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ISSR MARKERS AND POPULATION DIFFERENTIATIONS IN *ERODIUM CICONIUM* (L.) L'HÉR EX AITON

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Abstract

Erodium ciconium is an important grazing plant and a source of protein supplements to straw for ruminants in semideserts and wastelands of the Middle East. There is no information on its population genetic structure, genetic diversity, and morphological variability in Iran. We performed molecular data for knowing the population differentiation in this species. For this study, we used 110 randomly collected plants from 15 geographical populations in 6 provinces of Iran. AMOVA test revealed significant genetic difference among the studied populations and also revealed that, 63% of total genetic variability was due to within population diversity while, 37% was due to among population genetic differentiation. Mantel test showed positive significant correlation between genetic distance and geographical distance of the studied populations. Networking, STRUCTURE analyses revealed some degree of gene flow among these populations.

Introduction

Genetic diversity is a basic component of biodiversity and its conservation is essential for long term survival of any species in changing environments (Mills and Schwartz, 2005; Tomasello *et al.*, 2015). Change in environmental conditions often leads to variation in genetic diversity levels among different populations and populations with low variability are generally considered less adapted under adverse circumstances (Falk and Holsinger, 1991; Olivieri *et al.*, 2016). In the same way, most geneticists consider population size as an important factor for maintaining genetic variation (Ellegren and Galtier, 2016; Turchetto *et al.*, 2016). This is very important in fragmented populations because they are more vulnerable due to the loss of allelic richness and inbreeding depression (increases homozygosity within populations, Frankham, 2005). Therefore, knowledge of the genetic variability and diversity within and among different populations is crucial for their conservation and management (Cires *et al.*, 2012, 2013; Meloni *et al.*, 2015; Peñas *et al.*, 2016; Esfandani-Bozchaloyi *et al.*, 2018 a, b, c, d). In arid and semi-arid regions, the genus *Erodium* Aiton (Geraniaceae) includes 74 species and is distributed on all continents, excluding Antarctica (Fiz *et al.*, 2006). A major center of diversity is observed in the Mediterranean Basin (62 species). In Iran, *Erodium* is classified in two sections *viz.*, *Plumosa* Boiss. and *Erodium* Boiss. and three subsections namely, *Absinthioidea* Brumhard, *Malacoides* Lange and *Cicutaria* Lange (Schönbeck-Temesy 1970). *Erodium* species are found in different parts of Iran (Esfandani-Bozchaloyi *et al.*, 2017 a, b, c, d; Schönbeck-Temesy, 1970; Eig, 1931; Zohary, 1950; Leonard, 1989; White and Léonard, 1991; Akhani, 2007).

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Genus *Erodium* comprises 15 species in different parts of Iran (Schonbeck–Temesy, 1970). *Erodium ciconium* is distinguished from other members of its genus by its lobed cotyledons, with sinuses almost reaching the midvein and dense appressed hairs on the mericarp (Dahlgren, 1980). The tricolpate pollen grains have a striate-reticulate exine morphology (Verhoeven and Venter 1987; Perveen and Gaiser 1999; Shehata, 2008). Some species of *Erodium* are of medicinal importance while some are well known weeds.

Erodium ciconium (L.) L'Hér. is best adapted to Mediterranean climates, but is found globally in temperate areas with hot summers (Greuter *et al.*, 1986; Hulte´n and Fries, 1986). Although the species requires moisture from rainfall or irrigation for optimal germination (Blackshaw and Harker, 1998; Busso *et al.*, 1998; Brooks and Berry, 2006). *E. ciconium* has had some importance as a forage plant on ranges in California (Anonymous, 1939; Busso *et al.*, 1998; George *et al*., 2006); and is an important grazing plant and source of protein supplements to straw for ruminants in semideserts and wastelands of the Middle East (Al-Masri, 2007; Bilgir, 1982). The entire plant is edible with a flavor similar to sharp parsley if picked young (Camazine and Bye, 1980). Molecular markers play a significant role in protection of biodiversity, identification of promising cultivars, quantitative trait loci (QTL) mapping, etc. Different PCR based dominant markers, such as ISSR, SCoT, SRAP, etc. have been effectively used for quantification of genetic diversity (Anonymous, 1939; Busso *et al.*, 1998; George *et al*., 2006). Recent ISSR studies of natural populations have demonstrated the hypervariable nature of these markers and their potential use for population-level studies (Hulte´n and Fries, 1986). Limitations of the ISSR technique, as is the case for Random Amplification of Polymorphic DNA (RAPD; Esfandani-Bozchaloyi *et al*., 2019), are that the bands are scored as dominant markers and the genetic diversity estimates are based on diallelic characters. In the present study, ISSR markers were employed to analyze genetic diversity in 110 *E. ciconium* accessions belonging to 15 different populations for the first time in the Iran.

Materials and Methods

Plant materials

A total of 110 individuals were sampled representing 15 natural populations of *E. ciconium* from East Azerbaijan, Lorestan, Kermanshah, Mazandaran, Guilan and Ardabil Provinces of Iran during July-Agust 2018 (Table 1). For morphometric and ISSR analysis, we used 110 plant accessions (four to twelve samples from each populations) belonging to 15 different populations. More information about the geographical distribution of the accessions are given in Table 1. Different literatures were used for the correct identification of the samples of *E. ciconium* (Davis, 1967; Schönbeck-Temesy, 1970; Zohary, 1972; Janighorban, 2005).

Environmental variables

During this study, data on elevation, latitude and longitude etc. were recorded at each site using an electronic GPS. The climate variable data of mean annual temperature, mean maximum temperature (°C), mean minimum temperature (°C), annual rainfall (mm), number of frost days were collected from http://www.worldclim.org. (Table 1). Soil pH (1:2.5 v/v soil/water mixture; LY/T 1239–1999) for each population was measured using a digital pH meter (PHS-3C, Shanghai Leici Equipment Factory, China).

DNA extraction and ISSR assay

Fresh leaves were used randomly from four to twelve plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (Esfandani-Bozchaloyi *et al*., 2019). The quality of extracted DNA was examined by running it on 0.8% agarose gel. 10 ISSR primers *viz*., $(AGC)_5GT$, $(CA)_7GT$,

Table 1. Populations studied, their locality and ecological features.

p.no	Locality	No. of collected accessions	Mean maximum temp. (ºC)	Mean minimum temp. (ºC)	Annual temp.	pH	Annual rainfall (mm)	No. of frost days	Elevation (m)	Coordinates
1	Lorestan: Cheshmak	10	40.12	-18.12	13.4	7.8	325	77	1300	48° 51.778' E; 33° 13.175' N
2	Lorestan: Sepid-Dasht	5	38.45	-17,66	14.6	7.7	355	86	1280	48° 50.649' E; 33° 13.292' N
3	Lorestan: Dorud	6	35.55	-20.34	34	7.4	378	75	1100	48° 10.286' E; 33° 27.407' N
4	Mazandaran: Karaj-Chalus	4	41.34	-10.34	15.6	7.7	377	96	1370	48° 15.886' E; 33° 98.327' N
5	Lorestan: Borujerd	8	39.14	-17.55	18	7.6	390	73	110	47° 49.748' E; 33° 18.168' N
6	Lorestan: Nojian	7	30.34	-19.35	22	7.7	310	54	670	47° 30.663' E; 33° 4.840' N
7	Gilan: Langerud, Chaff	5	36.88	-11.23	16	7.5	320	76	940	47° 57.328' E; 33° 57.121' N
8	Lorestan: Visian	11	32.55	-22.45	18	7.8	334	88	800	47° 42.448' E; 33° 6.480' N
9	Kermanshah: Paveh	7	30.44	-18.66	15	8	229	120	360	45° 34.376' E; 34° 29.661' N
10	Kermanshah: Islamabad	6	32.88	-11.66	12	8.4	210	114	1474	46° 20.252' E; 35° 3.777' N
11	Kermanshah: Bijar	6	35.99	-8.44	10	8.3	250	167	1400	46° 20.396' E; 35° 1.812' N
12	Ardabil: Germi, 20 km from Germi to Pars-Abad	5	20.44	-25.66	10	8	478	220	380	48° 5.222' E; 39° 10.859' N
13	Guilan: Loleman	12	38.77	-5.66	20	7.5	550	30	230	49° 33.188' E; 36° 51.654' N
14	Guilan: Lahijan	7	35.87	-2.66	24	7.6	579	31	250	49° 8.158' E; 37° 10.483' N
15	Azarbaijan (E): Ahar, 45 Km from Meshkin-Shahr to Ahar	11	15.77	-26.88	5	7.4	467	170	1250	47° 17.038' E; 38° 23.792' N

$(AGC)_5GG$, UBC810, $(CA)_7AT$, $(GA)_9C$, UBC807, UBC811, $(GA)_9T$ and $(GT)_7CA$ commercialized by the University of British Columbia (UBC) were used. PCR reactions were performed in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The thermal program was carried out with an initial denaturation for 1 min at 94°C, followed by 40 cycles in three segments: 35 s at 95°C, 40s at 47°C and 55s at 72°C. The amplified products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

Molecular analyses

The ISSR profiles obtained for each samples were scored as binary characters. Parameter like Nei's gene diversity (He), Shannon Information Index (I), number of effective alleles, and percentage of polymorphism (P% = number of polymorphic loci/number of total loci) were determined (Weising *et al.*, 2005; Freeland *et al.*, 2011; Peakall and Smouse, 2006). Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland *et al.*, 2011; Huson and Bryant, 2006). Mantel test checked the correlation between geographical and genetic distances of the studied populations (Podani, 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al.*, 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software. AMOVA (Analysis of Molecular Variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse, 2006), and Nei's Gst analysis in GenoDive ver. 2 (2013) were used to show genetic difference of the populations (Meirmans and Van Tienderen, 2004). Moreover, populations' genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation (Hedrick, 2005), and D_est = Jost measure of differentiation (Jost, 2008).

To assess the population structure of the *E. ciconium*, a heuristic method based on Bayesian clustering algorithms were utilized. The clustering method based on the Bayesian-model implemented in the software program STRUCTURE (Pritchard *et al*., 2000; Falush and Stephens 2007) was used on the same data set to better detect population substructures. This clustering method is based on an algorithm that assigns genotypes to homogeneous groups, given a number of clusters (K) and assuming Hardy-Weinberg and linkage equilibrium within clusters, the software estimates allele frequencies in each cluster and population memberships for every individual (Pritchard *et al*., 2000). The number of potential subpopulations varied from two to ten, and their contribution to the genotypes of the accessions was calculated based on 50,000 iteration burn-ins and 100,000 iteration sampling periods. The most probable number (K) of subpopulations was identified following Evanno *et al.* (2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k (Meirmans, 2012). Gene flow (Nm) were calculated using POPGENE (version 1.31) program (Yeh *et al*., 1999).

Results and Discussion

Population's genetic diversity

Genetic diversity parameters were determined in 15 geographical populations of *E. ciconium* are presented in Table 2. The highest value of percentage polymorphism (47.18%) was observed in Gilan: Langerud, Chaff population number (Pop. No. 7), which shows high value for gene

diversity (0.144). and I (0.155). Population Mazandaran: Karaj-Chalus (Pop. No. 4) has the lowest value for percentage of polymorphism (8.44%) and the lowest value for I (0.049), and He (0.013).

Population genetic differentiation

AMOVA (PhiPT = 0.59, P = 0.0010) revealed significant difference among the studied populations (Table 3). It also revealed that 63% of total genetic variability was due to diversity within population and 37% was due to genetic differentiation among population.

Table 2. Genetic diversity parameters in the studied populations *E. ciconium*.

Pop	N	Na	Ne	I	He	UHe	%P
Pop1	10	0.388	1.081	0.068	0.046	0.056	19.76
Pop2	5	0.318	1.058	0.050	0.034	0.045	9.24
Pop3	6	0.835	1.206	0.179	0.119	0.132	35.12
Pop4	4	0.541	1.118	0.049	0.013	0.084	8.44
Pop5	8	0.718	1.162	0.147	0.097	0.106	29.41
Pop6	7	0.918	1.225	0.197	0.132	0.159	35.29
Pop7	5	0.576	1.144	0.155	0.144	0.095	47.18
Pop8	11	0.329	1.036	0.087	0.079	0.021	45.71
Pop9	7	0.647	1.182	0.152	0.103	0.111	27.06
Pop10	6	0.506	1.104	0.090	0.061	0.067	18.47
Pop11	6	0.694	1.131	0.126	0.081	0.087	27.06
Pop12	5	0.482	1.090	0.077	0.052	0.059	14.12
Pop13	12	0.459	1.115	0.089	0.062	0.068	12.29
Pop14	7	0.329	1.036	0.087	0.079	0.021	45.71
Pop15	11	0.718	1.162	0.147	0.097	0.106	29.41

N = number of samples, Na= Number of different alleles, Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

Table 3. Analysis of molecular variance (AMOVA) of the studied populations.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	12	496.576	38.327	4.062	37%	37%
Within Pops	60	594.767	8.530	8.630	63%	
Total	72	991.342		13.613	100%	

df: degree of freedom; SS: sum of squared observations; MS: mean of squared observations; EV: estimated variance; *Φ*PT: proportion of the total genetic variance among individuals within an accession (P < 0.001).

The pairwise comparisons of 'Nei genetic identity' among the populations of *E. ciconium* (Table 4) have shown a higher genetic similarity (0.91) between populations Lorestan: Borujerd (Pop. No. 5) and Kermanshah: Bijar (Pop. No. 11), while the lowest genetic similarity value (0.55) occurs between Lorestan:Visian (Pop. No. 8) and Mazandaran: Karaj-Chalus (Pop. No. 4).

Table 4. Pairwise Population Matrix of Nei Unbiased Genetic Identity.

pop1	pop2	pop3	pop4	pop5	pop6	pop7	pop8	pop9	pop10	pop11	pop12	pop13	pop14	pop15	
1.000															pop1
0.845	1.000														pop2
0.791	0.807	1.000													pop3
0.666	0.776	0.881	1.000												pop4
0.772	0.826	0.797	0.752	1.000											pop5
0.787	0.772	0.732	0.715	0.902	1.000										pop6
0.804	0.824	0.791	0.736	0.776	0.746	1.000									pop7
0.806	0.757	0.666	0.554	0.802	0.785	0.831	1.000								pop8
0.800	0.837	0.772	0.691	0.708	0.720	0.826	0.797	1.000							pop9
0.806	0.820	0.787	0.728	0.792	0.837	0.772	0.691	0.873	1.000						pop10
0.762	0.821	0.804	0.727	0.910	0.820	0.787	0.728	0.854	0.860	1.000					pop11
0.790	0.826	0.806	0.719	0.821	0.821	0.804	0.727	0.797	0.810	0.879	1.000				pop12
0.768	0.863	0.800	0.760	0.836	0.826	0.806	0.719	0.804	0.787	0.806	0.811	1.000			pop13
0.765	0.787	0.806	0.811	0.783	0.863	0.800	0.760	0.781	0.730	0.800	0.760	0.781	1.000		pop14
0.755	0.730	0.762	0.743	0.781	0.729	0.756	0.725	0.768	0.830	0.756	0.725	0.768	0.784	1.000	pop15

Population's genetic affinity

NJ tree and Neighbor-Net network produced similar results, and therefore, only Neighbor-Net network is presented and discussed (Fig. 1). We find almost complete separation of the populations in the network, supporting AMOVA result. The populations Lorestan: Borujerd (Pop. No. 5) and Guilan: Lahijan (Pop. No. 14) are distinct and stand separate from the other populations with great distance. The Pop. No. 3 and Pop. No. 6, as well as Pop. No. 11 and Pop. No. 13 show closer genetic affinity and are placed close to each other. In general, the findings of Fig. 1 is more or less consistent with Figure 3, but it is totally in conflict with STRUCTURE.

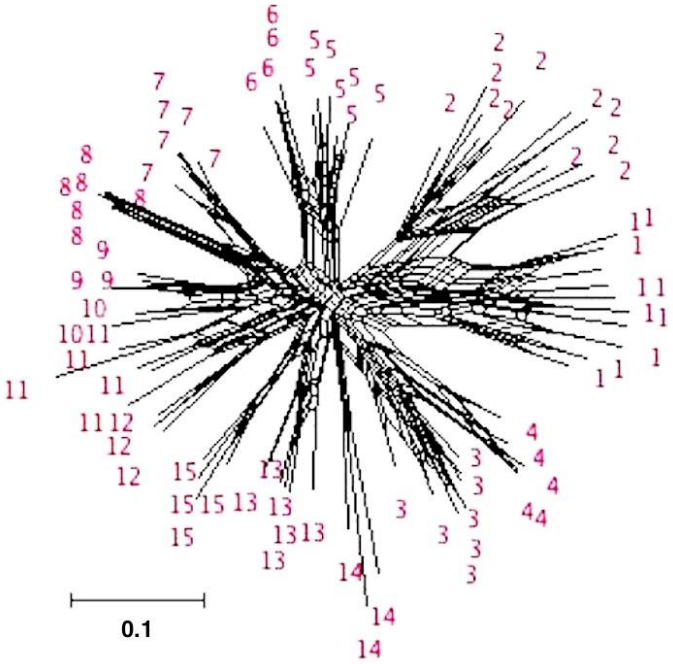

Fig. 1. Neighbor-Net network of populations in *E. ciconium* based on ISSR data.

Genetic divergence and separation of Pop. No. 1-6, as well as Pop. No. 11 and Pop. No. 15 from the other populations is evident in MDS plot of ISSR data after 900 permutations (Fig. 2). The other populations showed close genetic affinity. Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations ($r = 0.52$, $P = 0.001$). Therefore, the populations that are geographically more distant have less amount of gene flow and isolation by distance (IBD) in *E. ciconium.*

Population's genetic structure

K = 2 reveal the presence of 2 genetic groups. Similar result was obtained by Evanno test performed on STRUCTURE analysis which produced a major peak at k = 2 (Fig. 3). Both these analyses revealed that *E. ciconium* populations show genetic stratification. STRUCTURE plot based on k = 2 (Fig. 3), revealed genetic difference of populations (Pop. No. 1-7) (differently colored) with other populations. But it showed genetic affinity between populations 1-7 (similarly colored), as well as populations 8-15.

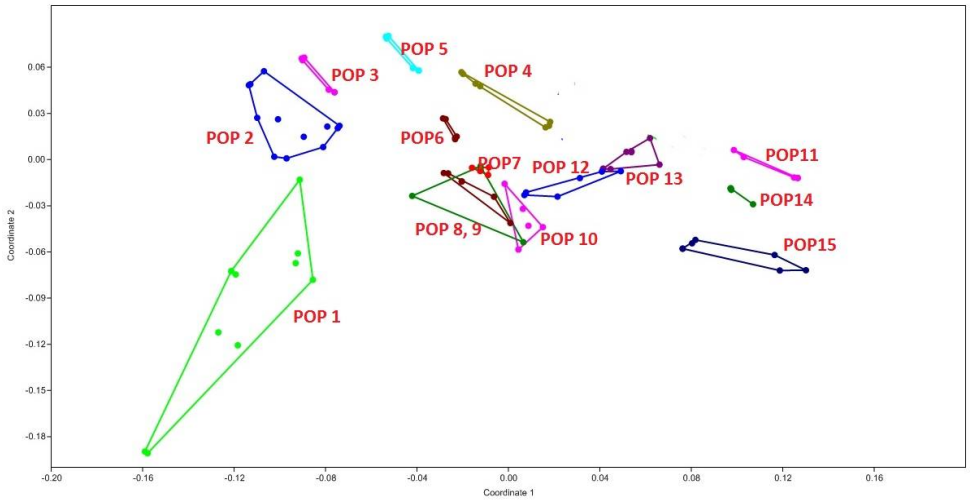

Fig. 2. MDS plot of populations in *E. ciconium* based on ISSR data.

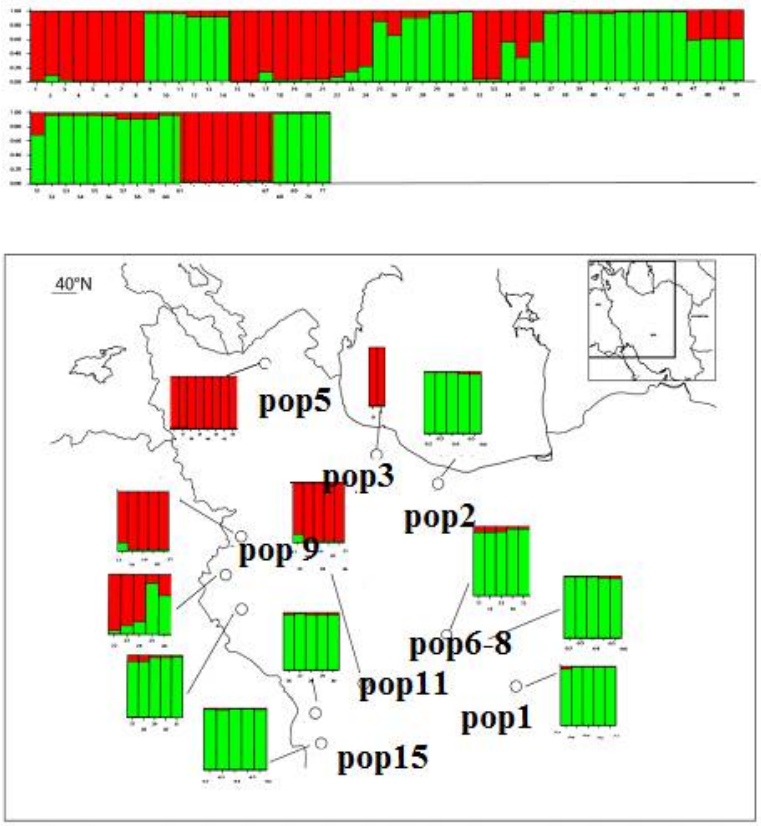

Fig. 3. STRUCTURE plot of *E. ciconium* populations based on k = 2 of ISSR data.

The mean Nm = 0.32 was obtained for all ISSR loci, which indicates low amount of gene flow among the populations and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. However, the reticulogram generated through the least square method (Fig. 4) revealed some amount of shared alleles among Pop. No. 5, 6 and Pop. No. 1, 2 and between Pop. No. 14 and Pop. No. 7 also between Pop. No. 11, and Pop. No. 9 and 10. This result is in conflict with grouping obtained from MDS plot, as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in these populations and all these results are not in agreement in showing high degree of genetic stratification within *E. ciconium* populations.

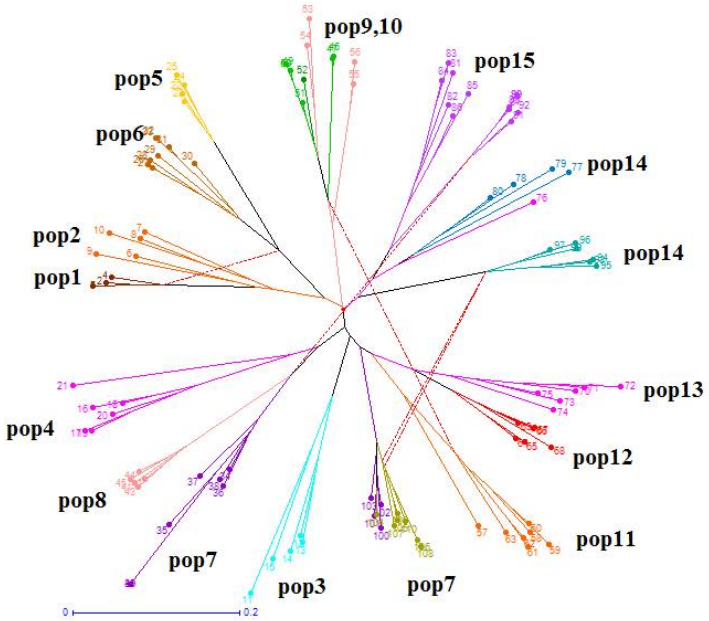

Fig. 4. Reticulogram of *E. ciconium* populations based on least square method analysis of ISSR data. (Population numbers are according to Table 1).

The present study provides interesting data on genetic variability, genetic stratification and morphological divergence in *E. ciconium* of north and west part of Iran. The studied populations have a low level of genetic diversity (He = 0.013-0.144). Low genetic variability may occur due to small size of the populations and genetic drift (Dahlgren, 1980). The Genetic diversity is of fundamental importance in the continuity of a species as it is used to bring about the necessary adaptation to the cope with changes in the environment (Warburg, 1938; Guittonneau, 1972). Degree of genetic variability within a species is highly correlated with its reproductive mode, and the higher degree of open pollination/cross breeding brings about higher level of genetic variability in the studied taxon (Knuth, 1908). Considerable morphological and genetic variability has previously been reported within *E. ciconium* (Webb and Chater, 1968; Dahlgren, 1980). Martin *et al.*, (1997) showed genetic diversity within and among populations of a threatened species *E. paularense* Fern. Gonz. & Izco using RAPD markers. Alarcón *et al.* (2012) based on AFLP data showed that the genetic diversity of the two *Erodium* lineages indicated two migration episodes from southern Iberia towards the north, with one lineage migrating via western Iberia and

the other via eastern Iberia. Geography appears to play an important role in isolation by distance, particularly for Mediterranean plants. Reductions in gene flow may lead to the appearance of new species or subspecies, with isolation in glacial refugia as a major promoter of such diversification (Esfandani-Bozchaloyi, *et al*., 2018a,b). *E. ciconium* is of wide spread in our country and it has several medicinal applications (Wiesnerova and Wiesner, 2004), however we had no information on its genetic structure and detailed taxonomic information. Our results revealed interesting data about its genetic variability, genetic stratification and morphological divergence in north and west part of Iran.

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