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Genetic Variation in Iranian Mints on the Bases of RAPD Analysis

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Abstract: In the present study, the genetic diversity and taxonomic relationships between 17 accessions of four *Mentha* species were examined using RAPD markers and analysed the data by both similarity methods and principal coordinate analysis. Out of 70 primers, 31 provided reproducible results. A total of 608 reliable and polymorphic RAPD bands were detected which used to estimate genetic similarity among pair-wise combinations of accessions. High genetic diversity was found within *Mentha* accessions. Jaccard's similarity value ranged from 0.13 to 0.69, with mean of 0.25, which reflects a rather high genetic variability among the accessions evaluated. The result indicated that RAPD as a molecular marker was useful for detecting the genetic variation among the accessions. According to our analysis, *M. piperita*14 is about 0.45 similar to *M. spicata*10 are clustered in the same group. The clustering between these two accessions is in agreement with the earlier observations.

Key words: Genetic diversity, *Mentha*, RAPD molecular marker

INTRODUCTION

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 produces secondary metabolites such as alkaloids, flavanoids, phenols, gummy polysaccharides, terpens and quinines that are used in food, pharmaceutical, cosmetics and pesticide industries (Khanuja *et al*., 2000). According to a high polymorphism in morphology and a great diversity in essential oil composition, the number of species of the genus *Mentha* L. has been a matter of speculation for many years. Harley and Brighton (1977) 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*, *M. longifolia*, *M. spicata*, *M. arvensis* and *M. aquatica*. Natural interspecific hybridization occurs with high frequency in section *Mentha*, this leads to a large diversity of chromosome numbers (24-120) and much of the taxonomy of section *Mentha* has been complicated by hybridization, by high morphological polymorphism, as well as polyploidy and vegetative propagation (Gobert *et al*., 2002).

The analysis of genetic variation both within and among plant materials is of fundamental interest to plant breeders. The accurate identification of plant materials is essential for effective germplasm characterization. It provides estimations of the extent of variation available, contributes to monitoring germplasm and can also be used to predict potential genetic gains (Moreno-Gonzalez and Cubero, 1993); without such information breeders have no means of selecting appropriate material for entry into breeding programme. However, due to stage specific expression of characters and influence of environment, morphological diversity estimates are less reliable. Moreover, at times there may be little morphological diversity among cultivars with related pedigrees (Choudhury *et al*., 2001).

The genetic structure of *Mentha* cultivars is imprecise and of heterozygote nature when compared to fixed populations such as inbreeds or cultivars from autogamous species. This obscures the determination of genetic diversity patterns based upon morphological and phenological or agronomic observations (Campos-de-Quiroz and Ortega-Klose, 2001).

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The assessment of genetic diversity at DNA level for these accessions has been considered as the desirable step in the process of developing taggable markers to aid genetic improvement in the variety development programme. In recent years, molecular markers have been applied to a wide number of genetic and breeding studies, including fingerprinting individuals and the positional cloning of important genes. One of the most extensively used molecular markers are RAPDs (Williams *et al*., 1990) which have been applied to address genetic diversity issues in plants (Vilanova *et al*., 2001; Choudhury *et al*., 2001; Campos-de-Quiroz and Ortega-Klose, 2001; Gichuki *et al*., 2003). They are especially suited to species with little molecular information such as mint due to the following attributes: 1) No previous knowledge of the genome is required; 2) Rapid results can be obtained when compared with alternatives such as RFLPs and 3) A universal set of primers which can be used for genomic analysis in any species is commercially available.

When compared with crops, the number of studies performed with RAPD or other molecular marker in mint is very limited. Molecular data regarding the structure and variability existing in breeding populations of this medicinal genus is quit limited. Hence, in the present study, the extent of genetic variation and taxonomic relationships between 17 accessions belonging to *Mentha spicata*, *M. piperita*, *M. suaveolens* and *M. longifolia* were assessed by RAPD fingerprinting.

MATERIALS AND METHODS

Plant materials: Four taxa of mints, namely *Mentha spicata* L., *M. piperita* L., *M. suaveolens* and *M. longifolia* (L.) Hudson were selected for RAPD analysis. These included nine accessions of *Mentha spicata* (1, 2, 3, 4, 7, 8, 9, 10, 12); four accessions of *Mentha piperita* (13, 14, 15, 16); three accessions of *Mentha longifolia* (5, 6, 11) and one accession of *Mentha suaveolens*. The plant materials were obtained from Agriculture Research station of Isfahan University of Technology, University Jehad Research Farm (sponsored by Shahid-Beheshti University) and Research Farm of Mayor in Isfahan. The plant materials were identified by Randy Olson, W.P. Fraser Herbarium, Canada. The plants analyzed in this study were maintained in the Shahrekord University glass house and leaf materials were taken for DNA isolation.

Total DNA isolation: Total cellular DNA was isolated from freshly germinated young leaves by following a modification of the CTAB method of Murray and Thampson (1980): about 3 g of fresh leaf tissue was crushed to powder in liquid nitrogen and transferred to pre-warmed (60°C) 2× CTAB buffer containing 1% PVP (w/v) (polyvinyl pirrolidone). DNA was resupended in TE buffer and stored at 4°C. DNA concenteration was quantified by gel electrophoresis and spectrophotometry.

RAPD fingerprinting and analysis of RAPD-PCR products: Thirty one arbitrary 10-mer primers (Genset, France) were used for PCR amplification of the total genomic DNAs (Table 1). Polymerase chain reaction was performed based on the protocol of Williams *et al*. (1990), with minor modification. Amplification were carried out in 25 μL of reaction mixture containing 2.5 μL of PCR buffer, 200 μM each of dATP, dCTP, dGTP, dTTP (Amersham biosciences), 15 ng of the primer, 0.7 unit of Smar*Taq* DNA polymerase (Cinnagen) and 25 ng of DNA template. DNA amplification was performed in a Eppendorf Mastercycler Gradiant programmed as follows: pre-denaturation 3 min at 94°C followed by 45 cycles, each of 1 min at 94°C, 1 min at 35°C, 2 min at 72°C, followed by one final extension cycle of 10 min at 72°C. The amplification products were size-separated by gel electrophoresis in 1.2% agarose gels with 0.5×TBE and stained with ethidium bromide. *Eco*R1/*Hin*dIII digested lambda DNA served as molecular size marker. PCR reactions were repeated at least twice to establish reproducibility of results. After AGE, the gel was photographed under UV light.

Data analysis: The molecular size of each fragment was calculated by using UVI Bandmap software 10.02. All the accessions were scored for the presence of band size (1) or its absence (0). Fingerprints were independently assessed by a second scorer to increase confidence in band scoring. Only those RAPD-PCR bands that appeared distinct in both of the replicate RAPD-PCR reactions were recorded. The data were analysed to generate Jaccard's (1908), Nei and Li's (1979) and simple matching's (Sokal and Michener 1958) similarity coefficients. These similarity coefficients were used to construct dendrograms using the unweighted pair group method with arithematic averages (UPGMA) employing the NTSYS-pc software, version 2.02. Similarity data were additionally analysed by principal coordinate analysis (PCoA) and graphically represented.

RESULTS

We obtained good amounts of DNA from mint and λ260/λ 280 values ranged from 1.8 to 1.93 which are considered appropriate. The DNA was readily amplified

Table 1: Number of polymorphic bands obtained from 31 primers among 17 *Mentha* accessions

No.	Primer sequence	No. of polymorphic bands	Plymorphism (%)	No.	Primer sequence	No. of polymorphic bands	Polymorphism (%)
1	TGC CGA GCT G	17	100	17	GTT TCG CTC C	24.00	100.00
2	AAT CGG GCT G	21	100	18	GGA CTG GAG T	7.00	87.50
3	AGG GGT CTT G	15	100	19	GGT GAC GCA G	7.00	87.50
4	GAA ACG GGT G	24	100	20	CTG CTG GGA C	18.00	100.00
5	GGG TAA CGC C	24	100	21	GTA GAC CCG T	27.00	100.00
6	GTG ATC GCA G	17	100	22	CCT TGA CGC A	29.00	100.00
7	CAG CAC CCA C	20	100	23	GGA GGG TGT T	20.00	95.23
8	AGG TGA CCG T	19	100	24	AGG GAA CGA G	11.00	91.66
9	CAA ACG TCG G	20	100	25	CCA CAG T 23	94.83	
10	CCG CCC AAA C	21	100	26	CCT GGG CCT A	10.00	91.01
11	AGC GAG CAA G	30	100	27	GGT GGC GGG A	28.00	100.00
12	GAA CAC TGG G	30	100	28	GAG GGC GGG A	26.00	100.00
13	CTC CTG CCA A	15	100	29	GAG GGC GTG A	32.00	100.00
14	GAG CGT CGA A	15	100	30	GAG CAC CAG G	16.00	100.00
15	CCC AGC TGT G	27	100	31	GGG CCC GAG G	0.00	00.00
16	CAC AGG CGG A	16	94.12				

Table 2: similarity matrix for Jaccard's coefficient of a total of 17 accessions of different species of *Mentha*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
*M. spicata*1	1.00																
*M. spicata*2	0.21	1.00															
*M. spicata*3	0.27	0.20	1.00														
*M. spicata*4	0.22	0.20	0.34	1.00													
*M. longifolia*5	0.20	0.19	0.29	0.54	1.00												
*M. longifolia*6	0.20	0.19	0.24	0.25	0.27	1.00											
*M. spicata*7	0.29	0.22	0.42	0.30	0.26	0.31	1.00										
*M. spicata*8	0.23	0.19	0.29	0.45	0.50	0.31	0.32	1.00									
*M. spicata*9	0.26	0.20	0.30	0.35	0.24	0.25	0.41	0.31	1.00								
*M. spicata*10	0.19	0.36	0.24	0.18	0.16	0.23	0.24	0.24	0.22	1.00							
*M. longifolia*11	0.21	0.39	0.25	0.22	0.22	0.27	0.28	0.27	0.23	0.69	1.00						
*M. spicata*12	0.32	0.21	0.23	0.20	0.21	0.22	0.34	0.22	0.25	0.23	0.29	1.00					
*M. piperita*13	0.15	0.13	0.22	0.18	0.21	0.18	0.21	0.25	0.23	0.18	0.20	0.14	1.00				
*M. piperita*14	0.17	0.28	0.24	0.18	0.18	0.23	0.27	0.23	0.21	0.45	0.52	0.29	0.18	1.00			
*M. piperita*15	0.16	0.17	0.20	0.21	0.22	0.19	0.25	0.25	0.25	0.21	0.22	0.21	0.34	0.23	1.00		
*M. piperita*16	0.20	0.18	0.26	0.25	0.21	0.24	0.36	0.27	0.30	0.23	0.26	0.25	0.2	0.22	0.19	1.00	
*M. suaveolens*17	0.17	0.20	0.19	0.14	0.18	0.19	0.24	0.15	0.18	0.24	0.25	0.24	0.15	0.24	0.18	0.19	1.00

1 = *M. spicata*, 2 = *M. spicata*, 3 = *M. spicata*, 4 = *M. spicata*, 5 = *M. longifolia*, 6 = *M. longifolia*, 7 = *M. spicata*, 8 = *M. spicata*, 9 = *M. spicata*, 10 = *M. spicata*, 11 = *M. longifolia*, 12 = *M. spicata*, 13 = *M. piperita*, 14 = *M. piperita*, 15 = *M. piperita*, 16 = *M. piperita*, 17 = *M. suaveolens*

Table 3: Range of similarity coefficient values within *Mentha* species

No.	*Mentha* species	Range
1	*M. spicata*	0.18-0.45
2	*M. piperita*	0.18-0.34
3	*M. longifolia*	0.22-0.28

with PCR. We checked the reproducibility of our RAPD procedures and found an appropriate level of robustness among duplicate reactions. Out of a total of 70 Genset primers evaluated, we selected 31 (44%) based on the quality and reliability of their amplification, which were applied to the mints under study. The amplification profiles produced by 31 primers gave a total of 617 bands, out of which only nine were monomorphic, while the rest 608 were polymorphic (Table 1). The number of bands generated per primer varied from 1(OPG70) to 32 (OPG68) and presented molecular weights between 409 to 3446 bp.

All mint accessions studied presented different patterns when amplified with the set of 31 primers selected. One of the main objectives of the study was to analyse the genetic similarity/distance between these species of mints. Amplified polymorphic DNA fragments were scored for analysis based upon Jaccard's, Nei and Li's and simple matcing's similarity coefficients.

As expected, Nei and Li's (1979) and simple matcing's value were higher than Jaccard's. The average Jaccard's, Nei and Li's and simple matching's coefficients across pairwise combinations were 0.25, 0.35 and 0.78 and ranged from 0.13 to 0.69 (Table 2), from 0.23 to 0.82 and from 0.64 to 0.91, respectively. In addition, several studies have indicated that the Jaccard's similarity coefficients is equal to and superior in some situations to other clustering methods such as simple matching in RAPD marker. We used Jaccard's similarity coefficients for clustering analysis.

Jaccard's similarity coefficients ranged from 0.13 to 0.68 across all 17 accessions. The range of similarity coefficients values within *M. longifolia*, *M.* ×*piperita* and

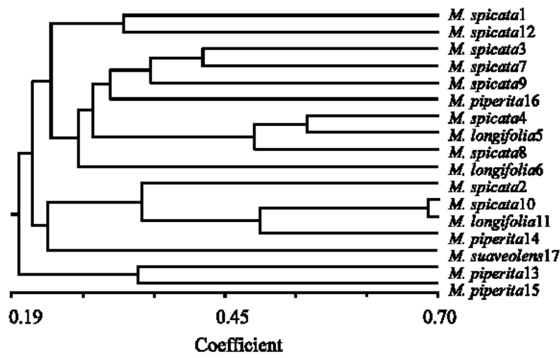

Fig. 1: Dendrogram generated using Jaccard's coefficient of *Mentha* accessions based on RAPD data

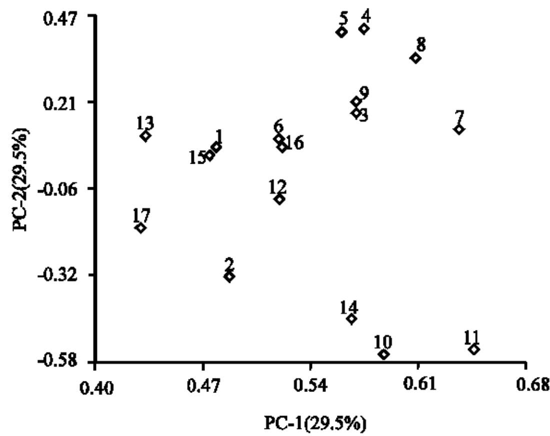

Fig. 2: Principal coordinate plot based of Jaccard's genetic similarity for the first two principal component

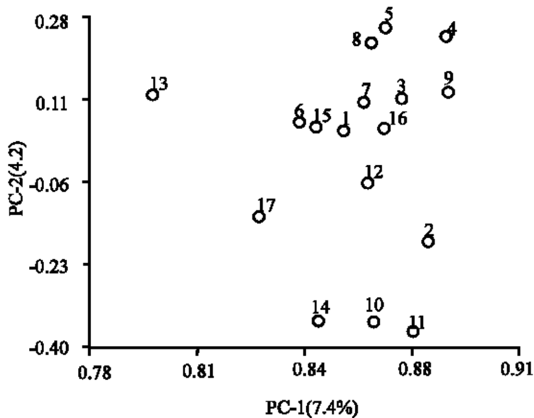

Fig. 3: Principal coordinate plot based of simple matching's genetic similarity for the first two principal component

M. spicata were 0.22-0.28, 0.18-0.34 and 0.18-0.45, respectively, (Table 3). We used UPGMA from the NTsys-Pc program to based Jaccard's, Nei and Li's and simple matching's genetic similarity coefficients to produce RAPD marker-derived dendrograms (Fig. 1). Dendrograms based on three similarity coefficients were showed similarity. However, at these dendrograms, two major clusters were formed at different genetic distances. Based on Jaccard's similarity coefficients 2 major clusters were formed at 80% genetic distance: (I) *M. piperita* 13 along with *M. piperita*15 were grouped into one and had 19.6% similarity with the rest of accessions; and (II) the rest of accessions. The most well known hybrid, *M. ×piperita* (peppermint) showed a range of similarity coefficients, from 0.18 to 0.34 and was separated by UPGMA into two clusters.

There were two distinct sub-clusters in cluster II: (1) *M. spicata*2, *M. spicata*10, *M. longifolia*11, *M. piperita*14 and *M. suaveolens*17 and (2) *M. longifolia*5, *M. longifolia*6, *M. piperita*16 and the rest of accessions of *M. spicata*. Within sub-cluster 2, two subgroups are clearly defined: (1) *M. spicata*1 and *M. spicata*12 and (2) *M. spicata*3, 4, 7, 8, 9, *M. longifolia*5, 6 and *M. piperita*16. In the subcluster1, maximum similarity observed between *M. spicata*10 and *M. longifolia*11 (0.69).

Jaccard's, Nei and Li's and simple matching's genetic similarity coefficients were used for principal coordinates analysis (Fig. 2 and 3). The first principal component of Jaccard's and Nei and Li's genetic similarity coefficients respectively explained about 29.5 and 42.9% of total variation among accessions. Whereas, The second principal component of Jaccard's and Nei and Li's coefficients explained about 9.2 and 8.09% of total variation among accessions, respectively. PCoA plots of Jaccard and Nei and Li were identical (Fig. 2). The first principal component of simple matching's genetic similarity explained about 74% of total variation among accessions, which separated *M. piperita*13 from the other accessions (Fig. 3).

DISCUSSION

It is essential to understand the extent and distribution of genetic variation within a breeding programme for best utilization of the germplasm available and to devise breeding strategies able to release new materials yet keeping appropriate levels of variability to support further genetic advances. Furthermore, information regarding genetic variability at the molecular level could be used to help identify and develop

genetically unique germplasm that complements existing cultivars. *Mentha*, a taxonomically complex section, was recently studied by Khanuja *et al*. (2000) by RAPD fingerprinting. This is the first report on the use of molecular markers for fingerprinting and evaluating genetic relationships of Iranian mints. The RAPD 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 degree of polymorphism displayed with RAPD in mint was high as expected from its allogamous nature. On average 19.9 bands were generated per primer a value higher than the 10.5 bands per primer reported by Khanuja *et al*. (2000) in other *Mentha* accessions.

The total number of polymorphic RAPD bands included in this study were 608 which is considered appropriate (Khanuja *et al*., 2000). The number of bands used in plants varies widely from 61 (Nienhuis *et al*., 1993) to 1205 (Smith *et al*., 1990). The percentage of polymorphic RAPD fragment detected in this collection of *Mentha* accessions is 98.7%, considerably higher than the 93.5% reported for a collection of diverse *Mentha* accessions (Khanuja *et al*., 2000), but lower than the 100% reported strawberry (Degani *et al*., 2001). RAPD-PCR analysis produces fingerprints with monomorphic bands and polymorphic bands. It is assumed that primer annealing sites are found at random throughout the genome (Caetano-Annolles and Gresshoff, 1997).

Although a larger of polymophic bands should provide better genome coverage, it would be more efficient to determine genetic relationships based upon the smallest set of bands providing enough reliability and discriminatory power. Reliability is an important issue when using RAPDs. Our RAPD results were reliable which is probably due to a combination of factors such as the careful preparation of reagent mixtures, the use of the same thermocycler across the study and also the volume of reaction, since reaction volumes under 25 μL do not generally provide an adequate reproducibility for RAPD reactions (Lowe *et al*., 1996). However, as it is difficult to check this, it is imperative that reaction conditions are specifically defined. Thus both the DNA isolation protocol and the PCR were accordingly optimised for RAPD-PCR analysis of *Mentha*.

Genetic similarity estimates based on molecular marker data have been described as a direct measure of genetic similarity (Graner *et al*., 1994, Russell *et al*., 1997). However, as with the coefficients of coancestry, the accuracy of genetic similarity estimates based on molecular data depends on several variable factors such as the number of markers analyzed, their distribution over the genome and the accuracy in scoring the markers (Schut *et al*., 1997). The number of markers analyzed affect the variance of the genetic similarity estimates, while distribution over the genome is expected to affect the variance of similarity estimate in the presence of significant linkage disequilibrium (Powell *et al*., 1996).

Analysis of these RAPD profiles for band similarity indices could clearly differentiate all the taxa of *Mentha* viz. *M. spicata*, *M. longifolia*, *M. ×piperita* and *M. suaveolens* from one another. *Mentha spicata* is a hybrid between *M. longifolia* and *M. suaveolens* (Harley and Brighton, 1977). These results, confirm the progenitor of *M. spicata*. Interestingly, *M. suaveolens*17, *M. piperita*14, *M. spicata*10, *M. spicata*2 and *M. longifolia*11 formed a subgroup in the cluster II with about 23% similarity. Our results, based on molecular markers showed that *M. suaveolens*, *M. longifolia*, *M. spicata* and *M. ×piperita* are closely related. In the view of taxonomists and studies on evolution of the mints, *M. ×piperita* is believed to be a hybrid between *M. spicata* and *M. aquatica* (Murray *et al*., 1972). *M. aquatica* is octaploid, while *M. spicata* is a tri or tetraploid. Therefore, two-thirds of the *M. ×piperita* genetic pool is composed of the *M. aquatica* genome. According to our molecular analysis, *M. piperita*13 and *M. piperita*15 showed the highest similarity with one of its octaploid progenitors, rather than with *M. spicata*. The primers provided enough RAPD polymorphisms to resolve genetic diversity between and within species. The similarity between these accessions was in agreement with their earlier observations. The PCoA results were in accordance with those of cluster analysis. Because of allogamy and polyploidy, according to UPGMA and PCoA analysis, a high degree of genetic diversity is expected among individual plants in mint accessions. Thus, these species are difficult to identify leading to taxonomic complexity. Cluster analysis proved to be more sensitive and reliable for detecting pedigree relationships among genotypes than PCoA when the first two or three PCS explained <25% of the total variation. In this to extract maximum information from the molecular marker data, that first two or three PCS explain >25% of the original variation PCoA can be used in combination with cluster analysis (Messmer *et al*., 1993).

The average genetic similarity coefficients 0.25 for Jaccard and 0.35 for Nei and Li among accessions suggested that the level of genetic diversity among *Mentha* accessions is too high which is probably due to

the allogamous nature of mint. Jaccard's genetic distance takes into consideration only matches between bands-alleles that are present and ignores pairs in which a band-allele is absent in both individuals. Nei and Li's genetic distance measures the proportion of bands-alleles shared as the result of being inherited from a common ancestor and represents the proportion of bands-alleles present and shared in both individuals divided by the average proportion of bands-alleles present in each individual. Simple matching's genetic distance takes into account mismatches and matches and gives equal weight to both in estimating genetic distance (Link *et al*., 1995; Johns *et al*., 1997). Whereas, the 1-1 matches in reality indicate more similarity than the 0-0 matches because there are many reasons for lack of amplification or absence of bands and a 0-0 match may not reflect identity by descent, but rather identity in state (Mohammadi and Prasana, 2003).

The degree of polymorphism displayed with RAPDs in *Mentha* was expected from a cross-pollinated nature. The analysis of molecular marker detected a larger fraction of genetic variation within species. This is in accordance with what would expected from a cross-pollinated species (Frankel *et al*., 1995). Phenetic analysis using UPGMA based on genetic similarity resulted in a highly resolved tree, that 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 RAPD markers, *M. ×piperita* is closer to *M. aquatica* than *M. spicata*. Thirty one primers provided enough RAPD polymorphisms to resolve genetic diversity between and whithin 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 desire genotypes (Gobert *et al*., 2002). 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 RAPD analysis in a larger collection seems evident.

The simultaneous use of several relationship measures such as AFLP (using selective primer pairs), RAPD and pedigree-based measures in a combined index as has been proposed by Cox *et al*. (1985) and Schut *et al*. (1997) would probably provide a more accurate measure of genetic relatedness than any of these measures alone.

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