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Use of RAPD and AFLP Markers to Identify Inter- and Intraspecific Hybrids of *Mentha*

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Abstract

Three controlled crosses were carried out involving *Mentha arvensis* and *Mentha spicata* [*M. spicata* CIMAP/C30 × *M. spicata* CIMAP/C33 (cv. Neera); *M. arvensis* CIMAP/C18 × CIMAP/C17 (cv. Kalka); and *M. arvensis* CIMAP/C17 × *M. spicata* CIMAP/C33]. The parents were subjected to random amplified polymorphic DNA (RAPD) analysis with 80 primers, and polymorphic primers were tested for detecting coinherited RAPD profiles among the progeny of these crosses. Of 50 seedlings tested from each intraspecific cross, all demonstrated dominant profiles with the selected RAPD primers except the detected hybrid from respective crosses. Coinherited markers could be detected with the primers OPJ 01, MAP 06, OPT 08, and OPO 20 for *M. arvensis*; OPJ 05, OPJ 14, OPO 19, and OPT 09 for *M. spicata*; and OPJ 07, OPJ 10, OPJ 11, OPJ 14, and OPO 02 for the cross *M. arvensis* × *M. spicata*. In our amplified fragment length polymorphism (AFLP) analysis, 40 coinherited marker fragments were identified for the cross involving *M. arvensis*, 32 for the cross involving *M. spicata*, and 41 for the interspecific cross between *M. arvensis* and *M. spicata*. In all crosses, similarity values between the parents were less than those between the parents and the hybrids. Although RAPD markers are generally considered dominant, it is possible to identify a few codominant markers that behave like restriction fragment length polymorphism (RFLP) markers. This molecular marker system may be helpful in rapidly screening out hybrids in crops where cross-pollination is a problem.

Plants belonging to the genus *Mentha* (Lamiaceae) show substantial variation in terms of their natural habitats, growth characteristics, and aromas. Essential oils produced by these plants are of particular economic interest because of the specific monoterpenes present in various ratios and combinations. Taxa of this genus have evolved in nature through natural hybridization and selection. Phylogenetic relationships among *Mentha* taxa have been reported by using random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analyses (Gobert et al. 2002; Khanuja et al. 2000). Fenwick and Ward (2001) used RAPD markers to analyze and identify 17 accessions of *Mentha* belonging to three species grown widely in the United States and also distinguished 8 peppermint accessions of different geographical origin. Regenerated shoots from somatic hybrids following protoplast fusions in vitro between *Mentha piperita* and *Mentha spicata* were characterized initially using a single RAPD primer (Krasnyanski et al. 1998).

Coinherited RAPD markers have also been reported from other plants. Mizuhiro et al. (2001) detected coinherited RAPD markers in interspecific somatic hybrids in *Primula* using OPK 07 primer. Comparison of the RAPD band patterns between an F_1 hybrid, *Papaver bracteatum*, and *Papaver pseudo-orientale* clearly showed that part of the bands of both parents were induced into an F_1 hybrid (Shoyama et al. 1998). RAPD patterns combined with southern hybridization were used to detect shared (inherited) genetic polymorphisms in the hybrid of *Paulownia fortunei* × *Paulownia tomentosa* (Kumar et al. 1999). Lee et al. (2005) investigated the segregation ratio of morphologic characters (4 qualitative and 15 quantitative) and RAPD markers in 55 interspecific hybrids between *Dianthus giganteus* and *Dianthus carthusianorum* and their parents to obtain complex inheritance information in the genus *Dianthus*. Using AFLP markers, Pooler et al. (2002) were able to verify 58 hybrids between *Tsuga caroliniana* and *Tsuga chinensis*, and one hybrid between *T. caroliniana* and *Tsuga canadensis*, but could find no definitive hybrids between *T. canadensis* and

T. chinensis. They have also discussed other marker systems, including RAPD, sequence characterized amplified regions (SCARs), internal transcribed spacers (ITS), and simple sequence repeats (SSRs).

The genus *Mentha* has attracted serious attention from plant breeders for genetic improvement of quality and yield traits. Selection programs for the isolation of desirable clones with improved monoterpene combinations and increased accumulation with superior agronomic traits are being pursued in several laboratories. Tyagi et al. (1992) published a critical review on work carried out in different places on the cytology, genetics, and breeding of commercially important *Mentha* species. However, due to the inherent problems with seed production (Caissard et al. 1996; Ono and Horio 1968; Ono and Ikeda 1969; Van Eck and Kitto 1990), conventional breeding programs face difficulties in making controlled crosses. Among the seeds that are set in bulk pollination, selection of actual hybrids through morphological trait and essential oil analyses remains a tedious proposition. The present investigation was aimed at identification of molecular markers to aid rapid screening for the detection of inter- and intraspecific hybrids in *Mentha* at an early seedling stage, so that only probable hybrids are further characterized in the breeding program.

Materials and Methods

Plant Material

Mentha spicata accessions CIMAP/C30 and CIMAP/C33 (cv. Neera) (Shasany et al. 2002), *Mentha arvensis* accessions CIMAP/C17 (cv. Kalka) and CIMAP/C18 (Khanuja et al. 2000), and the interspecific hybrid cv. Neerkalka between CIMAP/C17 and CIMAP/C33 (Patra et al. 2001) were obtained from the National Genebank for Medicinal and Aromatic Plants at the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. Seedlings for analysis of intraspecific hybridization were obtained by intentional cross-pollination (Patra et al. 2001). The plants to be cross-pollinated were planted in alternate rows in 5 m × 5 m plots with a row spacing of 60 cm. The florets of *M. arvensis* CIMAP/C18 were repeatedly dusted with manually collected pollen from *M. arvensis* CIMAP/C17, and florets of *M. spicata* CIMAP/C30 were dusted with pollen collected from *M. spicata* CIMAP/C33. The seeds collected from the female parents were sown in pots and the germinated seedlings were initially subjected to RAPD analysis. Seedlings showing coinherited RAPD fragments were then investigated by AFLP analysis.

Bulk seeds collected from the selectively pollen dusted mother plants of *M. arvensis* CIMAP/C18 and *M. spicata* CIMAP/C30 were sown separately in flat earthen pots. A total of 122 seedlings from *M. arvensis* CIMAP/C18 and 85 seedlings from *M. spicata* CIMAP/C30 were obtained. Fifty seedlings from each lot were randomly selected and transplanted to individual pots in the greenhouse. Plants of available interspecific hybrid Neerkalka (Patra et al. 2001) were raised vegetatively to serve as controls. Neerkalka was employed as a known genotype since it has been released as a variety and possesses characters of both *M. arvensis* and *M. spicata*, with a novel aroma of the essential oil (Patra et al. 2001; U.S. patent no. PP 12030).

Table 1. Primers synthesized in the laboratory

Primer no.	Primer sequence
MAP 01	AAATCGGAGC
MAP 02	GTCCTACTCG
MAP 03	GTCCTTAGCG
MAP 04	TGCGCGATCG
MAP 05	AACGTACGCG
MAP 06	GCACGCCGGA
MAP 07	CACCCTGCGC
MAP 08	CTATCGCCGC
MAP 09	CGGGATCCGC
MAP 10	GCGAATTCCG
MAP 11	CCCTGCAGGC
MAP 12	CCAAGCTTGC
MAP 13	GTGCAATGAC
MAP 14	AGGATACGTG
MAP 15	AAGATAGCGG
MAP 16	GGATCTGAAC
MAP 17	TTGTCTCAGG
MAP 18	CATCCCGAAC
MAP 19	GGACTCCACG
MAP 20	AGCCTGACGC

DNA Isolation and Polymerase Chain Reaction Amplifications

DNA was isolated from leaf tissue (1 month after transplanting in the main field from the parent plants and after germination from the seedlings) according to the protocol described by Khanuja et al. (1999) and quantified by fluorimetry using a DyNa Quant 200 fluorometer (Amersham Biosciences, Buckinghamshire, UK). Polymerase chain reactions (PCRs) for RAPD analysis were carried out in 25 µl volume. Each reaction tube contained 25 ng of DNA, 0.2 U of *Taq* DNA polymerase (Genei, Bangalore, India), 100 µM of each dNTP, 1.5 mM $MgCl_2$, and 5 pmol of a decanucleotide primer. The amplifications were carried out by using a DNA engine thermal cycler (MJ Research, Waltham, MA) programmed at 94°C for 5 min, followed by 45 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min, and stored at 4°C (Khanuja et al. 2000). The amplified products were separated on a 1.2% agarose gel containing 0.5 µg/ml of ethidium bromide and photographed with Image Master VDS (Amersham Biosciences, Buckinghamshire, UK). The bands were analyzed using Image Master 1D elite software (version 3.00; Amersham Biosciences, Buckinghamshire, UK). Custom-made decanucleotide primers (MAP01 to MAP20) were synthesized (Table 1) in the laboratory on a 392 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). Three other sets of primers were used: commercial kits OPJ, OPO, and OPT, each consisting of 20 random decamer primers (Operon Technologies, Alameda, CA). The amplifications with primers generating the coinherited

fragments were repeated three times for all the parents and the hybrids.

AFLP Analysis

Genomic DNA was restricted with two restriction endonucleases, *Eco*RI and *Tru*9I (an isoschizomer of *Mse*I), and double-stranded adaptors were ligated to the ends of DNA fragments, generating template DNA for subsequent PCR amplifications (preselective followed by selective). Restriction and ligation reactions were carried out simultaneously in a single reaction tube (Vos et al. 1995). To carry out the reaction, an enzyme master mix for 10 reactions was prepared containing 1 μl 10× T_4 DNA ligase buffer, 1 μl 0.5 M NaCl, 0.5 μl 1 mg/ml BSA, 1 μl *Tru*9I (10 U/μl), 4.25 μl *Eco*RI (12 U/μl), 0.5 μl T_4 DNA ligase (20 U/μl high concentration), and 1.75 μl water. The restriction-ligation reaction consisted of 300 ng of DNA (5.5 μl), 1 μl 10× T_4 DNA ligase buffer, 1 μl 0.5 M NaCl, 0.5 μl 1 mg/ml BSA, 1 μl *Mse*I adaptors (Applied Biosystems), 1 μl *Eco*RI adaptors (Applied Biosystems), and 1 μl enzyme master mix, as described above. The reaction mix was incubated overnight at room temperature and subsequently diluted 20-fold with $T_{10}E_{0.1}$ (10 mM Tris and 0.1 mM EDTA) buffer.

The ligated adaptors served as primer binding sites for low-level selection in preselective amplification of the restriction fragments. The *Mse*I complementary primer had a 3′-C and the *Eco*RI complementary primer a 3′-A. Only the genomic fragments having an adaptor on each end amplified exponentially during PCR. The preselective amplification mixture was prepared by adding 4 μl of 20-fold diluted DNA from the restriction-ligation reaction, 0.5 μl AFLP preselective primer (*Eco*RI, Applied Biosystems), 0.5 μl AFLP preselective primer (*Mse*I, Applied Biosystems), and 15 μl AFLP core mix. The preselective amplification was carried out in a thermal cycler programmed at 72°C for 2 min, followed by 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, 60°C for 30 min, and finally incubated at 4°C. The preamplified DNA was diluted 20-fold with $T_{10}E_{0.1}$ buffer and selective amplifications were carried out by using different *Mse*I and *Eco*RI primer combinations (Applied Biosystems). Primers chosen for the amplifications were from 16 available AFLP selective primers (8 fluorescently labeled *Eco*RI primers and 8 unlabeled *Mse*I primers). The *Eco*RI primers contained three selective nucleotides with the sequence 5′-[Dye-Primer-Axx]-3′, while the *Mse*I primers had the selective nucleotides starting with C, that is, 5′-[Primer-Cxx]-3′.

The "explorer" gel for all 64 reactions was run with accession *M. arvensis* (CIMAP/C17) to determine the most responsive primer pairs (generating more number of fragments) for the *Mentha* genome. Multiplexing of PCRs was designed to set up all 64 (8 × 8) reactions in 24 tubes. For selective amplification, the reactions were set up as follows: 3 μl of 20-fold diluted preselective amplification reaction product, 15 μl AFLP core mix, 1 μl *Mse*I primer 5′-[Primer-Cxx]-3′, 1.5 μl *Eco*RI primers 5′-[Dye-Primer-Axx]-3′ (0.5 μl of three *EcoR*I primers each were pooled here). Selective amplification was carried out in a thermal cycler programmed at 94°C for 2 min, followed by 10 cycles of 94°C for 20 s, 66°C (−1°C/cycle) for 30 s and 72°C for 2 min, and 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min with a subsequent hold for 30 min at 60°C and final incubation at 4°C. The samples were loaded on a 5% polyacrylamide gel on an ABI Prism 377 DNA sequencer (Applied Biosystems). The selective amplification reaction product (3 μl) was mixed with 4 μl of loading buffer (ROX500 size standard [10%], blue dextran [10%], deionized formamide [80%]) from which 1.5 μl was finally loaded on the gel. Six primer combinations—E-ACA/M-CTC, E-AGG/M-CTG, E-AGC/M-CTG, E-ACA/M-CAG, E-AAG/M-CTA, E-AGC/M-CTA—generating more fragments than others, were chosen after analyzing the explorer gel. All the accessions were then subjected to selective amplification with these primer combinations. Selective amplification products obtained with primers labeled with three different fluorescent dyes were pooled together along with a loading buffer containing a size standard for loading, as stated above. For AFLP reactions, the AFLP amplification modules and the guidelines supplied by Applied Biosystems were used.

Data Analysis

All the amplified fragments obtained in the RAPD profiles of the parents and the hybrids in three repetitions were scored for the primers showing coinherited patterns to estimate the percentage of reproducibility. The deviations were recorded by identifying the presence or absence of fragments in second and third repetitions compared to the fragments in the initial amplification. The AFLP profiles were analyzed using GeneScan analysis software (version 3.1; Applied Biosystems). For quantification of similarity, pairwise comparisons of banding patterns were made by calculating indices of similarity using the matching coefficient method of Nei and Li (1979).

Results and Discussion

In earlier studies, the parent plants were subjected to RAPD analysis (Khanuja et al. 2000; Shasany et al. 2002) and the most highly polymorphic primers were used to analyze the randomly selected seedlings described above. For each cross, a seedling showing polymorphisms common to both parents was further analyzed along with Neerkalka to detect coinherited markers, since coinheritance of RAPD bands had been reported in Neerkalka (Patra et al. 2001).

Earlier we detected a polymorphism of about 17% in RAPD profiles of *M. arvensis* parents (CIMAP/C17 and CIMAP/C18) and 67% between *M. arvensis* (CIMAP/C17) and *M. spicata* (CIMAP/C33), the parents for Neerkalka (Khanuja et al. 2000), using 60 primers. The percentage polymorphism was similar even when analyzed with an additional set of 20 primers (OPO 01 to OPO 20). Similarly the polymorphism among *M. spicata* parents (CIMAP/C30 and CIMAP/C33) was estimated to be 58% with 80 primers (Shasany et al. 2002). From the RAPD profiles analysis, 20 random primers—MAP 06; OPJ 01, 05, 07, 10, 11, 14, 17, 19; OPO 01, 02, 05, 08, 19, 20; OPT 03, 04, 08, 09,

Table 2. Presence and absence of bands in the parents and the hybrid of *M. arvensis* showing a coinheritance pattern

Primer	Band size (bp)	*M. arvensis* CIMAP/C 18	*M. arvensis* CIMAP/C 17	*M. arvensis* hybrid
OPJ 01	1600	+	+	+
	1400	+	−	+
	1250	−	+	−
	1200	+	+	+
	980	−	+	+
	900	−	+	+
MAP 06	1510	+	−	+
	1325	+	−	+
	1100	−	+	+
	975	+	+	+
	840	−	+	+
	750	+	+	+
OPT 08	1550	+	+	+
	750	−	+	+
	626	+	−	+
	590	−	+	−
OPO 20	1900	+	−	+
	1600	−	+	+
	1400	+	−	−
	1320	−	+	+
	1200	+	+	+
	950	+	+	+
	800	+	+	+

Table 3. Presence and absence of bands in the parents and the hybrid of *M. spicata* showing a coinheritance pattern

Primer	Band size (bp)	*M. spicata* CIMAP/C 30	*M. spicata* CIMAP/C 33	*M. spicata* hybrid
OPJ 05	1380	+	−	−
	1200	+	−	+
	1070	+	+	+
	800	−	+	−
	610	−	+	+
	325	+	−	+
	300	+	−	+
OPJ 14	1200	−	+	+
	780	−	+	−
	620	+	−	+
	520	+	+	−
	500	−	+	−
OPO 19	1400	+	−	−
	1200	−	+	+
	1100	+	−	−
	1050	+	−	−
	1025	−	+	−
	830	−	−	+
	750	+	−	+
	550	+	−	+
	500	+	−	−
	480	−	+	+
OPT 09	1400	−	+	+
	1120	+	−	+
	800	+	−	−
	600	+	−	−
	500	+	−	+
	475	+	−	−
	300	+	+	+

19—were selected for further analysis based on high polymorphism. Of these 20 primers, 4 (OPJ 01, MAP 06, OPT 08, and OPO 20) demonstrated a clear coinheritance pattern for RAPD marker bands in the intraspecific hybrid of *M. arvensis* (Table 2 and Figure 1), whereas dominant inheritance patterns were observed for the rest. Similarly, in the intraspecific hybrid of *M. spicata*, profiles with markers inherited from both parents were detected by primers OPJ 05, OPJ 14, OPO 19, and OPT 09 (Table 3 and Figure 2). For Neerkalka (*M. arvensis* × *M. spicata*), in addition to the reported primer MAP 03 (Patra et al. 2001), other primers for which the coinherited profiles could be detected were OPJ 07, OPJ 10, OPJ 11, OPJ 14, and OPO 02 (Table 4 and Figure 3). Similarity indices between inter- and intraspecific hybrids were calculated from the RAPD profiles generated by primers revealing coinherited banding patterns. For instance, the two parents of intraspecific hybrid of *M. arvensis* (CIMAP/C18 and CIMAP/C17) shared a similarity of 50%, but individually they showed 74% and 79%

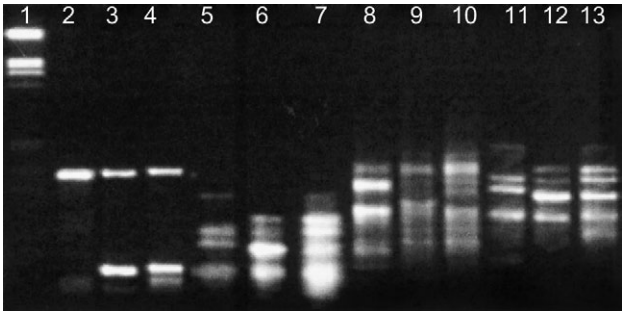

Figure 1. Coinheritance pattern in *M. arvensis*. Lane 1: Marker λ Hind III + *Eco*RI. Lanes 2–4: RAPD profiles of *M. arvensis* CIMAP/C18, *M. arvensis* CIMAP/C17, and the hybrid, respectively, with OPJ 01. Lanes 5–7: Profiles with MAP 06. Lanes 8–10: Profiles with OPT 08. Lanes 11–13: Profiles with OPO 20.

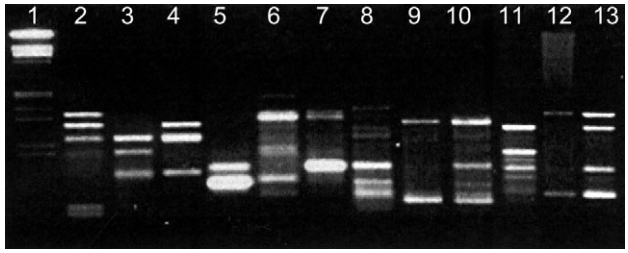

Figure 2. Coinheritance pattern in *M. spicata*. Lane 1: Marker λ Hind III + *Eco*RI. Lanes 2–4: RAPD profiles of *M. spicata* CIMAP/C30, *M. spicata* CIMAP/C33, and the hybrid, respectively, with OPJ 05. Lanes 5–7: Profiles with OPJ 14. Lanes 8–10: Profiles with OPO 19. Lanes 11–13: Profiles with OPT 09.

Table 4. Presence and absence of bands in the parents and the hybrid of *M. arvensis* × *M. spicata* showing a coinheritance pattern

Primer	Band size (bp)	*M. arvensis* CIMAP/C17	*M. spicata* CIMAP/C 33	Neerkalka
OPJ 07	970	−	+	+
	930	+	−	+
	850	+	+	+
	650	+	−	+
	600	−	+	+
	550	−	+	+
	325	−	+	−
OPJ 10	1100	−	+	+
	1000	+	−	+
	830	+	−	+
	775	−	+	+
	610	−	+	+
OPJ 11	1200	+	−	−
	1050	−	+	+
	950	+	−	−
	800	+	+	+
	750	−	+	+
	650	−	+	+
	475	+	−	+
OPJ 14	1275	+	+	+
	950	−	+	+
	800	+	−	−
	475	+	−	+
OPO 02	1300	+	−	−
	1200	−	+	+
	950	+	+	+
	775	+	−	−
	700	+	+	+
	550	−	+	+
	400	+	−	+

similarity, respectively, with the common hybrid. Similarly, in *M. spicata*, the two parents, CIMAP/C30 and CIMAP/C33, were only 20% similar, but the hybrids between them shared 56% and 49% similarity to the parents, respectively. Neerkalka, the interspecific hybrid, was similarly observed to be 58% closer to *M. arvensis* and 80% closer to *M. spicata*, whereas the two parents shared only 26% similarity.

Polymorphisms revealed through the RAPD technique may be caused by a number of events, such as a deletion eliminating the primer binding site, an insertion making a fragment too large for polymerization, nucleotide substitutions in the primer annealing site(s) leading to failure of polymerization, or a small addition or deletion leading to larger or smaller fragments, respectively. Though most of the time (95%) RAPDs behave as dominant markers (Fritsch and Rieseberg 1992; Williams et al. 1990), this technique can still be used to identify primers showing hybrid profiles with markers from both parents (coinherited markers) through which a large number of genotypes could be screened more quickly and cheaply than with other techniques. In our study, all seedlings other than the hybrids detected through the coinherited RAPD markers tested were found to be similar with the female parent.

Often RAPD markers have been considered as undependable due to their low reproducibility for being random

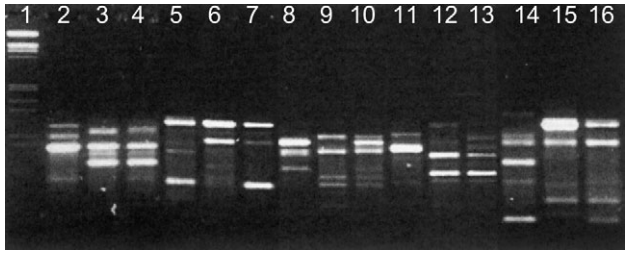

Figure 3. Coinheritance pattern in *M. arvensis* × *M. spicata* hybrid. Lane 1: Marker λ Hind III + *Eco*RI. Lanes 2–4: RAPD profiles of *M. arvensis* CIMAP/C17, *M. spicata* CIMAP/C33, and the hybrid, respectively, with OPJ 07. Lanes 5–7: Profiles with OPJ 10. Lanes 8–10: Profiles with OPJ 11. Lanes 11–13: Profiles with OPJ 14. Lanes 14–16: Profiles with OPO 02.

and because they are based on small nucleotide stretches (a decamer). For the detection and characterization of hybrids, researchers tend to employ more precise markers like RFLPs, AFLPs, etc. Since RAPD markers are not believed to reveal the codominant banding patterns, their utilization in hybridization programs is rare. Our results with *Mentha* species clearly indicate that RAPD markers can establish hybridity with great accuracy not only in interspecific clones but also in the intraspecific hybrid populations. As reviewed earlier, most of the hybrids detected using RAPD markers are interspecific in nature. This may be explained as expectance of higher polymorphism in interspecific rather than intraspecific hybridizations. So the prerequisite to identify coinherited markers for hybrid detection in intraspecific hybridization will be to screen large numbers of polymorphic markers between the parents. The extent of reproducibility of these coinherited RAPD markers was high in repeated experiments (98.04% and 99.29% for *M. arvensis* and *M. spicata* intraspecific hybrids, respectively, and 100% for interspecific hybrids), supporting the justification of using RAPD as a dependable marker with PCR conditions. Krasnyanski et al. (1998) identified and characterized *M. piperita* × *M. spicata* hybrid plants generated through somatic hybridization using the RAPD technique. After differentiating the parental DNA profiles amplified by the OPP-7 primer, the presence of distinct parental bands in the individual patterns of all hybrids, each of separate callus origin, confirmed their hybrid status. However, to establish the comparative advantage or disadvantage of RAPD, we further analyzed the above intraspecific hybrids, as well as the interspecific hybrid Neerkalka, through AFLP characterization.

To achieve this, we developed the "explorer" AFLP pattern by using 64 (8 *Eco*RI × 8 *Mse*I) combinations of primers and selected six paired combinations based on maximizing the polymorphisms observed in the initial explorer gel analysis with GeneScan software. The multiplexing of six paired combinations generated AFLP patterns for the parents and hybrids, which were further analyzed for coinheritance in relation to the parents.

Table 5. Similarity indices calculated through AFLP analysis for the intraspecific cross of *Mentha arvensis*

M. arvensis CIMAP/C 18	*M. arvensis* CIMAP/C 17	Hybrid
1.00		
0.58	1.00	
0.67	0.69	1.00

Table 7. Similarity indices calculated through AFLP analysis for the interspecific cross of *Mentha arvensis* and *Mentha spicata*

M. arvensis CIMAP/C 17	*M. spicata* CIMAP/C 33	Hybrid
1.00		
0.33	1.00	
0.48	0.43	1.00

Of the 237 bands obtained from the *M. arvensis* parental genotypes and the intraspecific hybrid, 51 were observed to be polymorphic, 80 monomorphic, and 106 unique (37 for *M. arvensis* CIMAP/C18, 46 for *M. arvensis* CIMAP/C17, and 23 for the hybrid). While 58% similarity was observed between the two *M. arvensis* parents (CIMAP/C17 and CIMAP/C18), the hybrid was much closer to both of these genotypes (67% and 69%, respectively) (Table 5). Of the 266 bands obtained in *M. spicata*, 42 were polymorphic, 80 monomorphic, and 144 unique (107 for *M. spicata* CIMAP/C30, 29 for CIMAP/C33, and 8 for the hybrid). Although the two parents showed similarity to only 50%, the hybrid showed 57% similarity to the female parent (*M. spicata* CIMAP/C30) and 76% to the pollen parent (*M. spicata* CIMAP/C33) (Table 6). In the case of the interspecific hybrid (Neerkalka) between *M. arvensis* CIMAP/C17 and *M. spicata* CIMAP/C33, of 362 bands obtained, 42 were polymorphic, 34 monomorphic, and 154 unique (56 for *M. arvensis* CIMAP/C17, 67 for *M. spicata* CIMAP/C33, and 31 for Neerkalka). As expected, the similarity between the two different species was low (33%), but the similarity between *M. arvensis* CIMAP/C17 and the hybrid was 48%, and that between *M. spicata* CIMAP/C33 and the hybrid was 43% (Table 7). In all the crosses, similarity values between the parents were understandably less than the similarity of the hybrids with their parents. As indicated in Tables 8, 9, and 10, respectively, 40 coinherited marker fragments were identified for the *M. arvensis* hybrid, 32 for the *M. spicata* hybrid, and 41 for the interspecific hybrid.

In the case of interspecific hybridization, RAPD may provide coinherited markers representing distinct genomic combinations in the hybrids. The frequency of coinheritance patterns in *Mentha* intraspecific hybrids was comparable to the interspecific hybrids. Natural interspecific hybridization occurs with high frequency in *Mentha*, both in wild populations and in cultivation. Most hybrids are sterile or subfertile, but vegetative propagation enables them to persist. Complex hybrid populations may arise, and if they are subfertile, may cross with parental or nonparental species. This leads to a large diversity of chromosome numbers (24 to 120), and much of the taxonomy of *Mentha* has been complicated by hybridization, by a high morphologic polymorphism, as well as by polyploidy and vegetative propagation (Gobert et al. 2002). Considering the complexity in this genus, the differences in the similar genomes that were detected through this

Table 6. Similarity indices calculated through AFLP analysis for the intraspecific cross of *Mentha spicata*

M. spicata CIMAP/C 30	*M. spicata* CIMAP/C 33	Hybrid
1.00		
0.50	1.00	
0.57	0.76	1.00

Table 8. Coinheritance pattern of the AFLP marker bands in *Mentha arvensis*

Fragment size (bp)	*M. arvensis* CIMAP/C 18	*M. arvensis* CIMAP/C 17	Hybrid
*Eco*RI ACA, *Mse*I CTC			
105	−	+	+
127	−	+	+
139	−	+	+
196	−	+	+
200	−	+	+
234	−	+	+
258	−	+	+
314	+	−	+
324	+	−	+
327	−	+	+
439	−	+	+
*Eco*RI AGG, *Mse*I CTG			
124	−	+	+
182	−	+	+
231	+	−	+
240	−	+	+
244	−	+	+
277	+	−	+
287	−	+	+
475	−	+	+
*Eco*RI AGC, *Mse*I CTG			
126	−	+	+
218	−	+	+
261	+	−	+
282	+	−	+
*Eco*RI ACA, *Mse*I CAG			
109	−	+	+
131	+	−	+
142	−	+	+
144	+	−	+
146	−	+	+
181	−	+	+
248	+	−	+
253	+	−	+
293	+	−	+
317	+	−	+
336	+	−	+
411	−	+	+
448	+	−	+
*Eco*RI AGC, *Mse*I CTA			
121	+	−	+
182	+	−	+
208	+	−	+
218	−	+	+

Table 9. Coinheritance pattern of the AFLP marker bands in *Mentha spicata*

Fragment size (bp)	*M. spicata* CIMAP/C 30	*M. spicata* CIMAP/C 33	Hybrid
*Eco*RI ACA, *Mse*I CTC			
154	−	+	+
174	+	−	+
175	+	−	+
185	+	−	+
343	+	−	+
*Eco*RI AGG, *Mse*I CTG			
181	−	+	+
183	−	+	+
240	−	+	+
275	+	−	+
276	−	+	+
286	−	+	+
*Eco*RI AGC, *Mse*I CTG			
110	−	+	+
119	+	−	+
166	+	−	+
173	−	+	+
180	−	+	+
306	−	+	+
310	−	+	+
*Eco*RI ACA, *Mse*I CAG			
101	+	−	+
249	+	−	+
252	−	+	+
264	−	+	+
278	+	−	+
291	+	−	+
303	+	−	+
310	−	+	+
332	−	+	+
364	−	+	+
439	−	+	+
496	+	−	+
*Eco*RI AGC, *Mse*I CTA			
208	+	−	+
310	−	+	+

Table 10. Coinheritance pattern of the AFLP marker bands in *Mentha arvensis* × *Mentha spicata*

Fragment size (bp)	*M. arvensis* CIMAP/C 17	*M. spicata* CIMAP/C 33	Hybrid
*Eco*RI ACA, *Mse*I CTC			
100	−	+	+
108	+	−	+
110	−	+	+
114	−	+	+
147	+	−	+
191	+	−	+
199	−	+	+
226	+	−	+
231	−	+	+
246	+	−	+
301	+	−	+
316	+	−	+
342	+	−	+
371	−	+	+
*Eco*RI AGG, *Mse*I CTG			
124	−	+	+
127	+	−	+
129	−	+	+
146	+	−	+
160	+	−	+
181	−	+	+
208	+	−	+
253	+	−	+
280	−	+	+
313	+	−	+
E*co*RI AGC, *Mse*I CTG			
120	+	−	+
153	−	+	+
219	−	+	+
306	+	−	+
*Eco*RI ACA, *Mse*I CAG			
130	+	−	+
146	−	+	+
149	+	−	+
163	+	−	+
233	+	−	+
247	−	+	+
253	+	−	+
271	−	+	+
293	−	+	+
303	+	−	+
306	+	−	+
331	−	+	+
364	−	+	+

analysis may be due to intermixing of genomes of various taxa of *Mentha* during evolution (Khanuja et al. 2000). The results obtained in our experiments indicate that through RAPD one can reveal hybridization patterns as distinctly as by AFLP, and therefore RAPD analysis may be applicable to various plant species where polyploidy has played a role in evolution and genomic constitution. An advantage of AFLP for plants such as *Mentha* could be the higher level of polymorphism achievable with fewer primer combinations, as is evident in our study. The number of primers required to reach similar interpretations on hybridization through RAPD analysis was much greater (80 primers) than through AFLP (6 primer pairs). Nevertheless, the rapidity and reproducibility of the detected RAPD markers cannot be ignored, as well as the fact that these specific RAPD markers can be utilized to develop SCAR markers (Hernandez et al. 1999, Lee et al. 2004; Paran and Michelmore 1993), adding a comparable precision for the probing of improved genotypes through hybridization programs.

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