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HYBRIDIZATION IN THE SECTION *MENTHA* (LAMIACEAE) INFERRED FROM AFLP MARKERS[1]

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The amplified fragment length polymorphism (AFLP) method was used to evaluate genetic diversity and to assess genetic relationships within the section *Mentha* in order to clarify the taxonomy of several interspecific mint hybrids with molecular markers. To this end, genetic diversity of 62 *Mentha* accessions from different geographic origins, representing five species and three hybrids, was assessed. Three *Eco*RI/*Mse*I AFLP primer combinations generated an average of 40 AFLP markers per primer combination, ranging in size from 50 to 500 base pairs (bp). The percentage of markers polymorphic ranged from 50% to 60% across all accessions studied. According to phenetic and cladistic analysis, the 62 mint accessions were grouped into two major clusters. Principal coordinates analysis separated species into well-defined groups, and clear relationships between species and hybrids could be described. Our AFLP analysis supports taxonomic classification established among *Mentha* species by conventional (morphological, cytological, and chemical) methods. It allows the assessment of phenetic relationships between species and the hybrids *M. spicata* and *M.* × *piperita*, largely cultivated all over the world for their menthol source, and provides new insights into the subdivision of *M. spicata*, based for the first time on molecular markers.

Key words: AFLP; DNA fingerprinting; genetic diversity; hybridization; Lamiaceae; *Mentha*; polyploid.

Mints are herbaceous plants and perennial aromatic herbs that are cultivated for their essential oils used both for medicinal and culinary purposes. These plants belong to the genus *Mentha* L. (Lamiaceae), which is a native from north temperate regions and occur in all five continents. According to a high polymorphism in morphology and a great diversity in essential oil composition, the number of species in the genus *Mentha* L. has been a matter of speculation for many years. Linneaus (1767) described the species of the genus based on inflorescence morphology. Several features have been used in the past to examine the diversity of *Mentha* using morphological (Malinvaud, 1880), cytological (Ruttle, 1931; Heimans, 1938; Morton, 1956; Sharma and Bhattacharyya, 1959; Harley, 1967, 1972; Harley and Brighton, 1977; Singh and Sharma, 1986), and chemical (Lawrence, 1978) markers. Harley and Brighton (1977) published a critical review of the chromosome numbers in relation to the taxonomy of the genus. They estimated that there are probably only 25 species and rather fewer hybrids. They recognized five sections (*Audibertia*, *Eriodontes*, *Pulegium*, *Preslia*, *Mentha*) on the basis of basic chromosome numbers and morphological features. There is no problem of identification for the first four sections because no example of natural interspecific hybridization exists. The fifth section, *Mentha*, includes five species: *M. suaveolens* Ehrh., *M. longifolia* (L.) Hudson, *M. spicata* L., *M. arvensis* L., and *M. aquatica* L. Chromosome counts for this section suggest a basic number of $x = 12$ with a range of numbers from diploid to octoploid. Plants of this section have a vigorous rhizome system as a means of spreading and dispersal and are self-compatible, and a high level of outbreeding is assured due to gynodioecy.

Natural interspecific hybridization occurs with high frequency in section *Mentha*, both in wild populations and in cultivation (Fig. 1). 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 chromosomes numbers (24–120), and much of the taxonomy of section *Mentha* has been complicated by hybridization, by a high morphological polymorphism, as well as polyploidy and vegetative propagation. The best known hybrids are *M.* × *piperita* (peppermint) and *M. spicata* L. (native spearmint), which are intensively cultivated for their essential oils. *Mentha* × *piperita* results from a cross between *M. aquatica* and *M. spicata*; *M. spicata* is the hybrid between *M. suaveolens* and *M. longifolia* (Harley and Brighton, 1977; Fig. 1). The great variability of *M. spicata* led several workers to establish a subdivision of this hybrid, and two subgroups were described based upon two features. Cytological studies (Ruttle, 1931; Morton, 1956) led to the conclusion that two *M. spicata* cytotypes exist, with $2n = 36$ and $2n = 48$ chromosomes, respectively. According to the cytotype implied in the cross with *M. aquatica*, two *M.* × *piperita* cytotypes result, with $2n = 66$ or $2n = 72$ chromosomes, respectively (Fig. 1). Moreover, morphological and chemical data divide *M. spicata* into two different subgroups according to the presence or absence of nonsecreting trichomes and the essential oil composition. Wild *M. spicata* is nearly always hairy, like its diploid parents, and can contain other terpenes that are found commonly in its diploid progenitors. Selected by man as an aromatic plant, *M.*

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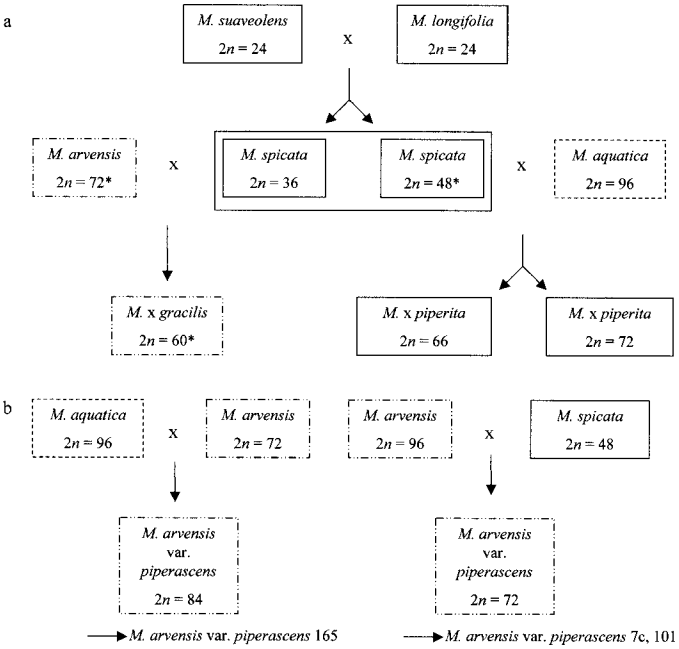

Fig. 1. (a) Relationships between species and hybrids based upon karyological data (Harley and Brighton, 1977). (b) *Mentha arvensis* var. *piperascens* could have resulted from two crosses (see DISCUSSION). An asterisk indicates species implied in the cross that led to *M.* × *gracilis*. Solid line = Spicatae; dashed line = Capitae; dashed-and-dotted line = Verticillatae.

spicata became glabrous with a characteristic odor due to carvone and menthone as the prevailing terpenes. *Mentha spicata* plants, introduced and distributed throughout the world, are often found as garden escapes. According to Lebeau (1974), it was essential to distinguish two *M. spicata* subspecies, *M. spicata* subsp. *spicata* and *M. spicata* subsp. *glabrata*, with and without nonsecreting trichomes, respectively, for the following reasons: (1) the presence or absence of nonsecreting trichomes led to a different aspect, (2) wide difference in perfume, and (3) difference in habitats. *Mentha spicata* propagates almost entirely by vegetative means. Lebeau (1974) and Harley and Brighton (1977) described some individuals of *M. spicata* that were close in appearance to its progenitor diploid species. They noted that *M. spicata* segregates parental characters in its progeny by selfing, which was impossible to distinguish from the hybrids it often forms with either *M. suaveolens* or *M. longifolia*. In some cases, such hairy *M. spicata* plants were confused with *M. longifolia*.

Over the past decade, several molecular techniques have been developed to provide information on diversity and genetic relationships. Two attempts to assess genetic relationships (Khanuja et al., 2000) and cultivar identity (Fenwick and Ward, 2001) based on RAPD markers have been undertaken in *Mentha* species. The complex systematics of section *Mentha* led us to choose amplified fragment length polymorphism (AFLP), based on the selective polymerase chain reaction (PCR) amplification of restriction fragments from a total digest of genomic DNA (Vos et al., 1995), to assess genetic diversity. It combines the specificity of whole-genome restriction fragment analysis and the selectivity of high stringency PCR amplification without prior knowledge of primer target sequences. A large number of markers can be obtained quickly compared to restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), or simple sequence repeat (SSR) (Powell et al., 1996; Pejic et al., 1998; Garcia-Mas et al., 2000). Because of its high repeatability and resolution, the AFLP technique has been used successively to assess genetic diversity, for example, in *Brassica juncea* (Srivastava et al., 2001) and to resolve phenetic relationships in *Dactylorhiza* (Orchidaceae) (Hedrén, Fay, and Chase, 2001) and in *Lactuca* sensu lato (s.l.) (Lactuceae, Asteraceae) (Koopman, Zevenbergen, and Van den Berg 2001).

The goal of the present study was to genotype diploid and polyploid species of section *Mentha* by AFLP to (1) assess the relationships among accessions of an extended collection representing legitimate species and the hybrids *M.* × *piperita* and *M. spicata*; and (2) check the existence of two *M. spicata* groups with molecular markers.

MATERIALS AND METHODS

Plant material—The National Clonal Germplasm Repository (NCGR, Corvallis, Oregon, USA), the Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben (IPK, Gatersleben, Germany), and the Conservatoire National des Plantes à Parfum, Médicinales, Aromatiques et Industrielles (CNPPMAI, Milly-la-Forêt, France) provided a total of 62 plants representing all five species and the hybrids *M.* × *piperita*, *M. spicata*, and *M.* × *gracilis* of section *Mentha* (for a complete list see http://ajbsupp.botany.org/v89/). The different accessions were chosen to cover a wide range of geographical origins as far as possible. Among these 62 accessions, 29 were collected from the wild, eight from breeding experiments, and ten from cultivated material.

Flow cytometry measurement—Nuclei were prepared from leaf tissue and stained according to Galbraith et al. (1983) and Marie and Brown (1993). About 4 cm^2 of *Mentha* leaves and 2 cm^2 of *Petunia hybrida* or *Lycopersicon esculentum* used as reference were chopped using a razor blade in 1 mL of modified Galbraith buffer (1% Triton X-100, 10 mmol/L metabisulfite, and 1% polyvinylpyrrolidone [PVP] 10 000). After filtration through a 30-μm nylon mesh, 10 mg/mL DNase-free RNase A (Boehringer, Paris, France) and 50 μg/mL propidium iodide (Sigma, Saint-Quentin Fallavier, France) were added. After an incubation for 20 min at room temperature, the fluorescence intensity was measured by flow cytometry (Elite cytometer, Beckman-Coulter, Roissy, France) at 488 nm. For each sample two nuclei preparations were analyzed, and for *M.* × *piperita* 38, eight repetitions were performed, randomly among all the other individuals. The DNA nuclear content was calculated by the ratio of the 2C peak positions, *Mentha*/reference, on the intensity fluorescence histograms. *Lycopersicon esculentum* or *Petunia hybrida* were chosen as references according to (1) the chromosome number of each *Mentha* species and (2) after pre-examination of one individual per species. Their 2C values were, respectively, 1.99 and 2.85 picograms (pg).

AFLP procedure—DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France) according to manufacturer's instructions using 20 mg of dried leaf material. DNA concentration was assessed by fluorimetry with the PicoGreen double strand DNA quantification Kit (Molecular Probes, Eugene, Oregon, USA). Genomic DNA was digested with *Eco*RI and *Mse*I, and the restricted fragments were ligated with *Eco*RI and *Mse*I adaptators (Vos et al., 1995) in the same reaction. Briefly, 5.5 μL (100 ng) extracted DNA was added to a 5.5 μL digestion-ligation solution containing T4 Buffer 1×, 50 mmol/L NaCl, 50 μg/mL bovine serum albumine, 1 unit (U) *Mse*I, 5 U *Eco*RI, 1 U T4-ligase, 0.9 μmol/L *Mse*I adapter, and 0.9 μmol/L *Eco*RI adapter. This resulting reaction mixture was then incubated at 37°C for 2 h and subsequently diluted tenfold in purified water. Preselective PCR by primers having one selective nucleotide each, namely E + 1 and M + 1 (Vos et al., 1995), was performed using 3 μL of digested-ligated DNA added to a 22-μL mixture containing Taq Buffer 1×, 150 μmol/L $MgCl_2$, 16 μmol/L of each dNTP, 0.2 μmol/L *Eco*RI primer (E/A), 0.2 μmol/L *Mse*I primer (M/C or M/G), and 0.5 U AmpliTaq Polymerase (Applied Biosystems

TABLE 1. Average number of peaks (fragments) obtained from three selective primer combinations among 62 *Mentha* accessions.

Primer pair	Average no. of peaks	% polymorphism
E-AGT/M-GTC	40	60%
E-ATC/M-GTC	40	50%
E-AGT/M-CAG	40	57.5%

Perkin Elmer, Courtaboeuf, France). Preamplification was performed using the following temperature profile: 2 min at 72°C, 25 cycles of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C, followed by one cycle for 10 min at 72°C. The preamplified DNA was then diluted in a ratio of 1 : 20 in purified water, and 5 μL of the diluted mixture were used as template for selective amplification and added to a 20-μL mixture containing Taq Buffer 1×, 250 μmol/L $MgCl_2$, 16 μmol/L of each dNTP, 0.04 μmol/L fluorescent *Eco*RI primer (E/AGA, E/AGT, E/ATC, E/ATG), 0.2 μmol/L *Mse*I primer (M/CAA, M/CAC, M/CAG, M/CAT, M/CTA, M/CTC, M/CTG, M/CTT or M/GAC, M/GTC) (Vos et al., 1995) and 1 U AmpliTaqGold Polymerase (Applied Biosystems Perkin Elmer). The preamplified DNA was amplified using one cycle of 10 min at 95°C, 36 cycles of 30 s at 94°C, 1 min at 65°C, and 1 min at 72°C, followed by one cycle for 10 min at 72°C. The annealing temperature was reduced every cycle by 0.7°C, and after 12 cycles it reached the annealing temperature of 56°C. This temperature was maintained for the subsequent 23 cycles. For gel electrophoresis, the amplified product (4 μL) was mixed with a 3.5-μL mixture containing formamide, loading buffer, and internal lane standard (GeneScan 500 Rox, Applied Biosystems Perkin Elmer), evaporated to a final volume of 3 μL and denaturated at 95°C for 2 min prior to loading on 64-lane 5% Long Ranger polyacrylamide gels on an automated DNA sequencer (ABI 377, Applied Biosystems Perkin Elmer).

Primer pair screening—Sixteen primer pairs were tested on four individuals in the selective PCR. Thirteen combinations gave too many bands for reliable analysis. A total of three AFLP primer combinations (E-AGT/M-CAG, E-AGT/M-GTC, and E-ATC/M-GTC) were chosen according to the number of fragments amplified, the level of polymorphism exhibited among closely related accessions, and the quality of the peaks produced on electrophenograms. Reliability of the technique was checked as following; two independent DNA extractions were performed for two accessions that were submitted to the whole AFLP procedure.

Phenetic analysis—AFLP electrophenograms were analyzed with GeneScan Analysis 3.1 (Applied Biosystems Perkin Elmer). All peaks that could be unambiguously read on the electrophenograms were treated as individual dominant loci and scored as either present (1) or absent (0) across all 62 accessions of *Mentha* for each primer-pair combination. Genetic similarities, based on Nei and Li's formula (1979) as $GS_{XY} = 2N_{XY}(N_X + N_Y)$, where N_{XY} is the number of peaks shared in accessions *X* and *Y*, N_X is the number of peaks in accession *X*, and N_Y is the number of peaks in accession *Y*, were calculated using the genetic distance estimation program of the TREECON software package (Van de Peer and De Wachter, 1994). We chose Nei and Li's formula to calculate distances between accessions because this method does not take into account shared absent fragments (0–0) and so reduces the amount of homoplasy. The genetic distance was computed as $GD_{XY} = 1 - GS_{XY}$, and dendrograms were generated by using the unweighted pair group mean average (UPGMA) and neighbor-joining methods in TREECON. The robustness of UPGMA and neighbor-joining trees was evaluated by bootstrapping (1000 bootstrap replicates) using TREECON.

Parsimony analysis—Determination of phylogenetic signal in the data set and cladistic analysis were performed using PAUP version 4.0b8 (Swofford, 1999). Phylogenetic signal was determined from the tree-length distribution of 100 000 trees, using the g1 statistic (Hillis and Huelsenbeck, 1992). The *Mentha* data set contained >25 taxa and >50 variable characters, and therefore the critical value of −0.09 was used. Data sets that produce g1 values less than −0.09 are significantly more structured than are the random data (Hillis and Huelsenbeck, 1992). An heuristic search comprised 1000 random-addition sequences, and tree bisection-reconnection (TBR) branch swapping was performed. Another search was run using successive weighting cycles. Characters were reweighted by the maximum value of the rescaled consistency index, and the search was conducted with 1000 random-addition sequences, followed by TBR branch swapping. Bootstrap values for the resulting tree were calculated using 1000 replicates.

TABLE 2. Range of similarity coefficient values and number of polymorphic peaks within *Mentha* species.

Mentha species	Range	No. of polymorphic peaks	No. of individual-specific peaks
M. aquatica	0.74–0.98	18	1
M. arvensis	0.46–0.89	40	12
M. spicata	0.61–0.98	27	3
M. suaveolens	0.66–0.98	36	8
M. longifolia	0.60–0.91	13	2
M. × *piperita*	0.65–0.98	39	8

Principal coordinates analysis—Principal coordinates analysis (PCoA) was performed to visualize interspecies relationships on the genetic distance (Nei and Li, 1979) matrix using the Ade-4 software (Thioulouse et al., 1997).

RESULTS

DNA content—DNA (2C) content ranged from 0.89 pg in *M. suaveolens* 10 to 0.96 pg in *M. suaveolens* 538 (http://ajbsupp.botany.org/v89/). A 4.2-fold amount was found in *M. suaveolens* 495 (3.87 pg). *Mentha longifolia* accessions have a DNA content that ranged from 0.84 pg in *M. longifolia* subsp. *typhoides* 20 to 0.99 pg in *M. longifolia* 533, while *M. spicata* accessions were subdivided into two groups. The first, composed of *M. spicata* 87, 694, 32, and 29, was characterized by a DNA content that ranged from 1.24 pg to 1.35 pg, while the second group consisted of *M. spicata* 96, 50, 84, 106, 54, 41, 363, 56, and *M. spicata* var. *crispa* 56 and was characterized by a DNA content that ranged from 1.64 to 1.83 pg. *Mentha aquatica* accessions have a DNA content that ranged from 2.83 to 2.96. Three groups were defined for *M. arvensis* accessions, characterized by 1.9 pg (*M. arvensis* 514, 515), 2.3 pg (*M. arvensis* var. *piperascens* 7, 101) and 2.57 pg (*M. arvensis* 165). Two groups were defined for *M.* × *piperita*, characterized with 2.3 pg (*M.* × *piperita* 19, 38, 138, 139, 140, 147, 156, 157) and 3 pg (*M.* × *piperita* 93, 110). *Mentha* × *piperita* 118 (1.3 pg) showed a decrease of the amount of 1.7-fold.

The AFLP polymorphism—Each primer combination generated an average of 40 bands per individual, ranging in size from 50 to 500 base pairs (bp). The percentage of polymorphic peaks for each primer combination varied from 50% to 60% (Table 1). Polymorphic peaks and individual-specific peaks were obtained among 62 accessions (Table 2). To analyze the genetic diversity of 62 *Mentha* accessions, we examined 67 polymorphic AFLP markers generated from three primer combinations.

Intraspecific genetic diversity—In the present study, Nei and Li's coefficient was chosen to estimate genetic similarity based on AFLP data. Similarity coefficients ranged from 0.11 to 0.98 across all 62 *Mentha* accessions. The range of simi-

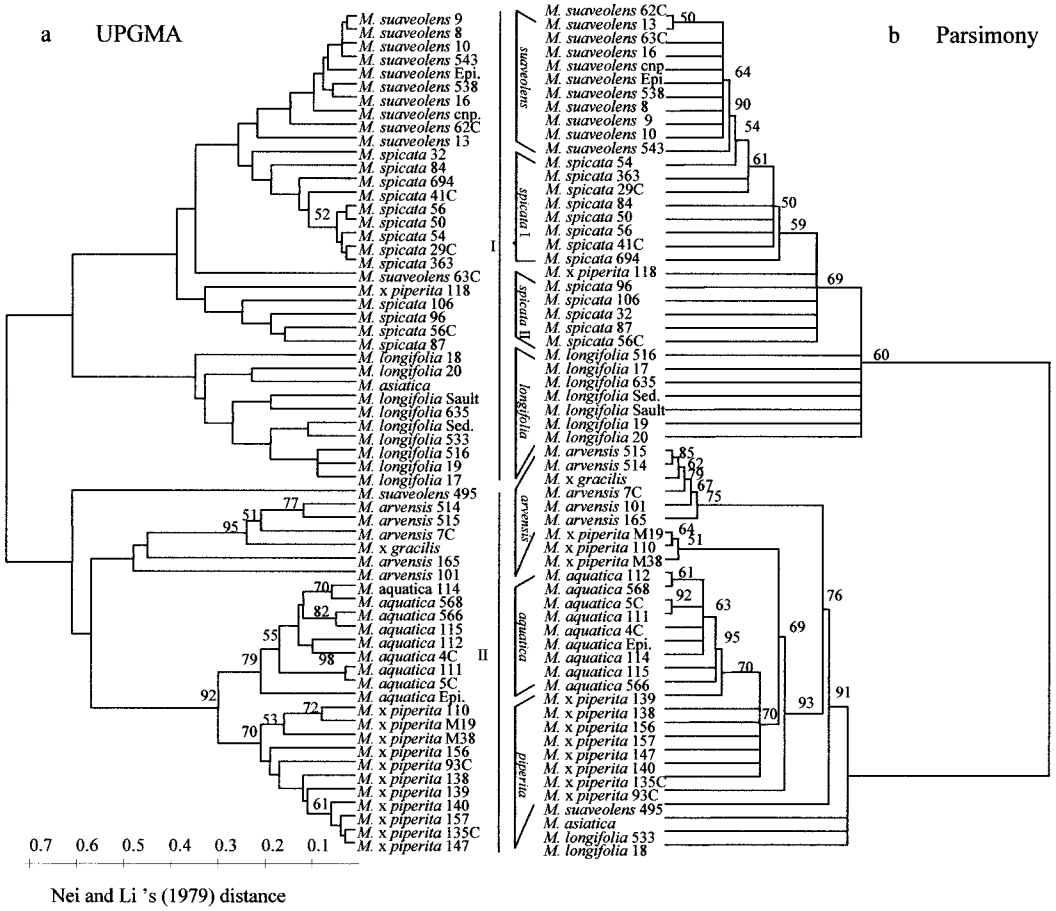

Fig. 2. Phenogram and cladogram of 62 *Mentha* accessions. (a) UPGMA phenogram based on Nei and Li's (1979) distance. Numbers above branches are bootstrap values. (b) Cladogram resulting from three successive weightings from 1000 random-addition sequences with TBR branch swapping and ''multrees'' switched off. Numbers on each branch are bootstrap values. Epi. = Episy; cnp. = cnppmai; Sed. = Sederon.

larity coefficients for each species is noted in Table 2. Among the hybrids, *M. spicata* is close to *M. longifolia* and *M. suaveolens*, *M.* × *piperita* is clustered with *M. aquatica*, and *M.* × *gracilis* shared a lot of similarity with *M. arvensis* accessions. Interspecific genetic diversity was estimated by cluster analysis to screen genetic relationships.

Cluster analysis—The pairwise genetic distances of the accessions were calculated to cluster the data among the *Mentha* accessions using the unweighted pair-group method with an arithmetic average (UPGMA) algorithm and neighbor-joining method. The phenogram in Fig. 2a divided accessions into two major clusters: (I) *M. suaveolens*, *M. spicata*, and *M. longifolia* and (II) *M. arvensis*, *M.* × *gracilis*, *M. aquatica*, and *M.* × *piperita*. There were three distinct subgroups in cluster I: (1) *M. suaveolens-M. spicata* I, (2) *M. spicata* II, (3) *M. longifolia* and two subgroups in cluster II: (1) *M. arvensis-M.* × *gracilis*, (2) *M. aquatica-M.* × *piperita* (well supported with a 92% bootstrap value). The range of similarity values among *M. spicata-M. suaveolens*, *M. spicata-M. longifolia*, and *M. suaveolens-M. longifolia* ranged from 0.48 to 0.88, 0.27 to 0.71, and 0.11 to 0.38, respectively. The UPGMA analysis showed that *M. arvensis* accessions formed a sister group to the *M. aquatica-M.* × *piperita* cluster. Within the *M. arvensis* group, two subgroups are clearly defined: (1) *M. arvensis* 514, 515, var. *piperascens* 7C, and *M.* × *gracilis* (95% bootstrap value), and (2) *M. arvensis* 165, var. *piperascens* 101. The most well-known hybrid, *M.* × *piperita* (peppermint) showed a range of similarity coefficients, from 0.65 to 0.98, and was separated by UPGMA into two clusters. The first is composed of cultivated *M.* × *piperita* from France (*M.* × *piperita* 19 and 38) and Bulgaria (*M.* × *piperita* 110), while the second cluster is mainly composed of wild accessions from the United States. *Mentha* × *piperita* 93C from the Ukraine appeared as a sister group to the American one. *Mentha* × *piperita* showed the highest similarity with one of its diploid progenitors, *M. aquatica* (0.6–0.8), rather than with *M. spicata* (0.3–0.5). *Mentha* × *piperita* var. *vulgaris* 118 is clustered in the *M. spicata* II subgroup, while *M. suaveolens* 495 appeared as a sister group to the *M. arvensis-M. aquatica-M.* × *piperita* cluster. The neighbor-joining method (data not shown) led to the same results except *M. arvensis* var. *piperascens* 101 and 7C were clustered in *M. spicata* I.

By deleting from the analysis the accessions suspected to be misidentified (*M. suaveolens* 495, *M.* × *piperita* 118), hypothetical hybrids and backcrosses (*M. suaveolens* 63C, *M. arvensis* 165, 101, 7C, and the *M. spicata* subgroup II [106, 96, 56C, 87]), *Mentha* species were assigned to one cluster with higher bootstrap values: *M. arvensis*, 99%; *M. aquatica-M.* × *piperita*, 98%; *M. suaveolens-M. spicata*, 97%; and *M. longifolia*, 95% (data not shown).

Parsimony analysis—Two cladistic analyses were performed. The first was run with all accessions. The g1 statistic for this data set was −0.31, indicating significant phylogenetic signal. The parsimony analysis yielded 197 most-parsimonious trees of 282 steps (retention index [RI] = 0.80, consistency index [CI] = 0.23, rescaled consistency index [RC] = 0.19). The search with three successive weighting steps yielded 240 trees of 52 steps (RI = 0.91, CI = 0.45, RC = 0.41). As with the phenetic analysis, (1) the same two major groups (I and II) could be described (Fig. 2b), and (2) *M.* × *piperita* var. *vulgaris* 118 was clustered in *M. spicata* II subgroup, while *M. suaveolens* 495 appeared as a sister group to the *M. arvensis*-*M. aquatica*-*M.* × *piperita* cluster. However, three accessions, *M. longifolia* 18, *M. longifolia* 533, and *M. asiatica*, were found in cluster II while these accessions were found in cluster I in the phenetic analysis. With regard to the two *M. spicata* subgroups, *M. spicata* 32 was clustered in subgroup II, while this accession was clustered in subgroup I in the phenetic analysis. *Mentha* species were assigned to one cluster with the following bootstrap values: *M. arvensis*, 75%; *M. aquatica*-*M.* × *piperita*, 93%; and *M. suaveolens*-*M. spicata*, 69%. The second analysis was performed without misidentified accessions, hypothetical hybrids, and backcrosses (data not shown). The g1 statistic for this second data set was −0.33, indicating significant phylogenetic signal also. Parsimony analysis yielded 251 most-parsimonious trees with 207 steps (RI = 0.85, CI = 0.32, RC = 0.27). The search with three successive weighting steps yielded ten trees of 53 steps (RI = 0.94, CI = 0.58, RC = 0.55). With regard to this second cladistic analysis, we obtained the same results as those noted in the phenetic analysis. *Mentha* species were assigned to one cluster with high bootstrap values: *M. arvensis*, 100%; *M. aquatica*-*M.* × *piperita*, 99%; and *M. suaveolens*-*M. spicata*, 99%.

Principal coordinates analysis—Nei and Li's genetic similarity coefficients were used for principal coordinates analysis. The variance of the first two principal coordinates accounted for 55% of the total variation (Fig. 3). The first axis, accounting for 38% of the variation, separated the *M. arvensis*-*M.* × *piperita*-*M. aquatica* group from the *M. suaveolens*-*M. spicata*-*M. longifolia* group. The second axis accounted for 17% of the variation and distinguished distinct groups for each species. The PCoA results are in accordance with those of the phenetic and cladistic analyses. The *M. asiatica* and *M.* × *gracilis* accessions were clustered with the *M. longifolia* and *M. arvensis* groups, respectively. *Mentha* × *piperita* 118 and *M. suaveolens* 495 were tightly clustered with the *M. spicata* II and *M. arvensis*-*M. aquatica*-*M.* × *piperita* groups, respectively. The PCoA (Fig. 3) showed *M. arvensis* accessions dispersed except *M. arvensis* 514 and *M. arvensis* 515, which were tightly clustered. *Mentha arvensis* var. *piperascens* 101 and 7C occupied an intermediate position between the two major groups, *M. aquatica*-*M.* × *piperita* and *M. suaveolens*-*M. spicata*-*M. longifolia*. *Mentha arvensis* 165 was clustered with the *M.* × *piperita* group.

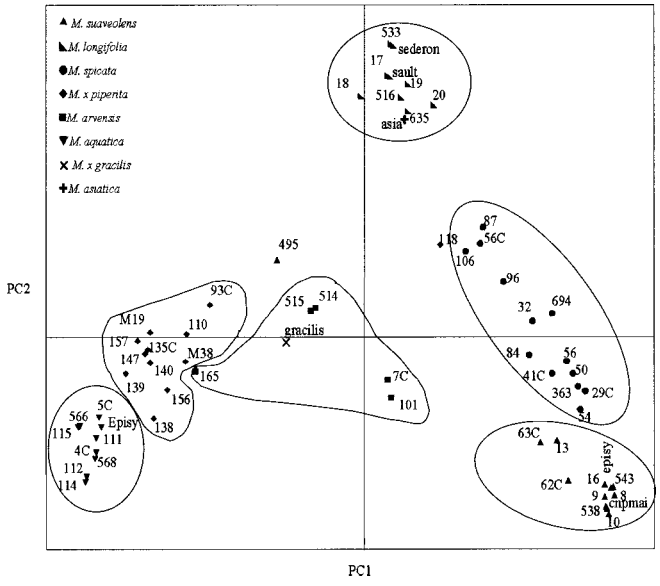

Fig. 3. Principal coordinates plot of 62 *Mentha* accessions for the first two principal components (55% of the total variation) estimated with 67 AFLP markers.

DISCUSSION

Mentha, a taxonomically complex section, was recently studied by Khanuja et al. (2000) by RAPD fingerprinting. Genetic diversity and genetic relationships were assessed among six *Mentha* taxa (*M. arvensis*, *M. spicata*, *M. spicata* cv. *viridis*, *M.* × *piperita*, *M.* × *piperita* cv. *citrata*, *M.* × *gracilis* Sole cv. *cardiaca*). Fenwick and Ward (2001) were the first to evaluate genetic diversity within the most widely grown peppermint (*M.* × *piperita*) and Scotch spearmint (*M.* × *gracilis*) cultivars by random amplified polymorphic DNA markers. Their results indicate a high degree of genetic uniformity in mint cultivars grown for oil in the United States. Effectively, new cultivars are selected as either spontaneous or induced variants or ''sports'' of existing cultivars and then clonally propagated. The lack of genetic diversity could result, for example, in a poor disease resistance as demonstrated by the widespread occurrence of verticillium wilt (caused by *Verticillium dahliae* Kleb.) (Fenwick and Ward, 2001). The AFLP method allowed us to assess genetic diversity between and within *Mentha* species. This assessment is fundamental because genetic diversity could be in future exploited through molecular approaches or plant breeding techniques to improve mint cultivars for disease resistance or to increase essential oil yield, for example. The major objectives of this study were to assess the phenetic relationships between species and hybrids of section *Mentha* and to have a better knowledge of the two *M. spicata* subgroups through use of molecular markers. For our purposes, the AFLP data will be discussed in the context of the classification described by Harley and Brighton (1977).

Genetic diversity in Mentha—*Cluster I: M. suaveolens*-*M. longifolia*-*M. spicata*—Based on inflorescence morphology, Linnaeus grouped these three species in the *Spicatae*. *Mentha longifolia*, a mountain plant, shows a great array of morphological diversity and has consequently been treated by some taxonomists as composed of numerous species, subspecies, varieties, and forms (Harley and Brighton, 1977). For example, among our accessions, *M. asiatica* is synonymous with *M. longifolia* and is not a *Mentha* species. In our collection, eight of the *M. longifolia* accessions originate from Europe, one from Nepal (*M. longifolia* subsp. *hymalaiensis* 635), and the last from Syria (*M. longifolia* subsp. *typhoides* 20). Plants from Afghanistan eastwards with petiolate leaves have been

referred to *M. longifolia* subsp. *hymalaiensis*, but according to Harley and Brighton (1977) most of those plants show the character to a much lesser degree and are closer to ''typical'' *M. longifolia*. Our results confirm this assessment because *M. longifolia* subsp. *hymalaiensis* 635 has a range of genetic similarity coefficient between 0.66 and 0.81 compared to typical *M. longifolia* accessions. With regard to *M. longifolia* subsp. *typhoides* 20 (0.60–0.72), it seems that the situation is identical. Clustering of *M. suaveolens* and *M. longifolia* diploid accessions into strictly separate group was apparent, while the tetraploid, *M. spicata*, is separated into two distinct groups, one a sister group to *M. suaveolens* accessions and the second forming a distinct cluster. According to cladistic, and phenetic analyses, our results, based on molecular markers, showed that *M. suaveolens*, *M. longifolia*, and their hybrid *M. spicata* are closely related. The PCoA clearly separated the two progenitors along axis 2 and hybrids are in between.

Cluster II: M. × piperita-M. aquatica-M. arvensis—This cluster is composed of species with larger genomes (hexaploid and octoploid) than the first one. *Mentha × piperita*, *M. aquatica*, and *M. arvensis* belong to the *Spicatae*, *Capitae*, and *Verticillatae* groups, respectively. With regard to the dichotomy noted in phenogram and cladogram, it seems that the section *Mentha* evolved into two lineages: (1) diploid species and (2) polyploid species. Progenitors of *M. arvensis* and *M. aquatica* have never been described. According to our results, two hypotheses could be assumed: (1) progenitors have evolved or disappeared or (2) polyploids have been subjected to hybridization and/or to successive polyploidization events and then progenitors have subsequently become difficult to find. According to Ruttle (1931), *M. aquatica* has an allopolyploid origin because 48 chromosome pairs were counted at meiotic mitosis in microsporocytes. *Mentha arvensis*, cultivated for its essential oils, shows a great natural diversity also revealed in the sampling used in this study. Two cytotypes exist, with $2n = 72$ and $2n = 96$ chromosomes, respectively. Moreover, *Mentha arvensis* could hybridize with all species of the section. For example, the cross between *M. arvensis* ($2n = 72$) and *M. spicata* ($2n = 48$) led to *M. × gracilis* L. According to the chromosome number counts of Harley and Brighton (1977), *M. × gracilis* is a pentaploid ($2n = 60$). Thus, *M. arvensis* contributes three of the five genomes of the hybrid, and it seems highly probable that *M. × gracilis* resembles *M. arvensis*, explaining why it is closely related to *M. arvensis* in the UPGMA, parsimony, and PCoA. Schürhoff (1929, in Ruttle [1931]) described *M. arvensis* var. *piperascens* as a sterile hybrid between *M. aquatica* and *M. arvensis*. Harley and Brighton (1977) noted it as an octoploid ($2n = 96$). According to our flow cytometry results, it seems that *M. arvensis* var. *piperascens* 101 and 7C are hexaploid ($2n = 72$). These accessions showed the highest similarity coefficients with other *M. arvensis* and *M. spicata* accessions while the similarity with *M. aquatica* is lower. According to UPGMA, parsimony, PCoA and flow cytometry analyses, it seems highly probable that *M. arvensis* var. *piperascens* 101 and 7C are the result of an hybridization event involving *M. arvensis* ($2n = 96$) and *M. spicata* ($2n = 48$) species (Fig. 1). This hypothesis is supported by neighbor-joining results, which clustered *M. arvensis* var. *piperascens* 101 and 7C in *M. spicata* I. *Mentha arvensis* 165 appeared as an hexaploid accession ($2n = 84$). According to the National Clonal Germplasm Repository, this accession gives almost no selfed seeds and is probably a male sterile due to the loss of a chromosome, but no count was done. In the PCoA, this accession is clustered in the *M. aquatica-M. × piperita* group, and the highest similarity coefficients were noted with *M. arvensis*, *M. × piperita*, and *M. aquatica* species. It seems that a hybridization event may have occurred between *M. arvensis* and *M. × piperita* or between *M. arvensis* and *M. aquatica*. The second hypothesis seems to be more probable than the first one because *M. × piperita* is a sterile hybrid. Similarity between *M. arvensis* 165 and *M. aquatica* and *M. × piperita* species could have resulted from common AFLP markers found in *M. aquatica* and its hybrid *M. × piperita*. In that case, *M. arvensis* 165 may be revisited as a *M. arvensis* var. *piperascens*. Finally, *M. arvensis* 514 and 515 appeared as tetraploid, while ''typical'' *M. arvensis* are hexaploid ($2n = 72$) or octoploid ($2n = 96$). These accessions are closely related with *M. × gracilis* in all our analyses. A great number of varieties have been described in *M. arvensis*, and *M. × gracilis*, one of its hybrids, shows a high variability. Thus, these species are difficult to identify leading to taxonomic complexity.

Close relationships between M. × piperita and M. aquatica—*Mentha × piperita*, which is intensively cultivated all over the world, results from a cross between *M. spicata* and *M. aquatica*. Actually, the latter is octoploid, while *M. spicata* is a tetraploid. Therefore, two-thirds of the *M. × piperita* genetic pool is composed of the *M. aquatica* genome. The PCoA showed that *M. × piperita* accessions are between its progenitors and as with UPGMA and parsimony analyses, *M. × piperita* is closer to *M. aquatica* than *M. spicata*. These results, based on molecular markers, confirm the two progenitors of *M. × piperita*, described on the basis of morphological and chemical features.

Genealogy of M. spicata: hybrid status and backcrosses—*DNA content*—Flow cytometry measurements are relatively consistent to previous cytological data. According to the basic chromosome number $x = 12$, it seems evident that the two *M. spicata* groups are characterized by $2n = 36$ and $2n = 48$ chromosomes, respectively. According to DNA content measurement by flow cytometry and the presence or absence of trichomes (data not shown), it seems that there is no relationship between these criteria and the two distincts groups of *M. spicata* resolved by UPGMA and principal coordinates analyses.

Thus it seems that the two *M. spicata* groups should not be defined based upon chromosome number or the presence/absence of nonsecreting trichomes but rather on the basis of molecular markers that cover the entire genome, such as AFLP markers. So, it seems evident that the *M. spicata* subdivision into two different subspecies, *M. spicata* subsp. *spicata* and *M. spicata* subsp. *glabrata*, by Lebeau (1974) should be revisited. Two hypotheses could explain the presence of two *M. spicata* groups: (1) *M. spicata* often forms backcrosses with its diploid progenitors, indeed, hybrids like *M. × villosa* Hudson (*M. spicata × M. suaveolens*) and *M. × villoso-nervata* Opiz (*M. spicata × M. longifolia*) are difficult to distinguish from ''typical'' *M. spicata* and (2) *M. spicata* occasionally segregates parental characters in its progeny on selfing (Harley, 1967). As a consequence, *M. spicata* plants are more and more closely related to *M. suaveolens* or *M. longifolia* during backcrossing or selfing. Harley found one specimen of *M. × villosa* in the southwest of England and said that it was re-

markable for its very close similarity to *M. suaveolens*. It seems that AFLP methods allow us to distinguish clearly *M. spicata* from its progenitors and *M. spicata* accessions that are closer to *M. suaveolens* or to *M. longifolia* as well.

Detection of misidentified accessions—*Mentha suaveolens* 495 and *M.* × *piperita* 118 were not found in their respective species clade in the UPGMA and principal coordinates analyses. *Mentha suaveolens* 495 is a seedling selection from another *M. suaveolens* accession that is not in our collection and *M.* × *piperita* var. *vulgaris* 118 comes from England. Morphologically, these accessions do not look like *M. suaveolens* or *M.* × *piperita*. Cytologically, *M. suaveolens* 495 and *M.* × *piperita* 118 have 3.87 and 1.31 pg of DNA, respectively, while other accessions of *M. suaveolens* and *M.* × *piperita* have a range of DNA from 0.9 to 1 pg and from 2.3 to 3.1 pg of DNA, respectively. It seems probable that *M. suaveolens* 495 was misidentified and then mislabelled before sampling. This accession came from breeding experiments and particularly from open pollination. It became apparent that identification of *M.* × *piperita* 118 as an accession of *M.* × *piperita* was in error. This plant is probably the result of hybridization, and AFLP analysis provided some information about the origin of its genetic pool.

In conclusion, the present study of AFLP analysis of mint accessions supports current taxonomic classification. Our data set was analyzed phenetically as well as cladistically, and the main well-supported clusters of the trees were comparable for both types of analyses. Phenetic analysis using UPGMA based on Nei and Li's (1979) genetic distance resulted in a highly resolved tree, while parsimony analysis showed phylogenetic signal and high bootstrap values. Finally, PCoA allowed us to easily visualize hybridization events. Actually, *M. suaveolens*, *M. spicata*, and *M. longifolia* species form a tight group and on the basis of AFLP markers, *M.* × *piperita* is closer to *M. aquatica* than *M. spicata*. The existence of polyploidy in section *Mentha* does not seem to represent a problem in AFLP analysis. The three primer combinations provided enough AFLP polymorphisms to resolve genetic diversity between and within species, allowing us to objectively identify species, hybrids, and accessions. This genetic variability could in future be exploited through molecular approaches for gene introgression in breeding programs to produce desired genotypes. While the number of mint accessions used for this study represent only a small sample of the available mint germplasm, the potential resolving power of AFLP analysis in a larger collection seems evident.

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