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Molecular techniques in the assessment of genetic relationships between populations of *Consolida* (Ranunculaceae)

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Abstract. Genetic diversity studies are essential to understand the conservation and management of plant resources in any environment. The genus *Consolida* (DC.) Gray (Ranuculaceae) belongs to tribe Delphinieae. It comprises approximately 52 species, including the members of the genus *Aconitella* Spach. No detailed Random Amplified Polymorphic DNA (RAPD) studies were conducted to study *Consolida* genetic diversity. Therefore, we collected and analyzed 19 species from 12 provinces of regions. Overall, one hundred and twenty-seven plant specimens were collected. We showed significant differences in quantitative morphological characters in plant species. Unweighted pair group method with arithmetic mean and principal component analysis (PCA) divided *Consolida* species into two groups. All primers produced polymorphic amplicons though the extent of polymorphism varied with each primer. The primer OPA-06 was found to be most powerful and efficient as it generated a total of 24 bands of which 24 were polymorphic. The Mantel test showed correlation (r = 0.34, p=0.0002) between genetic and geographical distances. We reported high genetic diversity, which clearly shows the *Consolida* species can adapt to changing environments since high genetic diversity is linked to species adaptability. Present results highlighted the utility of RAPD markers and morphometry methods to investigate genetic diversity in *Consolida* species. Our aims were 1) to assess genetic diversity among *Consolida* species 2) is there a correlation between species genetic and geographical distance? 3) Genetic structure of populations and taxa.

Keywords: Consolida, population structure, gene flow, network, genetic admixture.

INTRODUCTION

Genetic diversity is a vital feature that helps plant species survive in an ever-changing environment, and it sheds light on understanding the phylogenetic affinity among the species (Erbano *et al*. 2015; Ellegren and Galtier 2016; Turchetto *et al*. 2016). Quite a significant number of genetic resources and materials programs of plant species have been carried out to preserve

the plant species worldwide. Scientific data indicate that genetic diversity plays a pivotal role in conservation programs (Gomez *et al.* 2005; Frankham 2005; Cires *et al.* 2013).

The genus *Consolida* (DC.) Gray (Ranuculaceae) belongs to tribe Delphinieae. It comprises approximately 52 species, including the members of the genus *Aconitella* Spach. Iran is one of the richest countries for the genus in South-West Asia, since it has 24 species (Iranshahr *et al.,* 1992).

The genus *Consolida* S.F. Gray was considered as a separate genus based on one species (*C. regalis*) by Gray (1821), who worked on British flora. But some researchers considered *Consolida* as a section of *Delphinium* (De Candole 1824; Boissier 1867; Huth 1895; Nevskii 1937). Unlike the others based on annual life form, single spured petal, single follicle compared to 3 or 5 sessile follicles of *Delphinium* recognized *Consolida* as a separate genus (Tutin *et al.* 1964; Davis 1965; Munz 1967; Hayek 1970; Iranshahr 1992; Ertugrul *et al.* 2016; Khalaj 2013). Kemularia-Nathades (1939) recognized a new genus *Aconitopsis* from species of *Consolida* based on peculiar formation of the petal, upper sepal, and spur. The name *Aconitopsis* was later rejected by Sojak (1969) and being replaced by *Aconitella* because of nomenclature priority. Some researchers have studied these genera taxonomically (Soo 1922; Munz 1967 ; Davis 1965; Iranshahr *et al*., 1992; Constantinidis *et al*., 2001). *Consolida* has about 40 species, of which 19 have been recorded from Iran. *Aconitella* with ca. 10 species (3 species in Iran) and 31 species of *Delphinium* (species in Iran) are centred in Irano-Turanian and Mediterranean phytogeographic regions (Trifonova, 1990; Hasanzadeh *et al.* 2017).

Consolida has been separated from *Delphinium* by De Candolle based on single spurred petals, one follicle and annual life cycle and has occurred in separate section. Finally, it introduced as a separate genus by Gray in 1821 (Triffonova, 1990). Based on phylogenetic studies of Jabbour and Renner (2011), *Aconitella* is part of *Consolida*, both being embedded in *Delphinium*. The Jabbour & Renner (2011) results showed that *Consolida* diverged from *Delphinium* relatives in the Early to Middle Miocene, a period of increasing aridity, caused primarily by decrease in sea level in the Mediterranean (Hayek 1970; Iranshahr 1992; Ertugrul *et al.* 2016) and desertification in Asia (Triffonova 1990).

Some biosystematic studies have carried out in various field such as chromosomal studies (Trifonova 1990; Koeva 1992; Hong, 1986) chemical studies (Aitzetmuller *et al*.1999), palynological studies (Munz, 1967) and phylogenetic investigations by using DNA sequence data (Johansson 1995; RO *et al*.1997; Jabbour and Renner 2011; 2012; Yosefzadeh *et al.* , 2012). In the recent molecular studies (Jabbour and Renner 2001; 2012) it was showed that *Consolida* and *Aconitella* form a clade embeded in *Delphinium* and also *Aconitella* is embedded within *Consolida*. The Jabbour and Renner (2011) results showed that *Consolida* diverged from *Delphinium* relatives at least in the early of middle Miocene.

Genetic diversity studies are usually tapped due to molecular markers. Molecular markers are an excellent method to disentangle phylogenetic association between species and population. Among molecular methods or markers, RAPD (Random Amplified Polymorphic DNA) are sensitive to detect variability among individuals of species. RAPD method is cost-effective and can work with limited sample quantities. In addition to this, RAPD can amplify and target genomic regions with potential and several markers (Esfandani-Bozchaloyi *et al.* 2017a). Taxonomical Systematics studies were conducted in the past to identify the *Consolida* species. According to the best of our knowledge, there is no existing RAPD data on genetic diversity investigations in Iran. We studied one hundred and twenty-seven samples. Our aims were 1) to assess genetic diversity among *Consolida* species 2) is there a correlation between species and geographical distance? 3) Genetic structure of populations and taxa 4) Are the *Consolida* species able to exchange genes?

MATERIALS AND METHODS

Plant materials

19 *Consolida* species were collected from different regions of Iran (Table 1). These species were studied via morphological and molecular methods. 127 plant samples (10-25 per plant species) were examined for morphometry purposes (Figure 1). The random amplified polymorphic DNA analysis method was limited to 110 samples. According to previous references, all the species were identified (Iranshahr, 1992; Ertugrul *et al*., 2016; Khalaj, 2013). Voucher specimens were deposited in Herbarium of Azad Islamic University (HAIU).

Morphometry

We studied 18 qualitative and 7 quantitative morphological characters (Table 2). Data were transformed (Mean= 0, variance = 1) prior to ordination . Euclidean distance was implemented to cluster and ordinate plant species (Podani 2000).

Table 1. Location and herbarium accession numbers of the studied populations of *Consolida* species collected by Mehri in Iran.

No	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*C. tehranica* (Boiss.) Rech.f.	Tehran: Damavand	38°52'37"	47°23'92"	1144
		Tehran: Rodehen	32°50'03"	51°52'08"	1066
		Golestan, Ramian	35°50'03"	48°52'08"	1234
Sp2	*C. camptocarpa* (Fisch. &C.A.Mey.) Nevski	Khorassan: Sarakhs, 14 km to Mozduran	32°50'03"	51°24'28"	1990
Sp3	*C. lorestanica* lRANSHAHR,	Lorestan: 110 km Khorram abad	29°20'07"	51°52'08"	1610
		Markazi:Arak	36°14'14"	51°18'07"	1807
Sp4	*C. leptocarpa* Nevski	Golestan: Golestan national park, Mirzabailoo	38°52'37"	47°23'92"	1144
Sp5	*C. persica* (Boiss.) Grossh.	Fars: Bamo national park Fars: Shiraz Keramn: Jiroft Zanjan: Abhar	33°57'12"	47°57'32"	2500
Sp6	*C. aucheri* (Boiss.) Iranshahr	Khorassan: Neyshabur	34°52'373	48°23'92"	2200
Sp7	*C. anthoroidea* (Boiss.) Schrödinger	East Azerbaijan: kaleybar, Cheshme Ali Akbar	38°52'373	47°23'92"	1144
Sp8	*C. hohenackeri* (Boiss.) Grossh.	Arak: Komayjan, Pass of Chehregan village, the margin road	35°50'03"	51°24'28"	1700
Sp9	*C. stocksiana* Nevski	Golestan: Golestan national park, Mirzabailoo	36°14'14"	51°18'07"	1807
Sp10	*C. rugulosa* Schrödinger	Esfahan: Semirom to Keikha	32°36'93"	51°27'90"	2500
Sp11	*C. ambigua* (L.) Ball & Heywood	Tehran: Between Karaj and Eshtehard	37°07'02"	49°44'32"	48
Sp12	*C. orientalis* (Gray) Schrödinger	Azarbaijan: 20 km from Jolfa to Marand	28°57'22"	51°28'31"	430
Sp13	*C. regalis* S.F. Gray	Azarbaijan: Tabriz	30°07'24"	53°59'06"	2178
Sp14	*C. oliveriana* (DC.)Schrod.	Kermanshah: 31 km to Ghasre-shirin	28°57'22"	51°28'31"	288
Sp15	*C. flava* (DC.)Schrod	Khuzestan: Do-gonbadan	34°46'10"	48°30'00"	1870
Sp16	*C. trigonelloides* (Boiss.) Munz	Fars: Bamo national park	35° 37'77"	46°20'25"	1888
Sp17	*C. oligantha* (Boiss.)Schrod	Ardabil	33°47'60"	46°07'58"	1250
Sp18	*C. linorioides* (BOIss.) MUNZ,	Esfahan: Ghamishloo protected area	37°07'02"	49°44'32"	48
Sp19	*C. rugulosa* f. *paradoxa* (Bunge) Iranshahr	Hamedan: Khan Abad	28°57'22"	51°28'31"	288

Random Amplified Polymorphic DNA

We extracted DNA from fresh leaves. Leaves were dried. DNA extraction was carried out according to the previous protocol (Esfandani-Bozchaloyi *et al.* 2019; Niu *et al*., 2021; Sun *et al*., 2021). DNA quality was checked on an agarose gel to confirm the purity. We amplified the DNA with the aid of RAPD primers (Operon technology, Alameda, Canada). These primers belonged to OPA, OPB, OPC, OPD sets. We selected those primers (10) which could show clear bands and polymorphism (Table 3). Overall, the polymerase chain reaction contained 25µl volume. This 25 volume had ten mM Tris-HCl buffer, 500 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). We observed the following cycles and conditions for the amplification. Five minutes initial denaturation step was carried out at 94°C after this forty cycles of 1 minute at 94°C were observed. Then 1-minute cycle was at 52-57°C followed by two minutes at 72°C. In the end, the final extension step was performed for seven to ten minutes at 72°C. We confirmed the amplification steps while observing amplified products on a gel. Each band size was confirmed according to 100 base pair molecular ladder/standard (Fermentas, Germany).

Data analyses

Ordination methods such as multidimensional scaling and principal coordinate analysis were also performed (Podani 2000). The morphological difference among species and population was assessed through analysis of variance (ANOVA). PCA analysis (Podani 2000) was done to find the variation in plant population morphological traits. Multivariate and all the necessary calculations were done in the PAST software, 2.17 (Hammer et al. 2001). To assess genetic diversity, we encoded RAPD bands as present and absent. Numbers 1 and 0 were used to show the presence and absence of bands. It is essential to know the polymorphism infor-

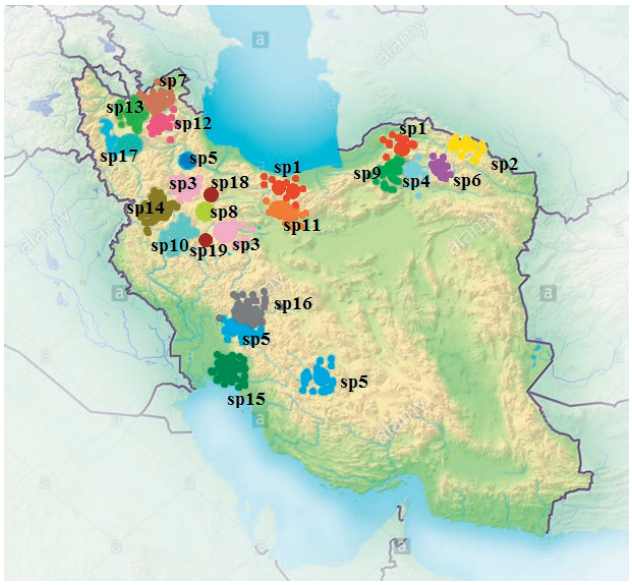

Figure 1. Map of distribtion of populations *Consolida* species in Iran; sp1= *C. tehranica;* sp2= *C. camptocarpa;* sp3= *C. lorestanica*; sp4= *C. leptocarpa;* sp5= *C. persica*; sp 6= *C. aucheri*; sp7= *C. anthoroidea*; sp8= *C. hohenackeri;* sp9= *C. stocksiana*; sp10: *C. rugulosa*; sp11: *C. ambigua ;* sp12= *C. orientalis*; sp13= *C. regalis;* sp14= *C. oliveriana;* sp15= *C. flava;* sp16= *C. trigonelloides;* sp17= *C. oligantha;* sp18= *C. linorioides;* sp19= *C. rugulosa* f. *paradoxa.*

mation content and marker index (MI) of primers because these parameters serve to observe polymorphic loci in genotypes (Ismail et al. 2019). Marker index was calculated according to the previous protocol (Heikrujam et al. 2015). Other parameters such as the number of polymorphic bands (NPB) and effective multiplex ratio (EMR) were assessed. Gene diversity associated characteristics of plant samples were calculated. These characteristics include Nei's gene diversity (H), Shannon information index (I), number of effective alleles (Ne), and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) (Shen et al. 2017). Unbiased expected heterozygosity (UHe), and heterozygosity were assessed in GenAlEx 6.4 software (Peakall and Smouse 2006). Neighbor-joining (NJ) and networking were studied to fathom genetic distance plant populations (Huson and Bryant 2006; Freeland et al. 2011). The comparison of genetic divergence or genetic distances, estimated by pairwise F_{ST} and related statistics, with geographical distances by Mantel test is one of the most popular approaches to evaluate spatial processes driving population structure. The Mantel test was performe as implemented in PAST. For this, Nei genetic distance was determined for RAPD data, while Geographic distance of PAST was determined for geographical data. It is calculated based on the sum of the paired differences among both longitude as well as latitude coordinates of the studied populations (Podani 2000). As we were interested in knowing the genetic structure and diversity, we also investigated the genetic difference between populations through AMOVA (Analysis of molecular variance) in GenAlEx 6.4 (Peakall and Smouse 2006). Gene flow (Nm) which were calculated using POPGENE (version 1.31) program [Yeh et al. 1999]. Gene flow was estimated indirectly using the formula: Nm = 0_25(1 _ FST)/FST.

We also did STRUCTURE analysis to detect an optimum number of groups. For this purpose, the Evanno test was conducted (Evanno et al. 2005).

RESULTS

Morphometry

Significant ANOVA results (P <0.01) showed differences in quantitative morphological characters in plant species. Principal component results explained 80% variation. Firs component of PCA demonstrated 57% of the total variation. Traits such as presence of petiole in caulin leaves, overtopping the bract from fruit, proportion of petal middle lobes to lateral lobes, presence of hair on the filament positively correlated with firs component (>0.7). The second and third components explained characters such as number of petal lobes, position of hair on filament, colour of anther, shape of follicle beak, shape of follicle. Unweighted pair group method with arithmetic mean (UPGMA) and principal component analysis (PCA) plots showed symmetrical results (Figure 2). Generally, plant specimens belonging to different species were separated from each other due to differences in morphology. Our PCA results also confirmed the application of morphological characters in separating and clustering the species in separate groups (Figure 2).

Species identification and genetic diversity

The primers, i.e., OPD-05, could amplify plant (*Consolida*) DNA (Figure 3). 133 polymorphic bands were generated and amplified. Amplified products ranged from 100 to 3000 bp. We recorded the highest polymorphic bands for OPA-06. OPD-08 had the lowest polymorphic bands. The average polymorphic bands ranged to 13.3 for each primer. The polymorphic information content (PIC) had values in the range of 0.38 (OPC-04) to 0.57 (OPB-02). Primers had 0.52 average polymorphic information content values.

Marker index (MI) values were 4.18 (OPD-05) to 8.87 (OPA-06), with an average of 6.87 per primer. Effec-

Table 2. Characters used in this study from Iran.

Character	Character states		
Length of basal leaves	0: <55 mm	1: <55mm	
Number of bracts	0: 0	1: 1	2: 2
Broad of petal	0: 3-9 mm	1: 8-16 mm	
Number of bracteole	0: variable	1: constant	
Length of bracteole	0: ≤ 9mm	1: ≥12 mm	
Length of spure	0: ≤ 25 mm	1: ≥ 25 mm	
Shape of spure	0: curved	1: erect	
Hair on lateral sepal	0: scattered	1: on the middle vein	
Number of petal lobes	0: 5	1: 3	
Proportion of petal middle lobes to lateral lobes	0: equal	1: shorter	2: longer
Hair on the filament	0: absent	1: present	
Hair on filament	0: wing	1: total of filament	
Colour of anther	0: brown	2: yellow	
Shape of follicle beak	0: erect	1: curved	
Shape of follicle	0: falciform	1: erect	
Hair on the follicle surface	0: absent	1: present	
Shape of fruit stalk	0: antrorse	1: erect	2: decurved
Proportion of pedicle to flower	0: shorter	1: longer	
Proportion of pedicle to fruit	0: shorter	1: longer	
Presence of petiole in caulin leaves	0: present	1: absent	
Presence of hair on the leaf surface	0: present	1: absent	
Overtopping the bract from flower	0:yes	1: no	
Overtopping the bract from fruit	0:yes	1: no	
Position of bract	0: near the flower	1: far from the flower	
Spure	0: present	1: absent	

tive multiplex ratio (EMR) values are useful to distinguish genotypes. In our study, we reported 9.34 (OPD-08) to 16.55 (OPA-05) EMR values. EMR values averaged 13.57 per primer (Table 3). All the necessary genetic features calculated of 19 *Consolida* species are shown (Table 4). *C. linorioides* depicted unbiased expected heterozygosity (UHe) in the range of 0.15. *C. orientalis* showed a 0.34. UHe value heterozygosity had a mean value of 0.23 in overall *Consolida* species. Shannon information was high (0.32) in *C. orientalis*. *C. linorioides* showed the lowest value, 0.20. Mean values for Shannon information was 0.22. The observed number of alleles (*N*a) ranged from 0.201 to 0.555 in *C. regalis* and *C. oligantha*. The effective number of alleles (Ne) was in the range of 0.67-1.876 for *C. flava* and *C. leptocarpa*.

Analysis of Molecular Variance (AMOVA) test highlighted genetic differences among *Consolida* species (P = 0.01). AMOVA revealed significant difference among the studied populations. It also revealed that, 46% of total genetic variability was due to within population diversity and 54% was due to among population genetic differentiation (Figure 4). Genetic similarity and dissimilarity assessed through Genetic statistics (GST) showed significant differences i.e., (0.77, P = 0.001) and D_est values (0.256, $p = 0.01$).

The neighbor-joining tree also revealed two major groups (Figure 5). The neighbor-joining tree also repeated the same pattern as indicated in figures 2. In current work, molecular findings also coincided with the traditional taxonomical (morphology) approaches for *Consolida* species.

The neighbor-joining tree divided *Consolida* species into two groups (Figure 4). Populations belonging to *C. tehranica; C. camptocarpa; C. lorestanica*; *C. aucheri*; *C. rugulosa*; *C. orientalis* and *C. hohenackeri* were in the first group. On the other hand, the second group consisted of two sub-groups. *C. stocksiana*; *C. ambigua ; C. oliveriana; C. flava* formed the first sub-group. *C. trigonelloides; C. oligantha; C. linorioides; C. leptocarpa* and *C. persica* formed the second sub-group. These groups

Table 3. RAPD primers and other parameters. Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPA-05	5'-AGGGGTCTTG-3'	15	15	100.00%	0.46	5.34	16.55	6.44
OPA-06	5'-GGTCCCTGAC-3'	24	24	100.00%	0.57	5.88	14.56	8.87
OPB-01	5'-GTTTCGCTCC-3'	22	22	100.00%	0.55	6.23	12.23	6.47
OPB-02	5'-TGATCCCTGG-3'	15	14	91.74%	0.57	5.66	14.56	5.67
OPC-04	5'-CCGCATCTAC-3'	13	12	92.31%	0.38	3.21	15.60	5.55
OPD-02	5'-GGACCCAACC-3'	14	13	97.74%	0.37	5.66	9.56	5.67
OPD-03	5'-GTCGCCGTCA-3'	13	12	92.31%	0.54	8.21	10.23	5.55
OPD-05	5' -TGAGCGGACA-3'	12	12	100.00%	0.47	7.32	11.55	4.18
OPD-08	5'-GTGTGCCCCA-3'	11	9	80.89%	0.43	6.56	9.34	7.18
OPD-11	5'-AGCGCCATTG-3'	10	10	100.00%	0.49	4.25	14.11	7.87
Mean		14.5	13.3	96.22%	0.52	6.32	13.57	6.87
Total		145	133					

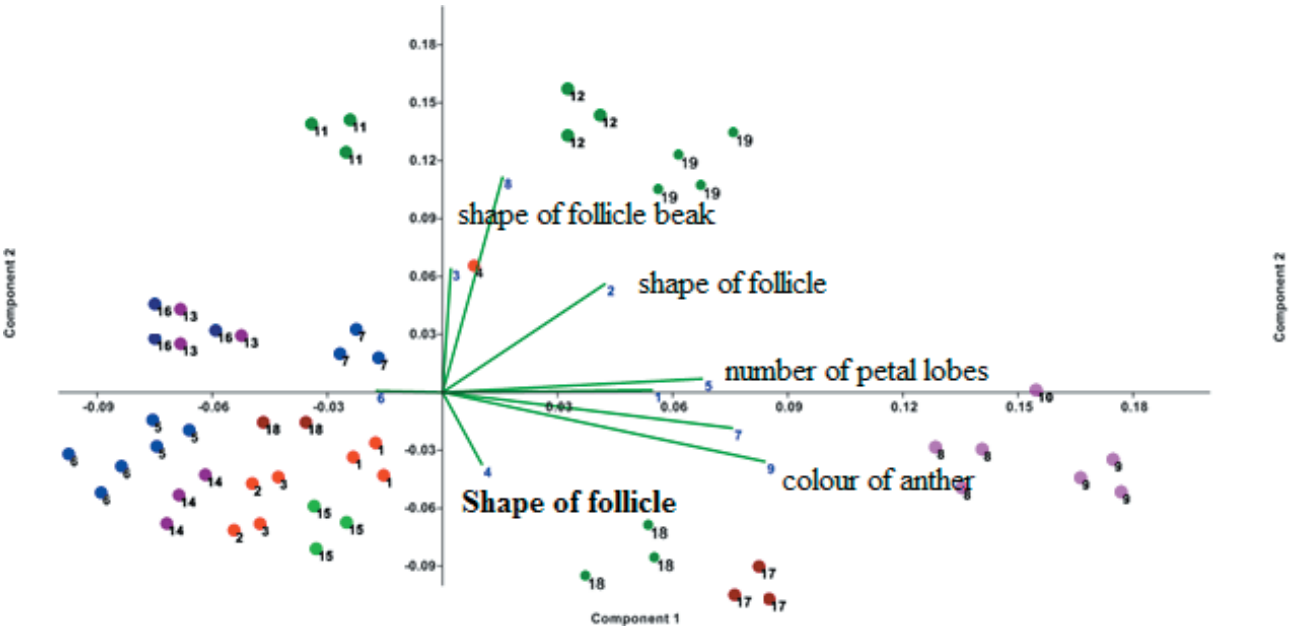

Figure 2. PCA plot of morphological characters revealing species delimitation in the *Consolida* species; sp1= *C. tehranica;* sp2= *C. camptocarpa;* sp3= *C. lorestanica*; sp4= *C. leptocarpa;* sp5= *C. persica*; sp 6= *C. aucheri*; sp7= *C. anthoroidea*; sp8= *C. hohenackeri;* sp9= *C. stocksiana*; sp10: *C. rugulosa*; sp11: *C. ambigua ;* sp12= *C. orientalis*; sp13= *C. regalis;* sp14= *C. oliveriana;* sp15= *C. flava;* sp16= *C. trigonelloides;* sp17= *C. oligantha;* sp18= *C. linorioides;* sp19= *C. rugulosa* f. *paradoxa.*

and sub-groups were formed due to molecular differences among the individuals of *Consolida*.

Gene flow (Nm) was relatively low (0.54) in *Consolida* species. Genetic identity and phylogenetic distance in the *Consolida* members are mentioned (Table 5). *C. camptocarpa* and *C. anthoroidea* were genetically closely related (0.907) to each other. *C. persica* and *C. rugulosa* were dissimilar due to low (0.702) genetic similarity. Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.34, P = 0.0002). Therefore, the populations that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in *Consolida* species**.** The most popular approaches for estimating divergence include calculation of genetic distances and variance partition-

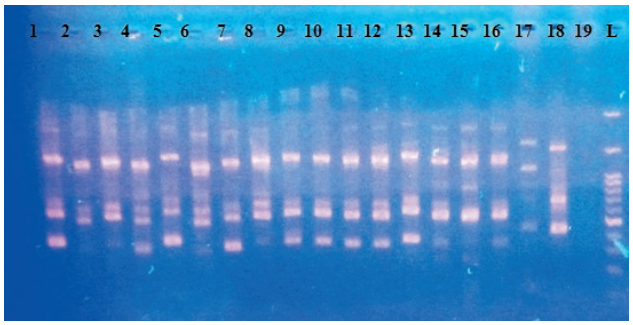

Figure 3. Gel Electrophoresis image of DNA fragments produced by OPD-03 of *Consolida* species. sp1= *C. tehranica;* sp2= *C. camptocarpa;* sp3= *C. lorestanica*; sp4= *C. leptocarpa;* sp5= *C. persica*; sp 6= *C. aucheri*; sp7= *C. anthoroidea*; sp8= *C. hohenackeri;* sp9= *C. stocksiana*; sp10: *C. rugulosa*; sp11: *C. ambigua ;* sp12= *C. orientalis*; sp13= *C. regalis;* sp14= *C. oliveriana;* sp15= *C. flava;* sp16= *C. trigonelloides;* sp17= *C. oligantha;* sp18= *C. linorioides;* sp19= *C. rugulosa* f. *paradoxa.* L = Ladder 100 bp. Arrows show polymorphic bands.

ing among and within populations using Wright's F_{ST} and other related statistics, such as G_{ST}, A_{ST}, R_{ST}, θ_{ST} and Φ_{ST}. For instance, the F_{ST} gives an estimate of the balance of genetic variability among and within populations, and is an unbiased estimator of divergence between pairs of populations under an island-model in which all populations diverged at the same time and are linked by approximately similar migration rates. However, migration rates usually vary proportionally with geographical distances, so that pairwise F_{ST} estimates between pairs of populations vary.

Evanno test performed on STRUCTURE analysis produced the best number of k = 10 (Figure.6). The STRUCTURE plot has revealed the allele combination difference among the studied populations and the occurrence of genetic admixture among them.

Inspite of genetic stratification and isolation by distance observed in *Consolida* species STRUCTURE plot (Figure 7) showed high degree of gene flow among the studied populations, Although the studied populations contained some specific alleles. For example populations 8-14 and 2,19 (differently colored segments in Figure.7), they shared some similar alleles too. For example, it showed genetic similarity between populations 3 and 4 (similarly colored), as well as 5, 6 and 15,16. The plants of population 1 had some alleles of population 10. Similarly, population 5,6 had some alleles of population 14.

Nonetheless, we were able to construct a consensus tree that agreed with our molecular (RAPD) and morphological findings (results not shown). The *Consolida* populations showed divergence due to genetic and morphological characters.

Table 4. Genetic diversity parameters in the studied *Consolida* species.

SP	N	Na	Ne	I	He	UHe	%P
C. tehranica	12.000	0.287	1.233	0.271	0.184	0.192	51.91%
C. camptocarpa	5.000	0.358	1.430	0.28	0.20	0.29	43.50%
C. lorestanica	6.000	0.299	1.029	0.231	0.18	0.23	44.38%
C. leptocarpa	5.000	0.462	1.876	0.288	0.29	0.28	62.05%
C. persica	8.000	0.399	1.167	0.24	0.21	0.113	52.88%
C. aucheri	5.000	0.336	1.034	0.23	0.25	0.19	51.83%
C. anthoroidea	4.000	0.344	1.042	0.28	0.23	0.27	57.53%
C. hohenackeri	5.000	0.455	1.234	0.277	0.24	0.22	55.05%
C. stocksiana	3.000	0.255	1.021	0.25	0.18	0.22	42.15%
C. rugulosa	3.000	0.288	1.024	0.23	0.35	0.30	64.30%
C. ambigua	5.000	0.462	1.095	0.288	0.25	0.27	62.05%
C. orientalis	8.000	0.399	1.167	0.322	0.398	0.344	65.77%
C. regalis	8.000	0.201	1.00	0.23	0.17	0.17	42.23%
C. oliveriana	5.000	0.341	1.058	0.24	0.27	0.20	53.75%
C. flava	5.000	0.455	0.67	0.277	0.24	0.22	55.05%
C. trigonelloides	8.000	0.499	1.067	0.24	0.13	0.24	49.26%
C. oligantha	6.000	0.555	1.020	0.22	0.25	0.28	43.53%
C. linorioides	10.000	0.431	1.088	0.20	0.12	0.15	41.53%
C. rugulosa f. *paradoxa*	3.000	0.255	1.021	0.25	0.18	0.22	47.15%

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

Table 5. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	ΦPT
Among Pops	20	1701.364	55.799	12.189	54%	54%
Within Pops	120	354.443	1.905	4.55	46%	
Total	150	2055.807		16.060	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, ($P < 0.001$).

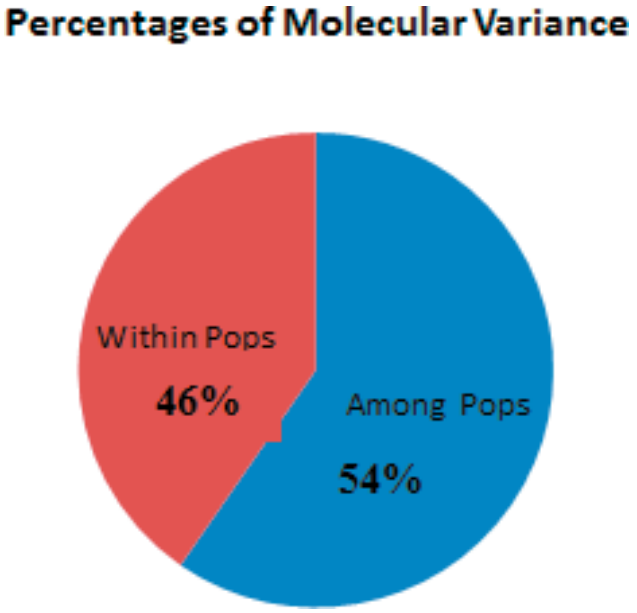

Figure 4. AMOVA test of the studied populations.

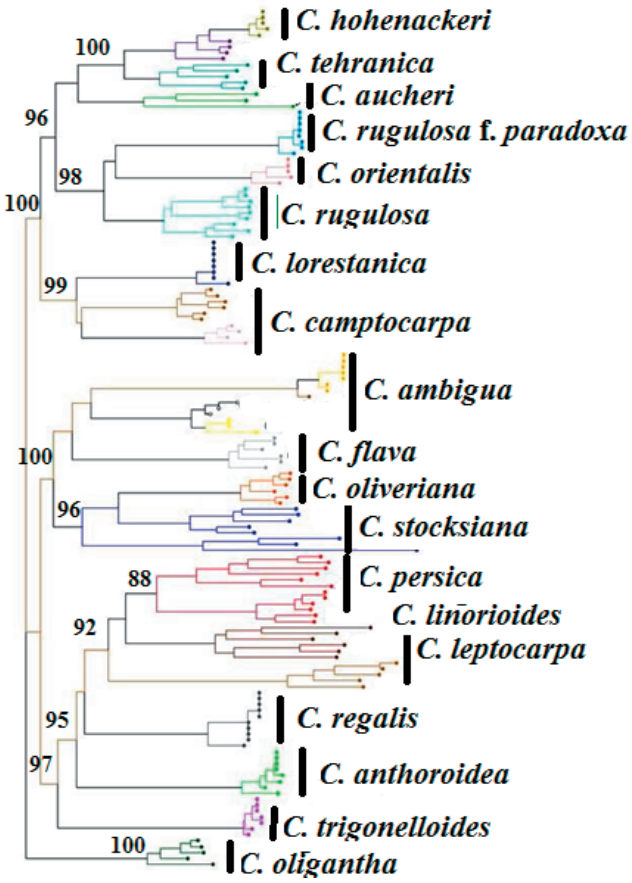

Figure 5. Neigbor-Joingin tree produced while using RAPD data. Branch support values are given as bootstrap (BP) value above branches.

DISCUSSION

The *Consolida* is a relatively complex taxonomic group, and several morphological characters make it difficult to identify and classify *Consolida* species (Ertugrul *et al.*, 2016). Given the complexity, it is necessary to explore other methods that could complement the traditional taxonomical approach (Erbano *et al.* 2015). Advent and developments in molecular techniques have enabled plant taxonomists to utilize molecular protocols to study plant groups (Erbano *et al.* 2015). *Consolida* is an evolved genus with precise synapomorphies (reduction of carpels from three or more to one, complete loss of lateral petals, spur consisting of one petal) that are not found in any other species of *Delphinium* and *Aconitum*. Most *Consolida* species are adapted to the Mediterranean type climate or more arid climate types of the Irano-Turanian zone (Ertugrul *et al.*, 2016). Pronounced periods of drought in these areas have certainly favoured the exclusive annual life cycle of *Consolida*. The biogeography of the genus indicates that Turkey, in particular Anatolia (c. 29 taxa) should be considered as the center of diversity, with further radiation of species into the Irano-Turanian area (c. 23 taxa), Greece (c. 10 taxa) and countries around the Mediterranean. *Consolida* forms a coherent, monophyletic clade with *Delphinium* and *Aconitum*. Some authors propose a direct evolution line of *Consolida* from *Delphinium* (Tamura 1966).

We examined genetic diversity in *Consolida* by morphological and molecular methods. We mainly used RAPD markers to investigate genetic diversity and genetic affinity in *Consolida*. Our clustering and ordination techniques showed similar patterns. Morphometry results clearly showed the utilization or significance of morphological characters in *Consolida* species. PCA results also confirmed the application of morphological characters to separate *Consolida* species. The present study also high-

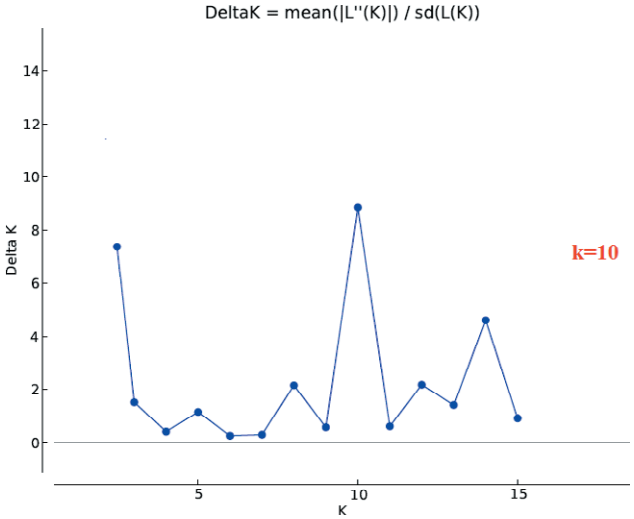

Figure 6. Delta k plot of Evanno's test based on STRUCTURE analysis.

lighted that morphological characters such as bract exerting from fruit, presence of spore, shape of spore apex, the number of petal, the number of petal lobes, could delimit the *Consolida* group. The *Consolida* species highlighted morphological differences. We argue that such a dissimilarity was due to differences in quantitative and qualitative traits. Present findings on morphological differences are in line with the previous studies (Iranshahr, 1992; Ertugrul *et al*., 2016; Khalaj 2013).

Genetic structure and gene flow

Polymorphic information content (PIC) values are useful to detect genetic diversity. The current study recorded average PIC values of 0.52. This value is sufficient to study genetic diversity in the population (Kempf *et al.* 2016). High genetic diversity among the *Consolida* population was reported in the present study. The previous scientific data (Kurata *et a*l. 2019) supports our current high diversity results. Genetic analysis conducted via analysis of molecular variance and STRUCTURE showed genetic differences among the species.

According to Bru¨tting *et al* (2012) sampled 53 populations from 6 arable plant species throughout Central Germany. Random amplified polymorphic DNA analyses (RAPD) were applied to calculate measures of genetic diversity at the population level and genetic differentiation. Their results showed that genetic diversity was found to be lowest in *Bupleurum rotundifolium* and *Anagallis foemina*, and highest in *Consolida regalis* and *Nigella arvensis*. The highest levels of genetic differentiation were observed among populations of *An. foemina* and *B. rotundifolium* but within populations in all other species. UST values differed strongly ranging between 0.116 for *C. regalis* and 0.679 for *An. foemina*. Patterns of genetic structure were related to the Red List status for all the species studied except *An. foemina*, for which it should consequently be raised. Them data confirm that even relatively recent threats are accompanied by detrimental genetic structure.

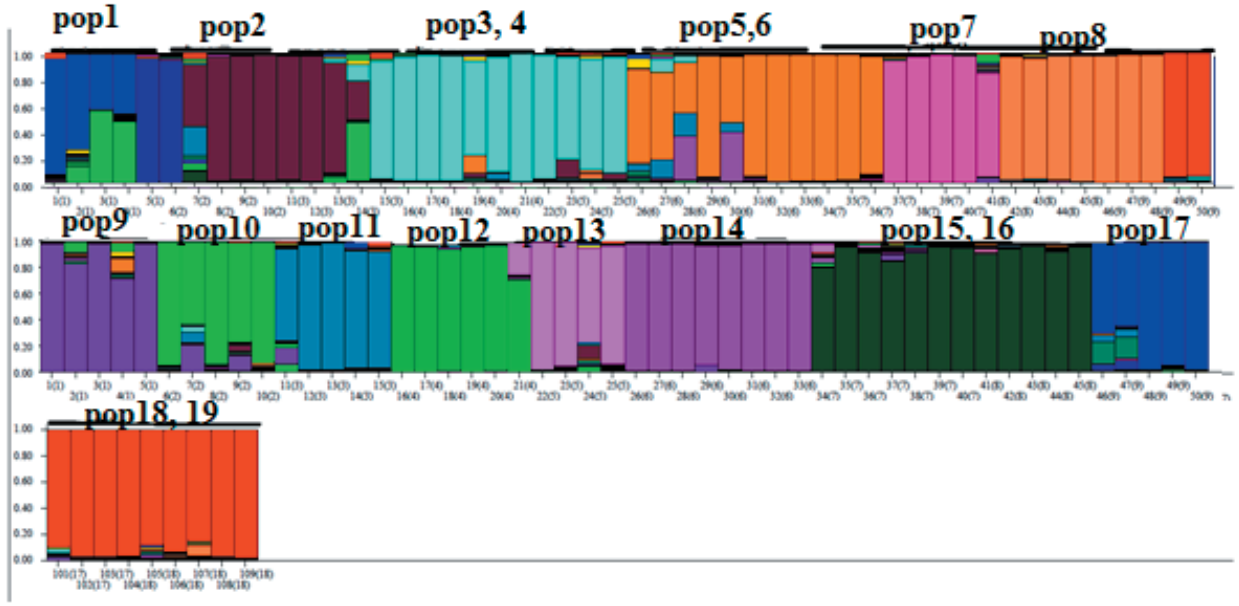

Figure 7. STRUCTURE plot of *Consolida* species based on k = 10 of RAPD data.

Table 6. The matrix of Nei genetic similarity (*Gs*) estimates using SCoT molecular markers among 19 *Consolida* species.sp1= *C. tehranica;* sp2= *C. camptocarpa;* sp3= *C. lorestanica*; sp4= *C. leptocarpa;* sp5= *C. persica*; sp 6= *C. aucheri*; sp7= *C. anthoroidea*; sp8= *C. hohenackeri;* sp9= *C. stocksiana*; sp10: *C. rugulosa*; sp11: *C. ambigua ;* sp12= *C. orientalis*; sp13= *C. regalis;* sp14= *C. oliveriana;* sp15= *C. flava;* sp16= *C. trigonelloides;* sp17= *C. oligantha;* sp18= *C. linorioides;* sp19= *C. rugulosa* f. *paradoxa.*

	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9	sp10	sp11	sp12	sp13	sp14	sp15	sp16	sp17	sp18	sp19
sp1	1.000																		
sp2	0.896	1.000																	
sp3	0.858	0.813	1.000																
sp4	0.846	0.836	0.842	1.000															
sp5	0.818	0.756	0.820	0.721	1.000														
sp6	0.821	0.867	0.725	0.835	0.793	1.000													
sp7	0.814	0.907	0.834	0.750	0.836	0.862	1.000												
sp8	0.838	0.782	0.768	0.775	0.881	0.794	0.828	1.000											
sp9	0.891	0.712	0.720	0.781	0.874	0.752	0.875	0.801	1.000										
sp10	0.826	0.798	0.854	0.759	0.702	0.742	0.770	0.754	0.880	1.000									
sp11	0.744	0.807	0.789	0.647	0.812	0.832	0.899	0.756	0.820	0.721	1.000								
sp12	0.701	0.812	0.832	0.703	0.787	0.768	0.766	0.767	0.725	0.835	0.839	1.000							
sp13	0.734	0.712	0.720	0.881	0.852	0.797	0.849	0.807	0.834	0.750	0.799	0.642	1.000						
sp14	0.744	0.826	0.705	0.742	0.745	0.775	0.807	0.789	0.747	0.812	0.832	0.799	0.756	1.000					
sp15	0.889	0.825	0.778	0.891	0.744	0.636	0.812	0.832	0.703	0.787	0.768	0.766	0.744	0.722	1.000				
sp16	0.743	0.838	0.739	0.738	0.787	0.768	0.712	0.720	0.881	0.852	0.797	0.649	0.807	0.797	0.891	1.0000			
sp17	0.782	0.891	0.771	0.794	0.852	0.797	0.826	0.805	0.742	0.745	0.775	0.817	0.782	0.798	0.888	0.757	1.000		
sp18	0.829	0.826	0.705	0.742	0.745	0.775	0.825	0.778	0.891	0.744	0.836	0.767	0.712	0.825	0.733	0.800	0.756	1.000	
sp19	0.889	0.825	0.778	0.891	0.744	0.936	0.838	0.739	0.738	0.787	0.768	0.773	0.826	0.705	0.742	0.745	0.775	0.854	1.000

Genetic diversity and population size

Our data suggest that the 19 study species differed highly in their genetic diversity. Populations of *C. rugulosa*; *C. ambigua* and *C. orientalis* showed the highest diversity, followed by *C. leptocarpa* and *C. anthoroidea*. Lowest values were found in *C. regalis* and *C. linorioides.*

It is widely accepted that the breeding system influences gene diversity dramatically (Mable and Adam 2007). For example Nybom and Bartish (2004) extracted from literature that selfing taxa have a mean He of around 0.09. In contrast, plant species with a mixed or outcrossing breeding system show an He of around 0.22 to 0.26. For our study species, *C. tehranica; C. camptocarpa; C. lorestanica*; *C. leptocarpa; C. anthoroidea*; *C. stocksiana*; *C. ambigua; C. orientalis* and *C. regalis* tend to have a mixed breeding system and that *C. oliveriana; C. flava; C. trigonelloides* are more outcrossing species. This assumption is certainly true for *C. regalis* because it is not self pollinating (Svensson and Wigren 1986). As inflorescences of outcrossing taxa are generally larger than inflorescences of selfing species (Hill *et al*. 1992), Lower genetic diversity could be an indication of higher fragmentation, as fragmentation leads to limited gene flow (Leimu *et al*. 2010). In fragmented populations pollinators struggle to reach the more distant populations and may even also decline in abundance (Potts *et al*. 2010). However, the relationship is consistent with population genetic theory, predicting that genetic drift is particularly important in small populations (Ellstrand and Elam 1993) and population size is positively correlated to genetic variation (Leimu *et al*. 2006). Molecular markers (RAPD) and morphometry analysis were useful to study genetic diversity and population structure in *Consolida* species identification. All the species had distinct genetic differentiation. Present results highlighted isolation and limited gene flow are the main deterministic factors that shape the *Consolida* population. We also reported high genetic diversity, which clearly shows the *Consolida* species can adapt to changing environments since high genetic diversity is linked to species adaptability.

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