrated on investigating variation among and within three *Monilinia* species using ITS sequence data, RAPD markers or arbitrarily primed PCR using microsatellite primers (Fulton et al. 1999; Snyder and Jones 1999). Unique RAPD fragments have also been used for specific primer design as a valuable identification tool (Schaad and Frederick 2002; Gell et al. 2007). The above mentioned RAPD markers and microsatellite primers amplify statistically distributed parts of the fungal genomes, compared to studies dealing with only one distinct DNA region. Based on AP-PCR results (Snyder and Jones 1999), little intraspecific variation among *M. fructicola* isolates was observed and the authors suggested the use of a

better genetic marker system to confirm this finding. One such marker system is the AFLP technique, which has been shown to be suitable for the analysis of genetic variation in fungi at species to strain level (Majer et al. 1996) and to reflect random variability over the complete fungal genome.

The main goal of the present study was an assessment of a fluorescent multi-locus AFLP marker system for variation analysis of *M. fructicola* isolates and comparison of AFLP fingerprints at the species level with related *M. laxa*.

2 Materials and methods

2.1 Fungal isolates and culture conditions

Since *M. fructicola* is a quarantine organism in Slovenia without known occurrence, all five studied isolates were acquired abroad. One isolate (Mfc07001, #1, isolated from *Prunus* spp.) was obtained from Instituto Agoforestal Mediterráneo, Universidad Politecnica de Valencia, and the remaining four from a commercial fungi collection Centraalbureau voor Schimmelcultures (CBS) in Utrecht, The Netherlands: one from Japan (CBS 101508, #2, isolated from *Prunus persica*); one from New Zealand (CBS 101510, #3, isolated from *Prunus armeniaca*) and two from the USA (CBS 329.35, #4, Virginia and CBS 203.25, #5, Washington both isolated from *Malus sylvestris*).

Long-term storage (glycerol or lyophilised) isolates were re-established by displacement to Petri dishes with potato dextrose agar medium (PDA) in the dark at 18–22°C. Two weeks later, the cultures were identified on the basis of classical morphological traits and also by using a simple molecular diagnostic tool with species-specific ITS rDNA primers (Ioos and Frey 2000).

Amplification of AFLP profiles were compared to three *Monilinia laxa* isolates from a study by Gril et al. (2008): isolate MLX0679 was a Slovenian isolate from apple tree, CBS 101507 was an Australian isolate from apricot and CBS 298.31 was an Irish isolate from *M. sylvestris*.

2.2 DNA isolation

Fungal isolates were grown for 5 days (25°C, 100 rpm) in 150 ml of malt extract broth [17 g l^{-1}] (Merck, Darmstadt, Germany) inoculated with three 4-mm-diameter fungal plugs. Fungal mycelium needed for DNA isolation was collected by vacuum filtering of the culture through Whatman filter paper. An amount of 0.5 g of harvested mycelium was wrapped in aluminium foil to avoid losing the material, and ground in a mortar with liquid nitrogen. Three ml of pre-warmed (68°C) CTAB extraction buffer [2% (w/v) CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl and 0.2% (v/v) ß- mercaptoethanol] were added to the completely crumbled mycelium. The suspension was distributed into four microcentrifuge tubes and incubated for 2 h at 68°C. Phenol:chloroform:isoamyl alcohol (PCI, 25:24:1) extraction was performed on samples by adding 700 µl of PCI solution, vigorous shaking (30 s) and centrifuging (Beckman centrifuge J2-HS centrifuge, rotor 18.1) for 15 min at 4°C at 13.000 rpm. After transferring the water phase into a new microcentrifuge tube, another extraction with 700 µl of chloroform:isoamyl alcohol solution (CI, 24:1) was performed, as described for PCI extraction. Afterwards, 70 µl of 3M Na-acetate (pH 5.2) and 700 µl of ice cold isopropanol was added to the supernatant for DNA precipitation and samples were further incubated at −20°C for 1 h. Precipitated DNA was collected by 15 min centrifugation (13.000 rpm, 4°C). The pelleted DNA was rinsed with 1 ml of 70% ethanol and resuspended in 30 µl of TE buffer [10 mM Tris-HCl pH 8.0, 1 M EDTA pH 8.0].

The integrity of the isolated DNA was checked on agarose gel electrophoresis (0.8% w/v) stained with ethidium bromide [0.5 µg ml^{-1}], the concentration was measured by fluorimetry (Amersham Biosciences DynaQuant).

2.3 AFLP analysis

AFLP reactions were repeated two times and performed as described in the variability analysis of *M. laxa* isolates (Gril et al. 2008). Briefly, primary template DNA was prepared in a two-step restriction-ligation reaction. Two hundred and fifty ng of fungal genomic DNA was digested with a frequent (*Msp*I) and rear (*Eco*RI) cutter (2.5 U of each enzyme, New England Biolabs) in a reaction volume of 40 µl for 3 h at 37°C, followed by ligation of 5 pmol of *Eco* and 50 pmol of *Msp* double stranded adaptors to the sticky ends of the previously cut genomic fragments using 1 Weiss U of T4 DNA ligase in a final volume of 50 µl at 37°C for 3 h. The acquired ligation reaction (5 µl) served as a template in a 50 µl PCR pre-amplification reaction with the following components: 1-fold PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 75 ng of *Eco*RI and *Msp*I pre-amplification primers without selective nucleotide (*Eco*RI + 0, *Msp*I + 0) and 1 U of Taq DNA polymerase. Fragments were preamplified with 20 PCR cycles in a GeneAmp PCR System 9700 (Applied Biosystems) using a temperature profile of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. The pre-amplification mixture was diluted 10-fold with sterile distilled water and 2 µl sub-samples were used for selective amplification, using the same core primers as in the pre-amplification, with an additional two selective nucleotides at the 3` end. The PCR amplification temperature profile was performed 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 min. The same reaction components as in the pre-amplification step were used, except for 0.3 U of Taq polymerase and 50 ng of Cy5-labelled *Eco*RI + 2 and unlabelled *Msp*I + 2 primers in a 10 µl PCR reaction mix. Twenty primer combinations (combinations of four *Eco*RI (E) and 10 *Msp*I (M) were chosen, based on the results from our *M. laxa* work (Gril et al. 2008) (Table 1).

The PCR products and added formamide loading buffer [5 mg dextrane blue in 1 ml of formamide] in 5 µl sub-samples were separated by electrophoresis using 5% polyacrylamide denaturating gel [5% acrylamide-bisacrylamide 19:1, 1x TBE, 7M urea] on an ALFexpressII (Amersham Biosciences) sequencer at a limiting power of 15W and constant temperature 55°C for 300 min. An external size standard ranging from 50 to 500 bp was run together with the samples to allow further sizing of the fragments.

2.4 Band scoring and genetic data analysis

Gels were manually analysed by using the Allele Locator 1.03 software package (Amersham Biosciences). Molecular standard fragments were added to the gel analysis to aid band sizing and all polymorphic bands in the resolved part of the gel were included in the analysis (up to 773 bp in the case of the E-AC+M-TC combination, Table 1). The presence (1) or absence (0) of all strong and reliably amplified markers in each of the five lanes was scored.

Monomorphic markers were excluded from further diversity assessment. The resulting binary matrices were prepared for transformation by the computer program NTSYSpc 2.02 (Rohlf 1998). The genetic similarity among isolates was calculated using Jaccard's similarity index, and a dendrogram was constructed (Fig. 1) using the unweighted pair group method with arithmetic averages (UPGMA) selected by the "FIND" option to detect all possible trees. Pairwise comparisons and cophenetic values were calculated by COPH and MXCOPM, subprograms of NTSYS.

Table 1: AFLP primer combinations used in the analysis of *Monilinia fructicola* isolates, number and range of length of all amplified DNA fragments, number of polymorphic fragments and percentage of polymorphism

Combination	Primer pair[1]		No. of all fragments	Fragment range length	No. of polymorphic fragments	Percentage of polymorphism
	E-	M-				
1	AC	TA	44	58 – 539	17	38.6
2	AC	GT	49	65 – 697	21	42.9
3	AC	TC	48	62 – 773	13	27.1
4	AC	CT	66	52 – 688	29	44.0
5	AC	TG	66	55 – 717	31	47.0
6	AC	AG	55	54 – 678	17	30.9
7	GA	CG	52	60 – 596	12	23.1
8	GA	CT	77	71 – 532	17	22.1
9	GA	AT	91	51 – 466	30	33.0
10	GA	TA	104	51 – 720	45	43.3
11	GA	AG	91	62 – 649	48	52.7
12	GA	TC	68	56 – 754	29	42.6
13	GA	GT	39	118 – 439	10	25.6
14	GT	CG	41	51 – 546	8	19.5
15	GT	GA	26	50 – 477	11	42.3
16	GT	GC	58	52 – 771	22	37.9
17	TC	TA	59	51 – 600	17	28.8
18	TC	GA	77	50 – 740	27	35.1
19	TC	AG	84	52 – 696	35	41.7
20	TC	CG	61	50 – 707	23	37.7
Sum			1256		462	
Average			62.8		23.1	35.8

[1] E for *Eco*RI and M for *Msp*I primer combination with two selective nucleotides.

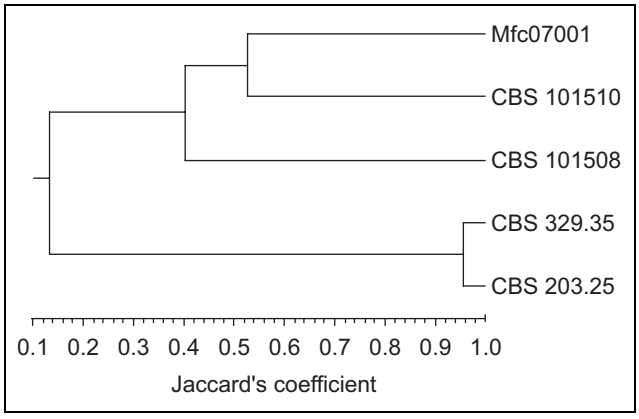

Fig. 1: UPGMA tree based on polymorphic AFLP markers for 5 isolates of *Monilinia fructicola*.

3 Results

AFLP analysis was conducted on DNA isolated from five fungal isolates of *M. fructicola* and a total of 1256 well defined bands were amplified. Primer combination E-GA+M-TA amplified the highest number of fragments (104), while the fewest bands (26) were observed with the E-GT+M-GA primer combination. On average, 62.8 AFLP markers were amplified per primer combination, in the size range from 50 to 773 bp.

A total of 462 polymorphic bands (36.78% polymorphism) were noticed across all five included isolates, which corresponded to an average of 23.1 polymorphic bands per primer combination. The highest number of polymorphic fragments (48) was scored by the E-GA+M-AG combination, while primer pair E-GT+M-CG amplified the fewest (eight). The average percentage of polymorphism ranged from 19.51% for the E-GT+M-CG combination to 52.75% for the E-GA+M-AG combination. The main AFLP characteristics for 20 primers are presented in Table 1.

Average Jaccard's similarity coefficient among the studied *M. fructicola* isolates was low, 0.309. The highest similarity coefficient, 0.95, was between the two US isolates (CBS 329.35 and CBS 203.25), the lowest 0.11 between the isolate from Spain (Mfc07001) and one from the USA (CBS 203.25).

UPGMA cluster analysis based on Jaccard's similarity coefficient showed that the isolates of *M. fructicola* separated into two main clusters (Fig. 1). The first cluster was formed by isolates from the USA (CBS 329.35, CBS 203.25). Isolates from Spain (Mfc07001), Japan (CBS 101508) and New Zealand (CBS 101510) formed the second cluster; although the Japanese isolate was more distinct from the other two. The clear clustering of *M. fructicola* isolates into two clusters according to the place of origin (US vs. other isolates) and host specificity (*Malus* vs. *Prunus*) demonstrates a possible geographic or host differentiation. A high cophenetic correlation coefficient, 0.998, between the clustering and the data matrix confirmed the clustering results.

The same primer combinations allow us to compare electropherograms of the same primer combinations of *M. fructicola* and *M. laxa* isolates (Gril et al. 2008) (Fig. 2). Completely different banding patterns, without noticeable fragments in common, were observed in the two *Monilinia* species in all 20 analyzed primer combinations.

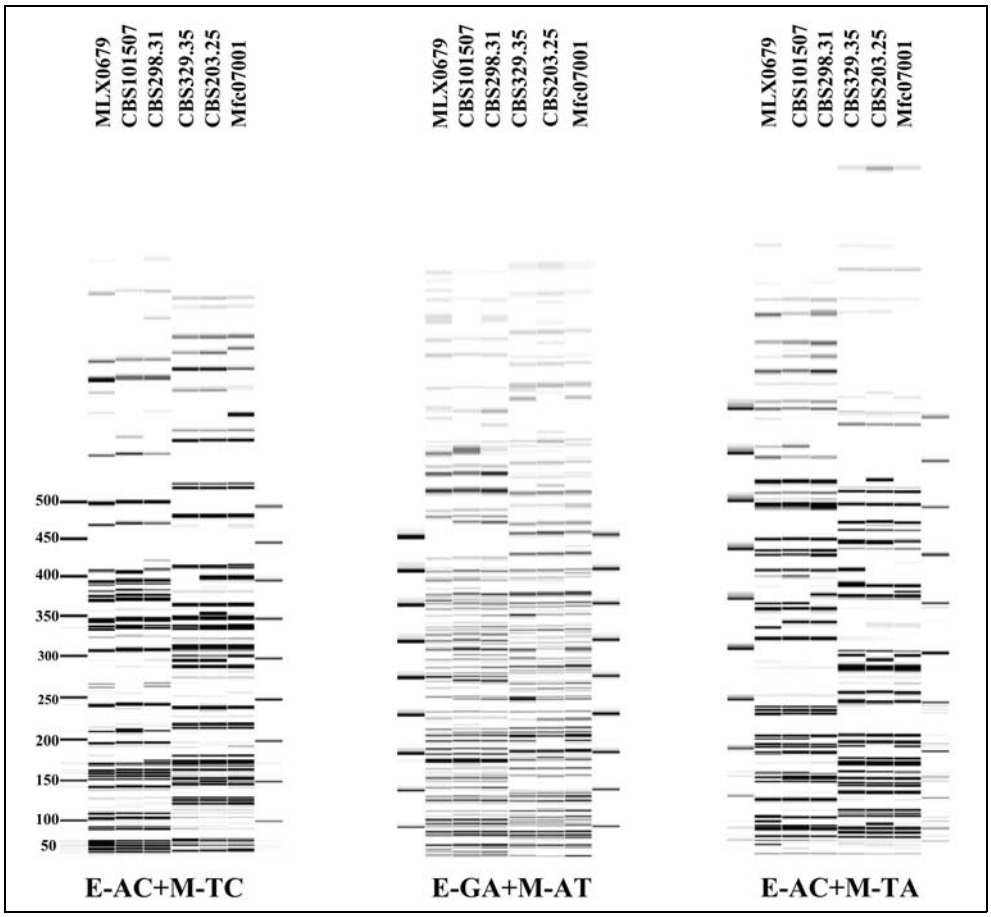

Fig. 2: Examples of completely different AFLP banding patterns of two brown rot fungi species, *Monilinia fructicola* (the last three columns of each electropherogram, isolates explained in Materials and methods section) and *M. laxa* (the first three columns of each electropherogram; isolates are MLX0679 – Slovenian isolate from apple tree, CBS 101507 – Australian isolate from Apricot and CBS 298.31 – Irish isolate from *Malus sylvestris*) (Gril et al. 2008). Three different amplification profiles are shown when three different selective primer combinations were used (E-AC+M-TC, E-GA+M-AT, and E-AC+M-TA).

4 Discussion

A number of recent plant pathogenic variability studies (Majer et al. 1996; Purwantara et al. 2000; Schnieder et al. 2001) have shown that AFLP molecular marker system can be successfully applied to reveal genetic variation of isolates or closely related fungal species that could not be distinguished by morphological or other molecular tools. The present study is the first report of the AFLP technique applied to *M. fructicola*. In contrast, a different marker system method, arbitrarily primed PCR, detected little interspecific variation in *M. fructicola* (Snyder and Jones 1999).

Our results also showed, as earlier observed by Majer et al. (1996), that there is a connection between the level of detected polymorphism and the chosen primer combinations. Comparison with the results obtained in an AFLP study of *M. laxa* isolates (Gril et al. 2008) shows that, in all cases, the primer pairs that detected the lowest number of total or polymorphic bands were the same in both studies (E-GT+M-GA for total bands and E-GT+M-CG for polymorphic bands).

When AFLP banding patterns of *M. laxa* (Gril et al. 2008) and *M. fructicola* were compared, completely different electropherograms were obtained (Fig. 2), suggesting that the AFLP technique is the appropriate tool for studying the closely related brown rot fungi group. AFLP is known to be extremely useful for genetic variability studies of closely related organisms, as has been shown on different fungal species (Majer et al. 1996).

The results of clustering based on Jaccard's coefficient clearly reveal that fungal isolates from different geographic origins are genetically distinct, since the neighbour-joining dendrogram clustered them into two well separated groups (Fig. 1). Two isolates from USA, obtained from *M. sylvestris*, were genetically very similar (Jaccard's pairwise similarity value 0.95) and clustered together, although they were from geographically different regions in the USA (Virginia and Washington). The clustering of the two USA isolates may be due to their host origin, since both isolates were collected from *Malus* trees. In the second cluster, geographically distant isolates (Spain, New Zealand and Japan) from *Prunus* hosts were clustered, also showing possible grouping according to host specificity. According to previous results of variability in SSU rDNA and based on similarities in RAPD patterns among *M. fructicola* isolates (Fulton et al. 1999), a possible common ancestor of isolates from New Zealand and Japan was proposed. Our results support these observations, since the two isolates from Japan and New Zealand (CBS 101510, CBS 101508) were placed together in the dendrogram in the second cluster (Fig. 1). Furthermore, AFLP polymorphisms clearly demonstrated the ability to distinguish *M. fructicola* isolates of different geographical origin.

We demonstrated that the fluorescent AFLP technique is sensitive and efficient for studying the genetic variation of *M. fructicola*. The results provide the basis for further studies of *M. fructicola* and resulting polymorphisms can be a source of new useful markers for diagnostic purposes.

Acknowledgements

The authors thank Dr. Ana Maria Perez from Instituto Agoforestal Mediterráneo, Universidad Politecnica de Valencia for kindly providing isolate Mfc07001. This work was supported by grant number 1000-06-310162 to Tjasa Gril and by P4-0077 from the Slovenian Research Agency.

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