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MOLECULAR CHARACTERIZATION OF *THYMUS* SPECIES USING ISSR MARKERS

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ABSTRACT

Thyme (*Thymus spp*.), a famous herb from Lamiaceae/Labiateae family is a valuable medicinal plant. In this study, fourteen accessions of *Thymus* species including *T. daenensis*, *T. kotschyanus* and *T. vulgaris* have been genetically fingerprinted by Inter simple sequence repeat (ISSR) markers. The 20 ISSR primers amplified 334 fragments, of which 325 were polymorphic. Number of amplified fragments ranged from 4 to 30 and their size was 200-2800 bps. Effective multiplex ratio (EMR) had wide-ranging data from 4 to 30 with an average of 15.84. Polymorphism information content (PIC) data were between 0.33 and 0.49. The mean of marker Index (MI) and resolving power (RP) were 6.63 and 10.64, respectively. The ISSR genotyping data were used to evaluate genetic diversity and the relationships among the accessions using UPGMA cluster analysis and principal coordinate analysis (PCoA). Both of the methods classified the 14 *Thymus* accessions in five sets and presented analogous grouping of the genotypes with some minor differences. The accessions were relatively grouped according to the location where they were collected. The analyzed ISSR markers created sufficient polymorphism and reproducible fingerprinting profiles and provided a powerful and reliable molecular tool for detecting genetic variation and relationships.

Key words: genetic diversity, genetic polymorphism, ISSR, marker parameters, medicinal plant, *Thymus*

INTRODUCTION

Some genera and species of medicinal plants such as *Thymus* are more important than other ones due to secondary metabolites and phytochemical compounds. The genus *Thymus* consists of about 300 species of herbaceous, perennials and shrubs or subshrubs distributed mainly over Mediterranean countries, Northern part of Africa and Southern Greenland (Sunar *et al*., 2009). This genus is usually used for flavoring agents, herbal tea, and medicine. The aerial parts and volatile constituents of thyme are used as a medicinal material (Stahl-Biskup and Saez, 2002). The aromatic and medicinal properties of *Thymus* have made it one of the most popular medicinal herbs. *Thymus* essential oil is among the world's best ten essential oils (Rahimmalek *et al*., 2009). At present, the demand of essential oils for this herb is raised for perfumery, cosmetic and medicinal use deprived of any breeding programs to select proper cultivars. In traditional herbal medicine, *Thymus* species are greatly used as tonic, antiseptic, antitussive and carminative (Amin, 2005; Ghasemi Pirbalouti *et al*., 2011). Two important species of *Thymus*, *Thymus daenensis* and *Thymus kotschyanus*, in Iranian folk medicine are more greatly used as fresh or dried for these objectives (Rahimmalek *et al.*, 2009).

Wild relatives of crops are usually used as principle resource of genetic variation for new breeding programs, while wild medicinal plants are actually in danger of extinction. Therefore genetic relationship of these plants is a critical subject (Fracaro and Echeverrigaray, 2006; Rahimmalek *et al*., 2009). Knowledge about genetic basis of medicinal plants populations threatened by extinction is an essential factor to perform the conservation programs. The unique genetic makeup of plant populations not only discriminates them from other populations, but also defines their ability to adapt to changing conditions and, potentially, to create new species. Many conservationists would discuss that the conservation of genetic diversity is the foundational basis of all conservation efforts because genetic diversity is requisite for evolutionary adaptation, and such adaptation is the key to the long-term survival of any species (Dyke, 2008). The habitat fragmentation and the spatial distance of populations increase genetic drift and differentiation among them, and reduce their future adaptation to environmental changes (Ben El Hadj Ali *et al*., 2012).

It is obvious that plant breeding, crop improvement and conservation programs depend on the availability of genetic diversity. Molecular markers can be used to study the genetic diversity and genetic relationships among *Thymus* ecotypes at DNA level. Study on population genetic diversity by means of various molecular marker systems can be useful for characterization and protection of genetic resources of important medicinal plants (Narasimhan *et al*., 2006; Padmesh *et al*., 2006). Molecular markers such as AFLP,

ISSR, RAPD and SSR are widely used marker systems in DNA fingerprinting. Inter-simple sequence repeats (ISSRs) are widely used in genetic diversity investigations since they require no prior DNA sequence information, their development costs are low and laboratory procedures can easily be transferred to any plant species (Barth *et al*., 2002).

A few reports are available about genetic diversity of thyme. Some species of this aromatic plant are endemic to Iran. The aims of this study were molecular characterization and detecting genetic diversity of *Thymus* accessions using ISSR markers.

MATERIALS AND METHODS

Plant materials: The seeds of thirteen ecotypes of *Thymus* species including *Thymus daenensis* and *Thymus kotschyanus* grown in different parts of Iran, including 5 accessions from North west of Iran, 4 from central regions, 3 from West of Iran, 1 from north of the country, and 1 exotic accession namely *Thymus vulgaris* from London, England were used in the present study (Table 1). The plant materials were provided by Research Institute of Forest and Rangelands, Tehran, Iran. The seeds were planted in glass vials under in vitro conditions in July 2010. The culture medium for seedling production was MS medium (Murasighe and Skoog, 1962). The aerial parts of the in vitro cultured plant samples were collected and stored at -80°C.

Table 1. Plant accessions of *Thymus spp.* collected from different parts.

Accession code	Gen bank code (RIFR)	Scientific name	Country	Province	Place of seed collection
1	18209	*T. daenensis*	Iran	Isfahan	Buin-Daran
2	21118	*T. kotschyanus*	Iran	Yazd	Galuyak farm – Nedushan
3	27471	*T. kotschyanus*	Iran	West Azarbaijan	Sardasht
4	27800	*T. kotschyanus*	Iran	Ardabil	Sarein
5	13206	*T. kotschyanus*	Iran	Gilan	Deilaman – Siahkal
6	27814	*T. kotschyanus*	Iran	Ardabil	Pars Abad e Moghan
7	15656	*Thymus sp.*	Iran	Markazi	Shahrak e Mohajeran
8	27221	*T. daenensis*	Iran	Isfahan	Chadegan
9	25951	*T. kotschyanus*	Iran	Kurdistan	Ghorveh
10	7507	*Thymus sp.*	Iran	Lorestan	Khoram Abad – Zagheh
11	14245	*Thymus sp.*	Iran	Lorestan	Khoram Abad
12	10126	*T. daenensis*	Iran	Isfahan	Fereidunshahr
13	–	*Thymus sp.*	Iran	Ardabil	Parchin village
14	14287	*T. vulgaris*	England	London	–

Table 2. ISSR markers used for analysis of genetic diversity of *Thymus* ecotypes.

Primer name	Sequence (3'-5')	Annealing temperature (°C)
S1	$(GGGT)_2G$	44
S3	$(GA)_8T$	44
UBC-112	$(GACA)_4$	52
UBC-804	$(ATG)_6$	48
UBC-811	$(GA)_8C$	48
UBC-814	$(CT)_8A$	54
UBC-815	$(CT)_8G$	52
UBC-822	$(TC)_8A$	55
UBC-824	$(TC)_8G$	55
UBC-825	$(AC)_8T$	48
UBC-826	$(AC)_8C$	55
UBC-827	$(AC)_8G$	55
UBC-834	$(AG)_8TT$	54
UBC-841	$(GACAC)_4$	52
UBC-845	$(CT)_8TT$	48
UBC-852	$(TC)_8AA$	48
UBC-864	$A(CTGA)_3CTG$	52
UBC-868	$(GAA)_6$	52
UBC-876	$(GATA)_2(GACA)_2$	48
UBC-880	$(GGAGA)_3$	52

DNA extraction and ISSR amplification: Young leaves and stems were harvested from all the ecotypes and used for DNA isolation using the CTAB method described by Murray & Thompson (1980). Twenty ISSR primers were used for screening all the accessions and revealing the genetic diversity (Table 2). PCR amplification was conducted according to Williams *et al*. (1990) with the exception that the reactions were performed in a volume of 25-µl in a CORBETT Research thermocycler. Amplified PCR products were run in 1.2% agarose gels. Gels were stained with ethidium bromide, visualized with a UV transilluminator and were used for data analysis.

Statistical Analysis: To calculate ISSR polymorphism, a binary data matrix was made based on the marker data. ISSR markers were scored for the presence (1) or absence (0) of amplified bands for each of 14 samples. The ISSR binary data matrix was used to calculate the Jaccard's similarity coefficients. Cluster analysis was performed via UPGMA method using NTSYS-pc software version

2.02 (Rohlf, 2000). Principal coordinate analysis (PCoA) was also carried out by this software.

For each ISSR marker, total amplified bands, number of polymorphic bands, and percentage of polymorphic bands (PPB) were recorded. To measure the informativeness of the ISSR markers to differentiate between the thyme genotypes, polymorphism information content (PIC), effective multiplex ratio (EMR), marker Index (MI) and resolving power (RP) were calculated. PIC was calculated according to the formula of Anderson *et al*. (1993), as PIC = $1-\sum p_i^{\ 2}$, where p_i is the frequency of the *i*th allele of the locus in the set of fourteen thymus accessions. EMR is the product of the fraction of polymorphic bands and the number of polymorphic bands (Kumar *et al*., 2009; Najaphy *et al*., 2011). MI was determined according to Powell *et al*. (1996) as the product of PIC and EMR. RP was calculated using the formula $RP=\sum I_b$, where I_b is band informativeness and $I_b= 1-[2\times(0.5-p)]$, where p is the proportion of genotypes containing the band (Altintas *et al.*, 2008).

RESULTS AND DISCUSSION

Marker polymorphism: The twenty ISSR primers amplified a total of 334 bands in the set of fourteen *Thymus* accessions, of which 325 bands showed polymorphism. The number of bands for each primer ranged from 4 for primer S3 to 30 for primer UBC-824 with sequences of $(GA)_8T$ and $(TC)_8G$, respectively. Percentage of polymorphic bands (PPB) ranged between 75 and 100 with an average of 96.76% (Table 3). Rahimmalek *et al*. (2009) reported that the number of products per primer varied from 11 in P9 with sequence of $(AG)_8T$ to 25 in P11 with sequence of $(CA)_8RT$, and the 15 ISSR primers chosen for analysis revealed 256 bands, of which 228 (88.9%) were polymorphic. Mean numbers of bands and polymorphic bands per primer were 16.7 and 16.25, respectively. The size of amplified bands varied from 200 to 2800 bps. The percentage of polymorphic bands (PPB) varied from 75% in primer UBC-822 with sequence of $(TC)_8G$ to 100% in 13 different primers with an average of 80.2% (Table 3). Such high level of polymorphism is comparable to the results of some similar molecular researches on medicinal plants of Lamiaceae family (Liu *et al*., 2006; Agostini *et al*., 2008; Trindade *et al*., 2008; Agostini *et al*., 2010).

The ISSR primers with dinucleotide motifs $(CT)_n$, $(AC)_n$, $(TC)_n$ and $(AG)_n$ yielded a high level of polymorphism (Table 3). The similar results were obtained in other researches, such as the SSR repeat units, TG, GT, GA, AG, AC, CA, TC, ACC and AGC were informative primers to amplify DNA of *Tadehagi*, a group of legume semi-shrubs in Southeast Asia. For instance, all primers with the repeat units of GT, TG, CA, AC and GA showed stable amplification and rich polymorphism (Liu *et al*., 2010). In our investigation, the primers containing TC and CT motifs generated sharper bands in *Thymus spp.* accessions than those with other motifs (Fig 1).

Table 3. Marker parameters of genetic variation calculated for ISSRs

Primer	Total amplified bands	No. of polymorphic bands	PPB[a]	PIC[b]	EMR[c]	MI[d]	RP[e]
S1	11	11	100	0.49	11	5.42	9.71
S3	4	4	100	0.49	4	1.99	3.85
UBC-112	14	14	100	0.36	14	5.17	6.85
UBC-804	13	13	100	0.48	13	6.27	10.57
UBC-811	10	10	100	0.40	10	4.01	5.57
UBC-814	16	16	100	0.45	16	7.30	11.28
UBC-815	11	10	90.90	0.49	9.09	4.54	9.71
UBC-822	8	6	75	0.47	4.5	2.12	7.42
UBC-824	30	30	100	0.33	30	10.02	12.71
UBC-825	7	6	85.71	0.49	5.14	2.55	5.57
UBC-826	24	24	100	0.38	24	9.34	12.71
UBC-827	24	23	95.83	0.45	22.04	9.99	16
UBC-834	15	15	100	0.47	15	7.07	11.42
UBC-841	17	17	100	0.35	17	6.04	7.85
UBC-845	13	13	100	0.38	13	5.04	6.85
UBC-852	24	24	100	0.35	24	8.53	11.57
UBC-864	25	23	92	0.43	21.16	9.20	14.71
UBC-868	23	23	100	0.39	23	9.07	12.42
UBC-876	24	23	95.83	0.48	22.04	10.71	19.14
UBC-880	21	20	95.23	0.43	19.04	8.25	13.42
Total	334	325					
Minimum	4	4	75	0.33	4	1.99	3.85
Maximum	30	30	100	0.49	30	10.71	19.14
Mean	16.70	16.25	96.52	0.42	15.84	6.63	10.46

[a] Percentage of polymorphic bands; [b] Polymorphism information content; [c] Effective multiplex ratio; [d] Marker index; [e] Resolving power

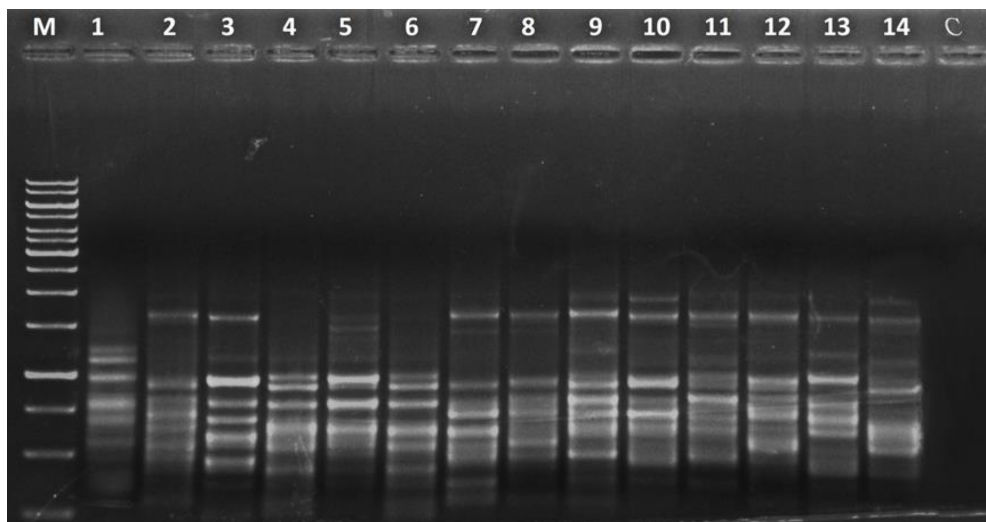

Fig. 1 Gel electrophoresis pattern of ISSR amplification using primer UBC-815.
M: Size marker; numbers 1 to 14 refer to accessions codes according to table 1; C: control (distilled water).

Polymorphism information content (PIC): Polymorphism information content is the probability of detection of polymorphism by a primer/primer combination between two randomly drawn genotypes and depends on the number of detectable alleles and the distribution of their frequency. Because of the high credibility of the PIC index, this parameter has been used widely in numerous genetic diversity researches (Hou *et al*., 2005; Kayis *et al*., 2010; Najaphy *et al*., 2011; Saleh, 2011; Dalamu *et al*., 2012; El-Awady *et al*., 2012; Noormohammadi *et al*., 2012; Sadeghi and Cheghamirza, 2012; Safari *et al*., 2012). The average of PIC values for the twenty primers was 0.43 and ranged from 0.33 to 0.49 (Table 3). Sixty percent of the primers (12 primers) had PIC values above 0.43 indicating that these primers are highly informative for determining *Thymus* accessions polymorphism. Comparison of the number of polymorphic bands with the PIC values revealed that a greater number of polymorphic bands were associated with lowers values of PIC. The lowest PIC index (0.33) was recorded for primer UBC-824.

Marker Index (MI) and Effective multiplex ratio (EMR): MI is a feature of a marker and was calculated for all the primers. The MI values ranged between 1.99 and 10.71 (Table 3). High MI values were scored with primers UBC-876 (10.71), UBC-824 (10.02) and UBC-827 (9.99). The lowest MI scores were obtained with ISSR primers S3 (1.99), UBC-822 (2.12) and UBC-825 (2.55). These primers amplified low number of PCR products. The effective multiplex ratio (EMR) is the number of polymorphic fragments detected per assay. EMR parameter varied from 4 to 24 with a mean value of 15.84. The primers that generated high number of bands had higher MI and EMR values. MI and EMR were positively correlated with RP ($r = 0.913$ and $r = 0.765$, respectively, $P < 0.01$) and negatively correlated with PIC. The high positive correlation between the values of PIC and MI, and the absence of significant correlation ($P > 0.05$) between the RP and MI was reported by Grativol *et al.* (2010). A positive correlation was found between EMR and MI ($r = 0.950$, $P < 0.001$). Parameters such as MI and EMR have been used for assessing the informative potential of molecular markers in various genetic diversity studies (Mondal *et al*., 2008; Datta *et al*., 2010; Kayis *et al*., 2010; Najaphy *et al*., 2011; Huang *et al*., 2012; Mirmajlessi *et al*., 2012; Sadeghi and Cheghamirza, 2012; Srivastava *et al*., 2012).

Resolving power (RP): An important feature of a good marker system is the capacity to distinguish among different accessions. The resolving power (RP) is a parameter that specifies the discriminatory potential of the primers chosen. Resolving power determines the ability of a primer/technique to generate optimally informative bands which were calculated per individual for each ISSR marker to determine their efficiencies. The estimates of RP varied from 3.85 to 19.14 with an average of 10.46 per primer. The highest RP values were recorded for the primers UBC-876 (19.14), UBC-827 (16), UBC-864 (14.71) and UBC-880 (14.28) (Table 3), suggesting that these primers was capable of distinguishing among different ecotypes. The lowest value (3.85) was scored with the primer S3. RP was positively correlated with total amplified bands, number of polymorphic bands, MI and EMR at $P < 0.01$. There are many researches containing RP index investigation (Pradeep Red dy *et al*., 2002; Gupta *et al*., 2008; Mondal *et al*., 2008; Shaw et al., 2009; Grativol *et al*., 2010; Kayis *et al*., 2010; Tonk *et a l*., 2011; Dalamu *et al*., 2012; Sadeghi and Cheghamirza, 2012). In the present investigation the RP values were found to be higher than

that of Gupta *et al*. (2008) and Shaw *et al*. (2009) and but lower than the values reported by Grativol *et al*. (2010).

Genetic relationships among *Thymus* ecotypes: The genetic similarity was calculated for all 91 combinations of fourteen ecotypes based on 325 polymorphic ISSR markers (Table 4). The similarities varied from 0.27 (ecotype 1 vs. ecotype 4) to 0.81 (ecotype 4 vs. ecotype 6) with an average of 0.46.

Table 4. Genetic similarity (Jaccard's) among fourteen ecotypes of *Thymus spp.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1.00													
2	0.44	1.00												
3	0.34	0.37	1.00											
4	0.27	0.32	0.31	1.00										
5	0.34	0.37	0.48	0.33	1.00									
6	0.30	0.31	0.30	0.81	0.32	1.00								
7	0.33	0.37	0.35	0.27	0.42	0.29	1.00							
8	0.48	0.52	0.37	0.29	0.38	0.29	0.42	1.00						
9	0.33	0.41	0.39	0.33	0.47	0.35	0.43	0.47	1.00					
10	0.34	0.42	0.34	0.29	0.38	0.29	0.42	0.47	0.48	1.00				
11	0.30	0.40	0.37	0.31	0.39	0.32	0.44	0.42	0.51	0.65	1.00			
12	0.39	0.54	0.34	0.29	0.40	0.30	0.43	0.55	0.45	0.49	0.45	1.00		
13	0.34	0.35	0.32	0.40	0.40	0.40	0.33	0.36	0.43	0.39	0.39	0.41	1.00	
14	0.35	0.33	0.32	0.35	0.32	0.32	0.31	0.34	0.33	0.37	0.33	0.36	0.39	1.00

A dendrogram based on UPGMA analysis with ISSR binary data (similarity matrix) was illustrated in figure 2. The fourteen ecotypes were grouped into five clusters (I. II, III, IV and V) with one, seven, two, three and one ecotypes, respectively. Cluster I consisted of ecotype 1. Cluster II contained central regions ecotypes (2, 8, 12, 9, 10, 11 and 7).

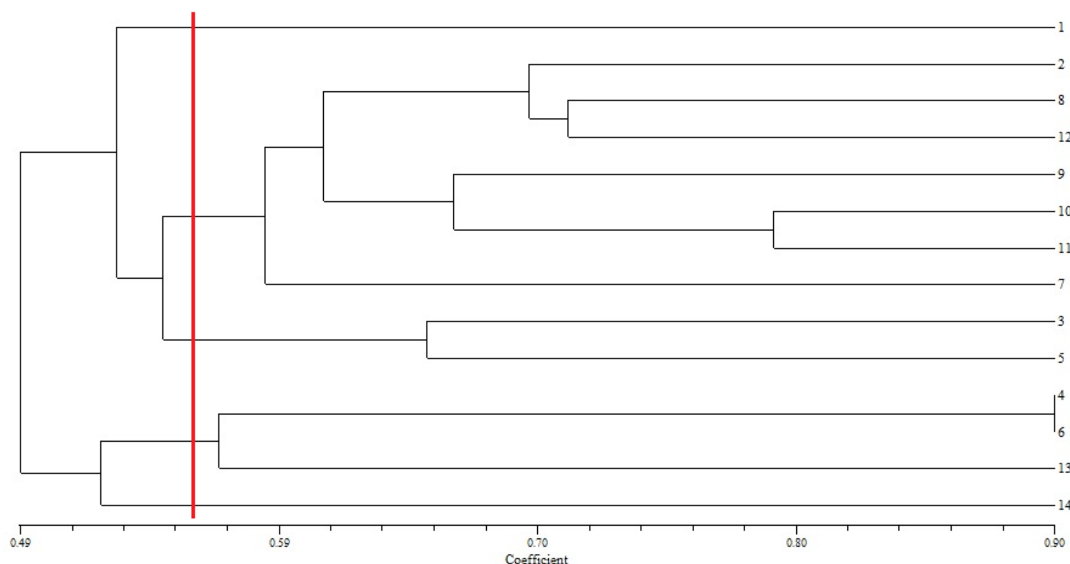

Fig. 2. UPGMA Dendrogram of 14 thyme ecotypes based on ISSR marker data. The accessions were grouped into five clusters (I. II, III, IV and V) with one, seven, two, three and one ecotypes, respectively.

Ecotypes 3 and 5 from north and northwest of Iran were placed together in cluster III. Cluster IV included ecotypes 4, 6 and 13 from Ardabil province. Finally, cluster V included ecotype 14 from England. Dendrograms in the current study indicated relatively clear configuration of clustering according to the geographic distribution patterns of the ecotypes. The highest cophenetic correlation coefficient (r = 0.87) was acquired for the clustering method representing a good fitness between the dendrogram clusters and the

similarity matrice. In some other related researches, a relationship between geographic distribution and genetic diversity has been reported (Fracaro and Echeverrigaray, 2006; Liu *et al*., 2006; Agostini *et al*., 2008). For example, Rahimmalek *et al*. (2009) by means of ISSR fingerprinting of Iranian accession of *Thymus daenensis*, found that two geographically diverse groups were generated by dendrogram. The Tc group contained the accessions collected from the center of the Zagros Mountains, and the Te group was collected from the extremes of the Zagros range. The proper selection of parental combinations is dependent upon the comprehending of relations between genotypes (Becelaere *et al*., 2005; Ali *et al*., 2008; Souza *et al*., 2012). In our collection of *Thymus* accessions, ecotypes 1 and 4 were the most divergent and therefore might have a larger possibility of heterosis in a breeding programs aimed to improve favorite medicinal properties or agronomical traits in *Thymus* species.

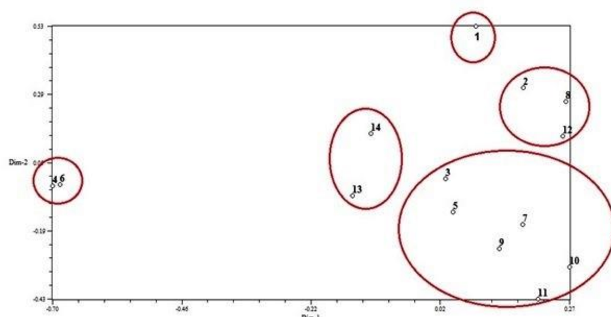

Fig. 3. Scatter plot of thyme ecotypes using principal coordinates analysis based on ISSR data. The fourteen ecotypes were classified into five groups based on the two components.

Principal coordinate analysis (PCoA), as a complementary technique for cluster analysis, is one of the important multivariate statistical approaches to group based on similarity coefficients. The dependability of the dendrogram and the principal coordinate analysis intensely supports the reliability of the marker system. The plot of the first and second principal components from principal coordinates analysis (accounting for 16.59 and 11.20 of variation, respectively) is depicted in Figure 3. Based on the results of PCoA, the first five most informative components explained 55.24% of the total variation. Similar to the UPGMA clustering pattern, the fourteen ecotypes of *Thymus* were grouped into five groups (clusters) based on the principal components analysis. Cluster I contained only ecotype 1. Group II consisted of ecotypes 2, 8 and 12. Ecotypes 3, 5, 7, 9, 10 and 11 were grouped together in cluster III. Group IV comprised of ecotypes 4 and 6. Cluster V included ecotypes 13 and 14. The results of PCoA and cluster analysis showed similar classification of the accessions with some minor differences in sub-groups.

The analyzed ISSR markers created sufficient polymorphism and reproducible fingerprinting profiles. They proved to be highly informative and provided a powerful and reliable molecular tool for detecting genetic variation and relationships of thyme accessions. In addition, this research provided information about geographic distribution and genetic similarity of the *Thymus spp*. The results of the PCoA corresponded largely to the results obtained through cluster analysis. Both PCoA and UPGMA cluster analysis approved the clustering of all 14 ecotypes into five groups, corresponding to the geographic distribution patterns of the ecotypes. The genetic variation data would be very useful for improvement of the thyme species through conventional breeding programs as well as molecular breeding approaches such as marker assisted selection.

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