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Molecular characterisation and similarity relationships among iranian basil *(Ocimum basilicum* L.) accessions using inter simple sequence repeat markers[1]

Caracterização molecular de acessos de *Ocimum basilicum* L. por meio de marcadores ISSR

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Abstract - The study of genetic relationships is a prerequisite for plant breeding activities as well as for conservation of genetic resources. In the present study, genetic diversity among 50 Iranian basil *(Ocimum basilicum* L.) accessions was determined using inter simple sequence repeat (ISSR) markers. Thirty-eight alleles were generated at 12 ISSR loci. The number of alleles per locus ranged from 1 to 5 with an average of 3.17. The maximum number of alleles was observed at the A7, 818, 825 and 849 loci, and their size ranged from 300 to 2500 bp. A similarity matrix based on Jaccard's coefficient for all 50 basil accessions gave values from 1.00-0.60. The maximum similarity (1.00) was observed between the "Urmia" and "Shahr-e-Rey II" accessions as well as between the "Urmia" and "Qazvin II" accessions. The lowest similarity (0.60) was observed between the "Tuyserkan I" and "Gom II" accessions. The unweighted pair- group method using arithmetique average UPGMA clustering algorithm classified the studied accessions into three distinct groups. All of the basil accessions, with the exception of "Babol III", "Ahvaz II", "Yazd II" and "Ardebil I", were placed in groups I and II. Leaf colour was a specific characteristic that influenced the clustering of Iranian basil accessions. Because of this relationship, the results of the principal coordinate analysis (PCoA) approximately corresponded to those obtained through cluster analysis. Our results revealed that the geographical distribution of genotypes could not be used as a basis for crossing parents to obtain high heterosis, and therefore, it must be carried out by genetic studies.

Key words - Basil. Cluster analysis. Molecular markers. Plant genetic diversity.

Resumo - O estudo das relações genéticas é um pré-requisito para atividades em reprodução de plantas assim como para conservação de recursos genéticos. Neste trabalho a diversidade genética entre 50 acessos de Manejericão Iraniano (*Ocimum basilicum* L.) foram determinadas usando marcadores de Seqüência Simples Repetida Interna (ISSR). Trinta e oito alelos foram identificados utilizando-se 12 locos ISSR. O número de alelos por lócus variou de 1 a 5 com uma média de 3,17. O máximo número de alelos foi observado em A7; 818; 825 e 849 locos, e seus tamanhos variaram de 300 a 2500 bp. Uma matriz de similaridade baseada no coeficiente de Jaccard para todas as 50 acessos obteve valores de 1,00-0,60. A máxima similaridade (1.00) foi observada nos acessos "Urmia" e "Shahr-e-Rey II" assim como entre os acessos "Urmia" e "Qazvin II". A menor similaridade (0,60) foi observada entre os acessos "Tuyserkan I" e "Gom II". O algoritmo de análise de agrupamentos foi o método da distância média usando a média aritmética (UPGMA) que classificou os acessos estudadas em três distintos grupos. Todos os acessos do manjericão com exceção de "Babol III", "Ahvaz II", "Yazd II" e "Ardebil I", foram localizadas nos grupos I e II. A cor da folha foi uma característica específica que influenciou o agrupamento dos acessos do Manjericão Iraniano. Devido a esta relação, os resultados da análise de coordenada principal (PCoA) aproximadamente corresponde à aquela obtida pela análise de agrupamentos. Os resultados revelam que a distribuição geográfica dos genótipos não pode ser usada como base para parentais cruzados com alta heterosis e, portanto devem ser conduzidos estudos genéticos para tais fins.

Palavras-chave - Manjericão. Análise de agrupamentos. Marcadores moleculares. Diversidade genética de plantas.

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Introduction

Ocimum basilicum L., known as common basil, is an annual aromatic herb belonging to the Lamiaceae family. It is a dicotyledonous and diploid plant with 2n = 48 chromosomes (PRAKASH, 1990). Among *Ocimum* spp., common basil is the most economic species to grow, and it is cultivated all around the world (MAROTTI *et al.*, 1996). Dried leaves of basil are used to flavour many products in the food industry. Its essential oil is used in the perfume and cologne, cosmetics, health, pharmaceutical and food industries (HARISARANRAJ *et al.*, 2008).

Based on Khosla's (1995) reports, the centre of basil diversity is in the tropical and subtropical regions of Africa, Asia and South America. Different chemotypes (plants of the same species that are chemically different but otherwise indistinguishable) and morphotypes have been reported in this species (MAROTTI *et al.*, 1996; PRAKASH, 1990).

To be most efficiently managed and effectively utilised, germplasm collections must be well characterised. In most cases, the identification of cultivars, lines and hybrids is based on morphological traits. However, the number of these traits is limited; they are unstable and they do not always enable one to distinguish between closely related accessions or cultivars (KONAREV *et al.*, 2000). Cultivar identification based on phenotypic traits is often influenced by the environment, making classification difficult.

On the other hand, basil taxonomy is complex because of the occurrence of inter-/intra-specific hybridisation and morphological similitude (DE MASI *et al.*, 2006). Sometimes, taxonomists defined a number of designations as synonymous and confused different varieties as homonyms.

The emergence of PCR-based molecular markers, such as randomly amplified polymorphic DNA (RAPDs), microsatellite or simple sequence repeats (SSRs) and amplified fragment length polymorphisms (AFLPs) has created the opportunity for fine-scale genetic characterisation of germplasm collections (POWELL *et al.*, 1996; SEMAGN *et al.*, 2006). The major limitations of these methods are the low reproducibility of RAPD, the high cost of AFLP and the need to know the flanking sequences to develop species-specific primers for SSR polymorphism.

The use of inter simple sequence repeat (ISSR-PCR) markers is a molecular technique that overcomes most of these limitations (PRADEEP REDDY *et al.*, 2002). It is rapidly being adopted by the research community in various fields of plant improvement (GODWIN *et al.*, 1997; RATNAPARKHE *et al.*, 1998). This technique is useful in the areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology and in a wide range of crop species. ISSR techniques are nearly identical to RAPD techniques except that ISSR primer sequences are designed from microsatellite regions, and the annealing temperatures used are higher than those used for RAPD markers. Based on the published, unpublished and in-progress studies that have been conducted using ISSR markers, it is clear that ISSR markers have great potential for studies of natural populations reviewed in (WOLFE *et al.*, 1998). ISSRs differentiate mostly as dominant markers following simple Mendelian inheritance (PRADEEP REDDY *et al.*, 2002; WANG *et al.*, 1998). These markers, due to their reproducibility, are becoming more popular and easier to use than other markers such as RAPD and AFLP (CHENNAOUI-KOURDA *et al.*, 2007).

Previous researchers utilised RAPD-PCR for the identification and characterisation of basil cultivars (DE MASI *et al.*, 2006; HARISARANRAJ *et al.*, 2008; IBTISAM, 2008; VIEIRA *et al.*, 2003). Harisaranraj *et al.* (2008) investigated the relationship between seven species of basil using 15 RAPD primers. They found close relation between *O. basilicum* and *O. tenuiflorum*.

The study of genetic relationships is a prerequisite for plant breeding activities as well as for conservation of genetic resources. In the present study, we report the genetic diversity among Iranian native basil accessions originating from different regions based on ISSR markers.

Materials and methods

Plant material and DNA extraction

Fifty basil accessions collected from different geographical regions of Iran (TAB. 1 and FIG. 1) were investigated. Basil accessions were grown in controlled conditions, and single-leaf samples from 2-week-old seedlings corresponding to 15 plants of each accession were taken for bulk DNA (DE MASI et al., 2006), frozen in liquid nitrogen and then maintained at -80 °C. Samples were ground to powder in the presence of liquid nitrogen. Genomic DNA was extracted using a CTAB-based method (DE MASI *et al.*, 2006). DNA concentration was measured at 260 nm in a spectrophotometer, and the quality of DNA was checked by running of 1 µl of genomic DNA on an 0.8% agarose gel prepared in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0).

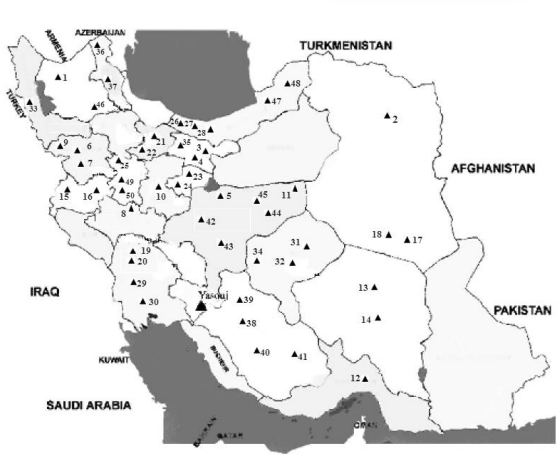
Table 1 - Name, type, and origin of 50 Iranian basil accessions used to evaluate their genetic diversity by inter simple sequence repeat (ISSR) marker

Code	Accession name	Elevation (m)	Latitude East	Longitude North	Special characters
1	Tabriz	1366	38.06	46.26	Green
2	Mashhad	980	36.19	59.37	Green
3	Shahr rey I	1100	51.40	35.70	Green
4	Shahr rey II	1100	51.40	35.70	Purple
5	Isfahan	1580	32.40	51.45	Green
6	Kordestan I	1800	35.18	47.06	Green
7	Kordestan II	1800	35.18	47.06	Purple
8	Lorestan	1147	33.26	45.17	Green
9	Kordestan	1800	35.18	47.06	Purple
10	Mahallat	1775	33.88	50.50	Green
11	Kashan	982	33.98	51.58	Green
12	Hormozgan	325	27.37	55.51	Purple
13	Kerman I	1755	30.30	57.10	Green
14	Kerman II	1755	30.30	57.10	Purple
15	Kermanshah I	1389	34.23	47.03	Green
16	Kermanshah II	1389	34.23	47.03	Purple
17	Birjand I	1491	59.13	32.52	Green
18	Birjand II	1491	59.13	32.52	Green
19	Dezful I	39	32.23	48.27	Green
20	Dezful II	39	32.23	48.27	Purple
21	Qazvin I	1800	36.15	50.01	Green
22	Qazvin II	1800	36.15	50.01	Purple
23	Gom I	928	34.49	50.56	Purple
24	Gom II	928	34.49	50.56	Green
25	Hamedan	1824	36.46	48.34	Green
26	Babol I	-14	36.33	52.42	Green
27	Babol II	-14	36.33	52.42	Purple
28	Babol III	-14	36.33	52.42	Green
29	Ahvaz I	39	31.24	48.49	Green
30	Ahvaz II	39	31.24	48.49	Purple
31	Yazd I	1230	32.00	54.40	Green
32	Yazd II	1230	32.00	54.40	Purple
33	Urmia	1267	37.34	44.58	Green
34	Yazd	1230	32.00	54.40	Green
35	Varamin	1100	35.19	51.39	Green
36	Ardebil I	1500	38.15	48.17	Green
37	Ardebil II	1500	38.15	48.17	Purple
38	Shiraz I	1486	29.39	52.35	Green
39	Shiraz II	1486	29.39	52.35	Green
40	Shiraz III	1486	29.39	52.35	Purple

Continuation da Table 1

41	Shiraz IIII	1486	29.39	52.35	Purple
42	Isfahan I	1580	32.40	51.45	Green
43	Isfahan II	1580	32.40	51.45	Green
44	Isfahan III	1580	32.40	51.45	Purple
45	Isfahan IIII	1580	32.40	51.45	Green
46	Maragheh	1267	46.16	37.23	Purple
47	Gorgan I	155	36.50	54.25	Purple
48	Gorgan II	155	36.50	54.25	Green
49	Tuyserkan I	1784	34.40	48.20	Purple
50	Tuyserkan II	1784	34.40	48.20	Green

Figure 1 - Geographical distribution of the 50 Iranian basil accessions used to evaluate genetic diversity using 12 ISSR markers. Numbers in the map show the basil accessions' codes. For genotype names corresponding to each code, see Table1



Polymerase chain reaction and data analysis

Polymerase chain reaction (PCR) was performed in a 25-µl volumes containing 12.5 µl of PCR master mix [200 mM Tris–HCl pH 8.55, 160 mM $(NH_4)_2SO_4$ 0.1% (v/v), 3.0 mM $MgCl_2$, 0.4 mM of dNTPs, 1.0 U of Taq DNA polymerase](CinnaGen Inc., Tehran, Iran), 2 µM ISSR primer (CinnaGen Inc., Tehran, Iran), 50 ng of genomic DNA and ddH_2O. DNA amplifications were performed using a GeneAmp PCR System 9700 Thermocycler (Perkin Elmer, Applied Biosystems, USA) programmed for a preliminary step of 3 min at 95 ºC, followed by 35 cycles of 93 ºC for 30 s, 35-58 ºC (depending on primer sequence) for 45 s and 72 ºC for 45 s. A final extension step of 10 min at 72 ºC was performed. The reaction products were then mixed with an equal volume of formamide dye [98% (v/v) formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol], resolved in a 1.5% (w/v) agarose gel in 0.5X TBE, visualised with ethidium bromide (1.0 μgml^{-1}) and photographed under UV light.

The PCR amplification products were scored for the presence (1) or absence (0) of each band marker across all 50 accessions, and the data were used to construct a binary data matrix. Different methods were used for constructing similarity matrices and dendrograms. The efficiency of clustering algorithms and their goodness of fit were determined based on the cophenetic correlation coefficient. In addition to this cluster analysis, a principal coordinate analysis (PCoA) was used to confirm the results of the cluster analysis. PCoA is a low-dimensional graphical plot that is used to depict the relationships among the genotypes studied. Data analyses were performed using NTSYS-pc version 2.11 software (ROHLF, 1998).

Results and discussion

Thirty-eight alleles were generated at 12 ISSR loci. The number of alleles per locus ranged from one to five, with an average of 3.17 (TAB. 2). The maximum number of alleles was observed at the 'A7', '818', '825' and '849' loci (TAB. 2), and their size ranged from 300 to 2500 bp.

Different methods were used for constructing similarity matrices and dendrograms (TAB. 3). The cophenetic correlation, a measure of the correlation between the similarity represented on the dendrograms and the actual degree of similarity, was calculated for each dendrogram (TAB. 3). Among the different methods, the highest value ($r = 0.63$; $P \leq 0.05$) was observed for UPGMA based on Jaccard's coefficient (TAB. 3). Therefore, the dendrogram constructed

based on this method was used for depicting the genetic diversity of accessions (FIG. 3). A similarity matrix based on Jaccard's coefficient for all 50 basil accessions gave values from 1.00-0.60. The average pairwise genetic similarity was 0.73. The maximum similarity (1.00) was observed between the "Urmia" and "Shahr-e-Rey II" accessions as well as between the "Urmia" and "Qazvin II" accessions. The lowest similarity (0.60) was observed between the "Tuyserkan I" and "Gom II" accessions.

Table 2 - Name, sequence and number of alleles for 12 ISSR primers

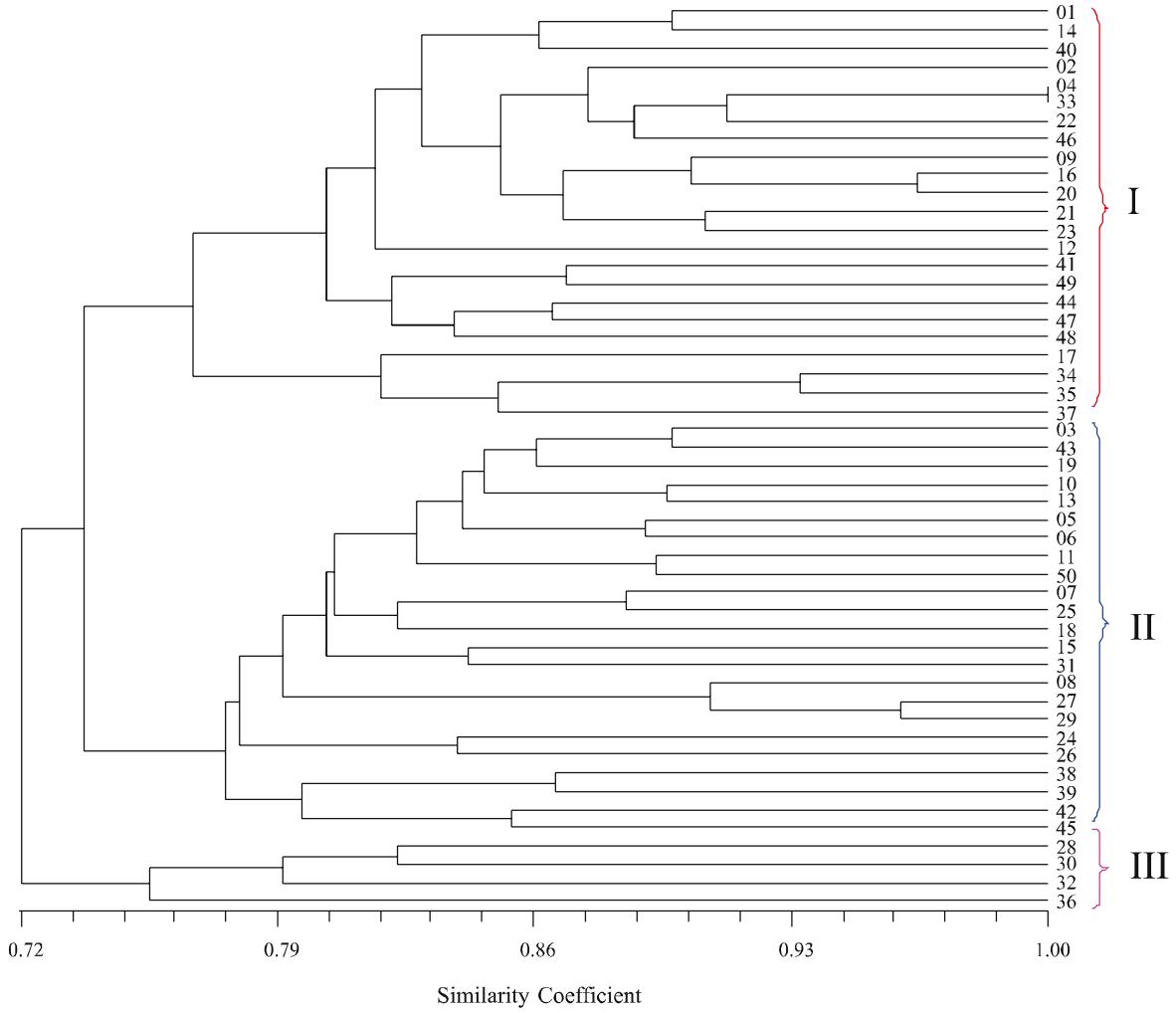
Primer code	Sequence (5´→3´)	Total bands	Polymorphic bands	Annealing Temperature
A7	agagagagagagagagagt	9	5	58 °C
A12	gagagagagagacc	8	1	52 °C
818	cacacacacacacag	8	5	56 °C
825	acacacacacacacact	8	5	55 °C
849	gtgtgtgtgtgtgtcg	7	5	55 °C
443	acacacacacacacacact	6	3	54 °C
426	caccaccaccaccaccaccact	6	2	54 °C
CA&AC	cacacacacacaac	2	1	35 °C
CAg5	cagcagcagcagcag	6	4	54 °C
CTC4RC	ctcctcctcctcrc	7	3	38 °C
CAA5	caacaacaacaacaa	6	3	41 °C
459	tgctgctgctgctgctgcc	5	1	54 °C
Total		78	38	
Mean		6.5	3.17	

Table 3 - Comparison of different methods for constructing the similarity matrix and dendrogram using the co-phenetic coefficient

Similarity matrices	Algorithm	Co-phenetic coefficient (r)*
D	Complete linkage	0.55
D	Single linkage	0.39
D	UPGMA	0.62
H	Complete linkage	0.56
H	Single linkage	0.34
H	UPGMA	0.59
J	Complete linkage	0.56
J	Single linkage	0.40
J	UPGMA	0.63
Phi	Complete linkage	0.27
Phi	Single linkage	0.36
Phi	UPGMA	0.48
SM	Complete linkage	0.56
SM	Single linkage	0.34
SM	UPGMA	0.59

*A measure of how successful cluster analysis has been in partitioning the data. D: Dic (NEI; LI, 1979); H: Hamann (HAMANN, 1961); J: Jaccard (JACCARD, 1908); Phi: Pearson's Phi coefficient (SOKAL; SNEATH, 1963); SM: Simple Matching (SNEATH; SOKAL, 1973). UPGMA: Un-weighted pair-group method using arithmetic average

Figure 2 - Dendrogram of 50 Iranian basil accessions generated by the UPGMA clustering method based on a Jaccard's coefficient of similarity matrix. Numbers in the dendrogram show the basil accessions' codes. For genotype names corresponding to each code, see Table1



The UPGMA clustering algorithm classified the studied accessions into three clusters (FIG. 2). The first, second and third groups comprised 46%, 46% and 8% of accessions, respectively (FIG. 2). All of the basil accessions, with exception of "Babol III", "Ahvaz II", "Yazd II" and "Ardebil I," were placed in group I or II. In group I, the highest similarity value (1.00) was observed between the "Urmia" and "Shahr-e-Rey II" accessions as well as between "Urmia" and "Qazvin II" accessions. Group I was further divided into two subgroups. In group II, the highest similarity value (0.96) was found between the "Babol II" and "Ahvaz I" accessions. Group II was further divided into three subgroups. In group III, the highest similarity value (0.82) was found between the "Babol III" and "Ahvaz II" accessions. Compared to group II and III, the pairwise similarity in group I was higher. In other words, the accessions in group I were clustered together at a higher similarity value.

Generally, accessions with purple leaves were located in the first group (FIG. 3c). Interestingly, the accessions with green leaves located in this group were not completely green-leaved but also included accessions that have green leaves with purple veins (FIG. 3b). Accessions with green leaves were located in the second group (FIG. 3a). Our results showed that accessions with purple and green leaves from the same location were classified into two separate groups. For instance, we studied two accessions from "Shahr-e-rey", one with purple leaves and another with green leaves. The purple-leaved accession was located in group I, and the green-leaved accession was classified

Figure 3 - Different types of basil accessions growing in Iran. a) Green-leaved accessions; b) Green-leaved accession with purple veins; c) Purple-leaved accession

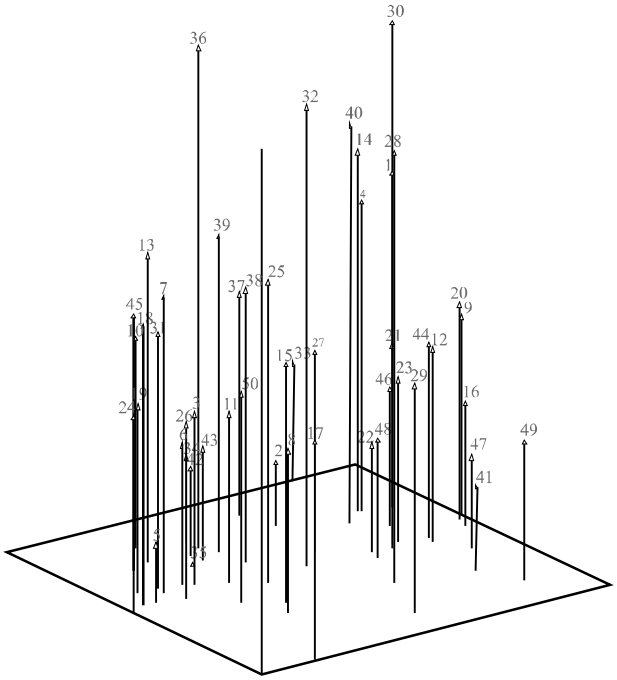
into group II. This was also seen for accessions from "Kordestan", "Kerman", "Kermanshah", "Birjand", "Dezful" and "Isfahan". De Masi *et al.* (2006) studied the genetic similarity among 12 basil genotypes with RAPD markers and identified two main groups, the first of which consisted of purple-leaf cultivars. Purple-leaf genotypes are used in food industries to aromatise food.

The clustering of a large number of genotypes in two groups (I and II) revealed low genetic variability that may be due to (a) the autogamous propagation of cultivated basil, which reduces genetic variability and (b) the biodiversity reduction in cultivars used in agriculture, as growers use fewer genotypes for seed production (DARRAH, 1980). In accordance with the above, in northwest Iran (Azerbaijan and Maragheh), the majority of cultivated genotypes have purple leaves, whereas in the centre (Tehran and its suburbs), green-leaved basil known as "Tehrani" basil is grown.

It was obvious that the genetic relationships among the studied accessions did not have a strong tendency to associate with their geographic origins. Murthy and Arunachalam (1966) showed that genetic drift and selection in different environments can cause greater diversity among genotypes than geographic distance. Therefore, selection of parental material in breeding programmes simply based on geographic diversity may not be rewarding. One possible reason for the genetic similarity among germplasm from different regions is that the materials might have originally been introduced from the same region. Crossing between clusters with maximum inter-cluster distance may be result in high heterosis. It is well documented that crosses between unrelated, and consequently genetically distant parents, show greater hybrid vigour than crosses between closely related genotypes (REIF *et al.*, 2007; SOLOMON *et al.*, 2007).

Principal coordinate analysis (PCoA) showed that the first three PCs explained 38.14% of the cumulative variation. These three PCs were then used to design a principal coordinate plot to identify the diversity pattern of studied genotypes (FIG. 4). The principal coordinate plot showed the close genetic relationship among the "Tabriz"(1), "Shahr-e-Rey II"(4), "Kerman II"(14) and "Shiraz III"(40) accessions and among the "Kordestan III"(9), "Kermanshah II"(16) and "Dezful II"(20) accessions, which had also been observed in cluster analysis (FIG. 2). Our results showed that the PCoA data approximately corresponded to those obtained through cluster analysis. This is in agreement with other findings (DARVISHZADEH *et al.*, 2010; KUMAR *et al.*, 2009; SORKHE *et al.*, 2007).

Figure 4 - Three-dimensional graph from the principal coordinate analysis of 12 ISSR markers of 50 Iranian basil accessions. Genotype codes: see Table 1



Conclusions

1. In the present study, we characterised and identified the genetic relationships of 50 Iranian basil accessions using ISSR markers for the first time. We found relatively acceptable genetic diversity within available basil accessions. The studied accessions were classified into three distinct groups. Some accessions share specific characteristics that influence their clustering;

2. Assessment of the genetic variability within plant species is a prerequisite for plant breeding programs and has an important role in the conservation of plant genetic resources. It is particularly useful in the characterisation of individual accessions and cultivars, in detecting duplications of genetic material in germplasm collections, and as a general guide in the choice of parents for hybrid breeding programs.

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