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 homozygosy 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., Plamosa 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; Hultén 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 (Hultén and Fries, 1986). Limitations of the ISSR technique, as is the case for Random Amplification of Polymorphic DNA (RAPD; Esfandani-Bozhaloyi 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-August 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-Bozhaloyi et al., 2019). The quality of extracted DNA was examined by running it on 0.8% agarose gel. 10 ISSR primers viz., (AGC)\textsubscript{3}GT, (CA)\textsubscript{3}GT,
Table 1. Populations studied, their locality and ecological features.

| p.no | Locality                      | No. of collected accessions | Mean maximum temp. (°C) | Mean minimum temp. (°C) | 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                  | 18.13        | 7.7 | 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: Bonujerd            | 8                          | 39.14                   | -17.55                  | 15.78        | 7.6 | 390                 | 73              | 110           | 47° 49.748'E; 33° 18.168'N          |
| 6    | Lorestan: Nojian              | 7                          | 30.34                   | -19.35                  | 18.76        | 7.7 | 310                 | 54              | 670           | 47° 30.663'E; 33° 4.840'N           |
| 7    | Gilan: Langerud, Chaff        | 5                          | 36.88                   | -11.23                  | 16.17        | 7.5 | 320                 | 76              | 940           | 47° 57.328'E; 33° 57.121'N          |
| 8    | Lorestan: Vissian             | 11                         | 32.55                   | -22.45                  | 18.53        | 7.8 | 334                 | 88              | 800           | 47° 42.448'E; 33° 6.480'N           |
| 9    | Kermanshah: Paveh             | 7                          | 30.44                   | -18.66                  | 15.82        | 8.1 | 229                 | 120             | 360           | 45° 34.376'E; 34° 29.661'N          |
| 10   | Kermanshah: Islamabad         | 6                          | 32.88                   | -11.66                  | 12.28        | 8.4 | 210                 | 17              | 1474          | 46° 20.252'E; 35° 3.777'N           |
| 11   | Kermanshah: Bijar             | 6                          | 35.99                   | -8.44                   | 10.35        | 8.3 | 250                 | 167             | 1400          | 46° 20.396'E; 35° 1.812'N           |
| 12   | Ardabil: Geri, 20 km from Geri to Pars-Abad | 5 | 20.44                   | -25.66                  | 10.88        | 8.4 | 478                 | 220             | 380           | 48° 5.222'E; 39° 10.859'N           |
| 13   | Guilan: Loleman               | 12                         | 38.77                   | -5.66                   | 20.53        | 7.5 | 550                 | 30              | 230           | 49° 33.188'E; 36° 51.654'N          |
| 14   | Guilan: Lahijan               | 7                          | 35.87                   | -2.66                   | 24.66        | 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                  | 5.4          | 7.4 | 467                 | 170             | 1250          | 47° 17.038'E; 38° 23.792'N          |
(AGC)$_5$GG, UBC810, (CA)$_2$AT, (GA)$_3$C, UBC807, UBC811, (GA)$_3$T and (GT)$_2$CA 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 MgCl$_2$; 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 ($H_e$), Shannon Information Index (I), number of effective alleles, and percentage of polymorphism ($P\% = \frac{\text{number of polymorphic loci}}{\text{number of total loci}}$) 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 $G'ST$ = 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; ΦPT: 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 |
|----|------|------|------|------|------|------|------|------|------|-------|-------|-------|-------|-------|-------|
| pop1| 1.000|      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| pop2| 0.845| 1.000|      |      |      |      |      |      |      |       |       |       |       |       |       |
| pop3| 0.791| 0.807| 1.000|      |      |      |      |      |      |       |       |       |       |       |       |
| pop4| 0.666| 0.776| 0.881| 1.000|      |      |      |      |      |       |       |       |       |       |       |
| pop5| 0.772| 0.826| 0.797| 0.752| 1.000|      |      |      |      |       |       |       |       |       |       |
| pop6| 0.787| 0.772| 0.732| 0.715| 0.902| 1.000|      |      |      |       |       |       |       |       |       |
| pop7| 0.804| 0.824| 0.791| 0.736| 0.776| 0.746| 1.000|      |      |       |       |       |       |       |       |
| pop8| 0.806| 0.757| 0.666| 0.554| 0.802| 0.785| 0.831| 1.000|      |       |       |       |       |       |       |
| pop9| 0.800| 0.837| 0.772| 0.691| 0.708| 0.720| 0.826| 0.797| 1.000|       |       |       |       |       |       |
| pop10| 0.806| 0.820| 0.787| 0.728| 0.792| 0.837| 0.772| 0.691| 0.873| 1.000|       |       |       |       |       |
| pop11| 0.762| 0.821| 0.804| 0.727| 0.910| 0.820| 0.787| 0.728| 0.854| 0.860| 1.000|      |       |       |       |
| pop12| 0.790| 0.826| 0.806| 0.719| 0.821| 0.821| 0.804| 0.727| 0.797| 0.810| 0.879| 1.000|      |       |       |
| pop13| 0.768| 0.863| 0.800| 0.760| 0.836| 0.826| 0.806| 0.719| 0.804| 0.787| 0.806| 0.911| 1.000|      |       |
| pop14| 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|       |
| pop15| 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|
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.

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. cicionium*.

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. cicionium* 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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