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Morphology and assessment of genetic structure and diversity of *Dionysia revoluta* (Primulaceae) in Iran

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Abstract. *Younesi S, Assadi M, Nejadsattari T, Mehregan I. 2017. Morphology and assessment of genetic structure and diversity of* Dionysia revoluta *(Primulaceae) in Iran. Biodiversitas 18: 1173-1178. Dionysia revoluta* Boiss. (Primulaceae), the most widespread species of the genus in Zagros Mountains of western Iran, is studied morphologically and genetically. Clustering analysis of morphological characters did not match the biogeography of the populations. Cluster analysis separated two subspieces of *D. revoluta* i.e. subsp. *revoluta* and subsp. *canescens* (Boiss.) Wendelbo. Factor analysis (FA) showed that petal size and length of the corolla tube were the only two factors with the slightest variation. Amplified Fragment Length Polymorphism (AFLP) fingerprint technique was used to evaluate the genetic structure and diversity among 14 populations of *D. revoluta.* The results of AFLP analysis of *D. revoluta* individuals showed that 67% of the total diversity was that of within-population diversity, 17% was among-population diversity and 16% was among-region diversity. AFLP data suggest that amount of genetic variation of populations growing in localities with other *Dionysia* species in sympatry is higher than those populations growing allopatric. This implies the hybridization as a possible important factor in the evolution of the group. It was also concluded that hybridization would result in higher genetic diversity within those populations of *D. revulota* occur with other species in sympatry.

Keywords: AFLP, biogeography, genetic structure, Iran, Primulaceae

INTRODUCTION

The genus *Dionysia* Fenzl., one of the larger genera of Primulaceae Batsch ex Borkh., has more than 50 species with a concentration in the Zagros Mountains of Iran (Lidén 2007; Younesi et al. 2015). The generic name "*Dionysia*" was first applied to the material collected from Kurdistan (N Zagros, Lidén 2007). Later on, the number of known species of the genus raised dramatically (see Wendelbo 1961, Lidén 2007 and Borjian et al. 2014). *Dionysia* is well known for its narrow endemic species distributed mainly in allopatry (Wendelbo 1961, Lidén 2007). So far, different phylogenetic schemes are presented for the genus (Melchior 1943, Wendelbo 1961, Mast et al. 2001 and Trift et al. 2004). As most recent, Thrift et al. (2004) illustrated the phylogenetic relationships in *Dionysia,* using Parsimony, Jackknifing and Bayesian inference of nuclear DNA sequences (internal transcribed spacer, ITS) and two chloroplast regions (*rps*16 intron and *trn*L-*trn*F). Their phylogeny showed a biogeographical rather than a taxonomic pattern and despite major grouping of the species, the relationships between species in each group belonging to adjacent areas remained unclear (Thrift et al. 2004).

Dionysia species are suffrutescent herbs in the form of loose tufts or dense cushions, scattered mainly in crevices of rocks and cliffs (Wendelbo 1961, Lidén 2007). Most of the species have limited distribution with distinct and narrow ecological needs. *Dionysia revoluta* Boiss. is the most common and widespread species in the Zagros Mountains. It is characterized by lush cushions, 5-10 mm long and 0.5-2.5 mm wide leaves, which have strongly revolute margins. Leaves are densely glandular with 5-8 crenations. The species has two subspecies. Leaves of *D. revoluta* subsp. *revoluta* are green, 4-7.5 mm long and 0.6-2.5 mm wide, and covered with short hairs and glands. Leaves of *D.* subsp. *canescens* (Boiss.) Wendelbo are gray, 5-10 long and 0.5-1 mm wide, and covered with dense long hairs (Jamzad 1996). Both subspecies have sessile flowers, 1-2 per inflorescence, and linear bracts. Due to the wide distribution of this species and its fairly large ecological amplitude, Lidén (2007) suggested that different populations may show genetic diversity.

Since evolutionary processes, such as natural selection, adaptation, and genetic drift depend on a species' population structure, knowing a species' population structure is highly necessary for an understanding of evolutionary processes (Meirmans 2012). Genetic variation within a species supply the basis for any evolutionary change and is, therefore, the most fundamental level of biodiversity (May 1994). It has also been shown to be important for the fitness of individuals as high levels of heterozygosity may increase the fitness of individuals (Chapman et al. 2009). It is an important factor for survival of populations facing environmental changes. The first and an important step in managing species are to collect information on inter- and intra-populational variations (Abdemishani and Shah Nejad Bushehri 1998).

Different methods of molecular fingerprinting techniques are used to assess the genetic diversity, most of

them use polymerase chain reaction (PCR) for detection of fragments. Some methods are based on the amplification of random genomic DNA fragments generated by arbitrarily selected PCR primers (Vos et al. 1995). In comparison with other genotype finger-printing (such as random amplified polymorphic DNA (RAPD), DNA amplification fingerprinting (DAF), arbitrarily primed PCR (AP-PCR), pulse field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) and amplified ribosomal DNA restriction analysis (ARDRA)), Amplified Fragment Length Polymorphism (AFLP) is suggested to be most informative (Jones et al. 1997, Vaneechutte 1996). It is a method with high repeatability, and often able to show patterns below the species level. AFLP can also be used to assess gene flow, gene distribution and outcrossing, and hybridization (Vos et al. 1995, Winfield et al. 1998, Arens et al. 1998, Beismann et al. 1997).

In this study, we aimed to evaluate the genetic structure and relationships within and between populations of *D. revoluta* growing wild in W Iran using AFLP fingerprinting technique.

MATERIALS AND METHODS

Plant materials

Dionysia revoluta in Iran is represented by its two subspecies i.e. subsp. *revoluta* and subsp. *canescens*. Our study included samples from both subspecies. Fresh samples were collected from the Zagros Mountain range in provinces "Isfahan" and "Chaharmahal va Bakhtiari" during April 2014. Fresh leaves of five individuals from each population, with at least 20 m distant from each other, were collected, and immediately dried in Silica Gel (Chase and Hill 1991). Vouchers were deposited at the herbarium of Islamic Azad University, Science and Research Branch, Tehran, Iran (IAUH) (Table 1).

DNA extractions

Total DNA was extracted from silica gel dried leaves of 65 individuals following a modified CTAB protocol of Doyle and Doyle (1990) using NucleoSpin® Plant II Kit (Machery-Nagel, Dueren, Germany) after manufacturer′s protocol. The quality of extracted DNA was checked on a 1 % agarose gel and spectrophotometry.

AFLP

AFLP was performed according to Vos et al. (1995) and Scalone et Albach (2012) with some modifications; ca. 500 ng of total genomic DNA was digested by 5 unit *Eco*RI (Thermo Scientific, Waltham, USA) and 1 unit *Mse*I (Thermo Scientific, Waltham, USA) restriction enzymes and simultaneously ligated to 0.5 µL 5 pmol *Eco*RI (5'-CTC GTA GAC TGC GTA CC-3'; 5'-AAT TGG TAC GCA GTC TAC-3'; Metabion, Martinsried, Germany) and 0.5 µL 50 pmol *Mse*I (5'-GAC GAT GAG TCC TGA G-3';

Table 1. Sampling localities of *Dionysia revoluta* populations examined and analyzed in this study (Herbarium acronym after Thiers, 2017). All material is collected by S. Younesi

Pop. no.	Pop. code	Location	Alt. (m)	Longitude	Latitude	Herbarium no.	Species in sympatry
1	PLK	Iran: Bakhtiari, Semirom-Yasoudj road, 5 km after Pol-e Gharah, Dashtak, Pol-e Kataa	1610	51° 14.922	31° 11.479	14460 (IAUH)	*D. bryoides*
2	GRD	Iran: Bakhtiari, 5 km from Gooshaki towards Gandomaan, Gerdebishe	1890	51° 12.397	31° 34.806	14463 (IAUH)	*D. ceaspitosa*, *D. zagrica*
3	MLK	Iran: Bakhtiari, Semirom-Yasoudj road, 10 km before Malkhalifeh	1950	51° 19.528	31° 12.714	14464 (IAUH)	-
4	MLL	Iran: Bakhtiari, Semirom-Yasoudj road, 15 km before Malkhalifeh	2180	51° 18.753	31° 12.913	14465 (IAUH)	-
5	KHF	Iran: Esfahan, Khafr	2250	51° 28.255	31° 0.011	14466 (IAUH)	*D. bryoides*
6	KHA	Iran: Esfahan, Khafr, Abshaar	2340	51° 27.107	30° 9.981	14467 (IAUH)	-
7	TNL	Iran: Bakhtiari, Zardkouh, Lordegaan, Ardal tunnel	1820	50º39.468	32° 3.333	14473 (IAUH)	-
8	TCH	Iran: Bakhtiari, Lordegaan, Kouh-e Rig, opposite to the village Tchamanbid	2260	51° 0.368	31° 23.16	14477 (IAUH)	*D. zagrica*, *D. bryoides*
9	CHR	Iran: Bakhtiari, Lordegan, Monj, Baadaamestaan, mt. Chahaartagh	2130	50° 36.08	31° 29.992	14480 (IAUH)	-
10	GHR	Iran: Bakhtiari, Lordegaan, Ghorogh-e Kohyaan	2140	50° 33.08	31° 15.672	14481 (IAUH)	*D. bryoides*, *D. zagrica*, *D. ceaspitosa*
11	SHR	Iran: Bakhtiari, Boroudjen, Saraastaaneh, Kaleh-Gandomaan, Shouraab	2350	51° 5.24	31° 45.96	14486 (IAUH)	-
12	KHN	Iran: Bakhtiari, Lordegaan, between Tchelehgaah and Kohyaan	2130	50° 36.09	31° 29.99	14487 (IAUH)	-
13	BST	Iran: Bakhriari, Lordegaan, Behesht-Abaad	1680	50° 37.79	32° 1.83	14492 (IAUH)	different sub-species of *D. revoluta*
14	DNO	Iran: Bakhriari, Boroujen, Sarastaneh	2350	51° 5.24	31° 45.96	14486 (IAUH)	-

5'-TAC TCA GGA CTC AT-3'; Metabion, Martinsried, Germany) adapters by 0.004 µL T4 DNA ligase (10WU/µL, Thermo Scientific, Waltham, USA) for 3 h at 37° C. The reaction tube also contained 1 µL T4 DNA ligase buffer (Thermo Scientific, Waltham, USA), 1 µL 0.5 M NaCl, 0.25 µL 2mg/ml BSA (Bovine Serum Albumin, Thermo Scientific, Waltham, USA), and 1.4 µL ddH2O. A Labcycler® Thermocycler (Sensoquest, Göttingen, Germany) was used for performing pre-selective and selective amplifications with PCR protocols following Vos et al. (1995) including 2 min initial denaturation at 72° C, 20 cycles of 20 sec denaturation at 94° C, 30 sec annealing at 56° C, 2 min extension at 72° C, and a 30 min final extension at 60° C. Pre-selective amplification contained 6.5 µL PCR-mix (Ampliqon, Odense, Denmark; including 2× PCR buffer, 2 mmol MgCl2, 1% Tween 20, 0.2 mmol dNTPs), 0.125 µL 10 pmol primer E01 (5'-GAC TGC GTA CCA ATT CA-3'), 0.125 µL 10 pmol primer M02 (5'-GAT GAG TCC TGA GTA AC-3'), 3.25 µL ddH2O and 3 µL 3-fold ddH2O-diluted product of the digestion-ligation reaction.

The PCR program for selective amplification followed Westberg and Kadereit (2014). Selective amplification contained 7.5 µL PCR-mix (Ampliqon, Odense, Denmark; including 2× PCR buffer, 2 mmol MgCl2, 1% Tween 20, 0.2 mmol dNTPs), 0.18 µL 10 pmol florescent-labeled selective E primer E01, 0.25 µL 10 pmol selective M primer, 2.07 µL ddH2O and 5 µL 10-fold ddH2O-diluted product of the pre-selective PCR. Four primers (Biolegio, Nijmegen, Netherlands) combinations were used for the selective PCR. These are: E-38 (5'HEX- GAC TGC GTA CCA ATT CAC T-3') and M-57 (5'- GAT GAG TCC TGA GTA ACG G-3'); E-45 (5'FAM-GAC TGC GTA CCA ATT CAT G-3') and M-54 (5'-GAT GAG TCC TGA GTA ACCT-3'); E-40 (5'NED-GAC TGC GTA CCA ATT CAGC-3') and M-55 (5'-GAT GAG TCC TGA GTA ACGA-3'); E-31 (5'PET-GAC TGC GTA CCA ATT CAA A-3') and M-59 (5'-GAT GAG TCC TGA GTA ACT A-3'). Applied Biosystems standard dye set D-33 was used for the genotyping. PCR products were then pooled equally. 2 µL of equally combined PCR products for each sample was run with 7.75 µL of HiDi formamide and 0.25 µL internal size standard Gene Scan ROX on an ABI-Hitachi 3730 automated capillary DNA analyzer.

Software package GeneMarker 2.4.1 (SoftGenetics, State College, PA, USA) was used to analyze and score fragments. Fragments in a readable region of bands from 60 to 500 bp in length were scored as present (1) or absent (0). The mismatch error rate was calculated based on six supplementary re-extracted samples.

Software package Structure 2.3.4 (Pritchard et al. 2000) was used to investigate the Bayesian estimation of genetic structure. The first step was to calculate the likelihood of the data for a range of K=1 to K=9 values by creating posterior probabilities of K (true number of clusters; Porras-Hurtado et al. 2013). In order to estimate the number of genetically homogeneous groups of individuals, we implanted a Bayesian algorithm in the software STRUCTURE first described by Evanno et al. (2005). We repeated the process 20 times with 60,000 iterations and burned the first 10,000 iterations.

The genetic diversity and AMOVA (Analysis of Molecular Variance; Li, 1976, Meirmans, 2012) test for individuals, populations, and regions were performed using GenAlEX 6.5 package embedded in Microsoft Excel software.

Morphometrical analysis

Plant material from both subspecies *D. revoluta* subsp. *revoluta* and subsp. *canescens* were examined under a binocular. Measurements were made for 16 quantitative characteristics. Those were vegetative and reproductive characters including 1. Length of Internode; 2. Leaf length; 3. Leaf width; 4. Number of teeth on each leaf; 5. Bract length; 6. Bract width; 7. Calyx length; 8. Calyx width; 9. Petal length; 10. Petal width; 11. Anther length; 12. Style length; 13. Ovary diameter; 14. Number of seeds in ovary; 15. Number of pollens in each anther; 16. Tube length.

SPSS v. 18 (IBM) software package was used to perform multivariate analyses. Hierarchical cluster analysis of morphological data was performed with the ward method using squared Euclidean distance after standardization of measurements to 0-1 range.

Factor analysis was performed to reduce the dimensions and identify the source of changes. Principal Component Axes (PCA) analysis was performed with Varimax rotation to obtain eigenvalues greater than 1.

RESULTS AND DISCUSSION

AFLP analyses included 65 individuals from 17 populations of 14 regions (Table 1). Fragment analyses of the selective PCR products yielded 101 bands in a readable region of 60 to 500 bp in length. Table 2 shows the results of AMOVA test. According to the AMOVA, out of total diversity, 67% belonged to within-population diversity, 17% of among-population diversity and 16% to among-region diversity (Figure 1). As seen in Figure 1, within-population diversity is significantly high. Using the method of Evanno et al. (2005) based on an ad hoc statistic DeltaK between successive K=1 to K=9 values, STRUCTURE software package detects the uppermost hierarchical level of structure for K=2 (Table 3). Then, we run STRUCTURE with 1,200,000 iterations and burned the first 500,000 iterations. Genetic structure of individuals and populations are shown in Figure 2 with different clusters shown by different colors. Some populations show alleles mainly belonging to one cluster (TNL, DNO, SHR, CHR, KHM, TCH, MLL and MLK). Those populations grow with no other *Dionysia* species in sympatry (exp. TCH; Table 1). Other six populations (BST, GRD, GHR, PLK, KHF and KHA) consist of individuals with alleles from two different clusters. These six populations plus population TCH grow with other *Dionysia* species in sympatry or with obvious morphological variation (Table 1). In localities where *Dionysia* species are sympatric, we observed specimens with obvious intermediate forms, especially in floral parts.

Flowers of all *Dionysia* species (except for *Dionysia involucrata* and *D. teucrioides*) are known to be heterostylous (Trift et al. 2004), and it enhances the chance of hybridization. *Dionysia revoluta* has flowers with yellow corollas, while those of *D. bryoides* have purple corollas (Wendelbo 1961; Lidén 2007). We observed flowers with mixed colored corollas in the areas with both two species in sympathry, same as phenomenon described by Lidén (2007). These highly support the possibility that introgression may have contributed to genetic and morphological variation and occurrence of hybrids between *D. revoluta*, *D. bryoides* and other species (Figure 2, Table 1).

The results of the morphometrical analysis showed that populations of two subspecies have similarities and differences based on the studied characteristics. Significant differences in flower morphology of all samples were observed in the factor analysis of 16 qualitative characteristics. Separation of *D. revoluta* into two subspecies is mainly based on the leave morphology (Wendelbo 1961; Jamzad 1996). Despite this, our results showed that petal length and width and tube length showed the greatest impact and slightest changes in the separation of populations (Table 4). It means that floral morphology has more advantage than leaf morphology in classification of the genus *Dionysia*.

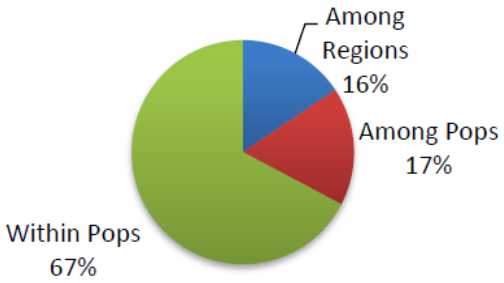

Figure 1. Result of AMOVA test showing the percentage of molecular diversity in *D. revoluta* (within-population, among populations, and among regions)

Table 2. Results of Analysis of Molecular Variance (AMOVA) to calculate the molecular variation of the populations of *D. revolute*

Source	Df	SS	MS	Est. Var.	%
Between regions	3	135.624	45.208	1.987	16
Between populations	9	171.513	19.057	2.169	17
Within populations	50	427.498	8.550	8.550	67
Total	62	734.635		12.706	100

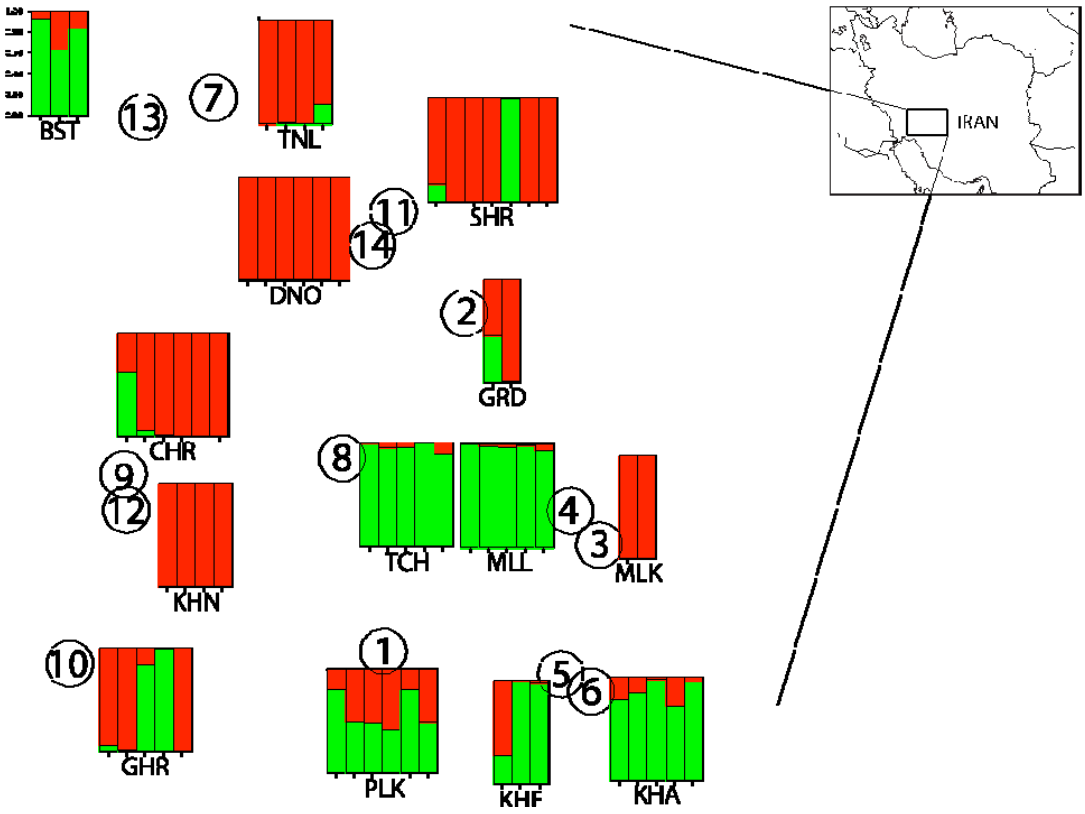

Figure 2. Genetic structure of individuals and populations of *D. revoluta* with K set to 2.

Table 3. Calculation of Delta K to determine the true number of clusters in populations of *D. revoluta* (method after Evanno et al. 2005)

Number of K	Ln (K)	Number of repetitions	St-Dev. [Ln (K)]	L' (K)	L" (K)	L"/ St-Dev. [Ln (K)]	[Delta K]
1	**-2445.4**	**20**	**0.14**				
2	-1998.378	20	0.57	447.02	447.02	-787.98	**787.98**
3	-1820.32	20	0.76	178.06	-268.96	-355.58	355.58
4	-1803.675	20	5.63	16.65	-161.41	-28.67	28.67
5	-1747.9	20	5.45	55.78	39.13	7.18	7.18
6	-1724.15	20	18.74	23.75	-32.03	7.17	7.17
7	-1696.495	20	10.27	27.66	3.91	0.38	0.38
8	-1785.545	20	17.62	-89.05	-116.71	-6.62	6.62
9	-1793.357	20	28.04	-7.81	81.24	2.90	2.90

Table 4. Factor analysis of the characteristics of the species *D. revoluta* subsp. *revoluta* and *D. revoluta* subsp. *canescens* Boiss

Rotated Component Matrix[a]	Component					
	1	**2**	**3**	**4**	**5**	**6**
The length of Internode	-.312	.169	.068	.119	.522	-.176
Leaf length	-.128	.861	.113	-.049	-.084	.058
Leaf width.	.100	.771	-.081	.148	.040	.084
The number of teeth on each leaf	-.128	.069	.122	.143	-.755	-.014
Bract length	.315	.230	.512	.020	-.008	.356
Bract width	.116	-.012	.090	.244	-.014	.740
Calyx length	.165	.028	.788	-.051	-.069	.129
Calyx width	-.081	-.020	.774	.076	.134	-.192
Petal length	.818	.098	.235	.111	-.030	.094
Petal width	.779	-.081	.009	.147	-.005	-.015
Anther length	.427	.517	.227	.090	.327	-.043
Style length	.025	-.002	.136	-.058	.646	.278
Ovary diameter	-.056	.116	-.065	-.163	.136	.724
The number of seeds in each ovary	-.118	.043	.088	.794	-.156	-.048
The number of pollen in each anther	.204	.089	-.066	.806	.047	.100
Tube lentgth	.732	.055	-.012	-.152	.025	.030

Extraction Method: Principal Component Analysis.
Rotation Method: Varimax with Kaiser Normalization.
Rotation converged in 6 iterations.

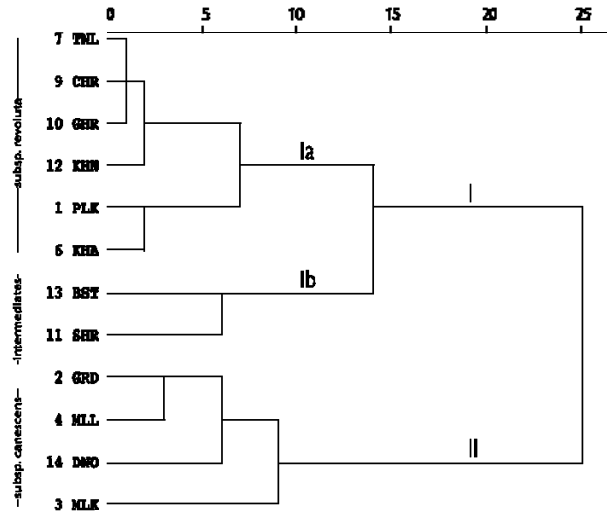

Figure 3. Dendrogram obtained from clustering analysis of 16 morphological characters of different populations of *D. revoluta* using Ward Linkage method.

In the clustering analysis, populations were grouped into two main clusters at phenon line 25 (clusters I and II; Figure 3). Cluster I was then divided into two sub-clusters at phenon line 14 (Ia and Ib). Grouping based on the morphological characters was clear with sub-cluster Ia composed of populations of *D. revoluta* subsp. *revoluta*, cluster II composed of *D.* subsp. *canescens* populations, and subcluster Ib composed of populations with intermediate morphology (Figure 3). In contrast to most of *Dionysia* species having limited distribution with distinct and narrow ecological needs (Lidén 2007), *D. revoluta* is the most widespread species in the Zagros Mountains. *Dionysia* species normally grow on poor limestone rocks and *D. revoluta* is the only species that also grow on the rich soils. Our samples are collected from the localities with a relatively wide range of ecological conditions. It is plausible that the morphological diversity in *D. revoluta* could be related to the phenotypic plasticity, which often makes a strong contribution to phenotypic trends associated with contemporary climate change (Merila and Hendry 2014; Franks et al. 2014).

Results of clustering analysis are not congruent with results of AFLP. Despite the morphological differences between the sections and subsections of the genus in current taxonomies, Trift et al. (2004) found no section and subsection with more than one species of *Dionysia* to be monophyletic. Instead, they found that species often belong to clades corresponding to geographical proximity. Regarding that fact, and also the higher rate of within-population diversity observed in *D. revoluta* populations (67%) in our survey, it is highly plausible that sympatric species can easily do genetic exchanges. This implies the hybridization as a possible important factor in the evolution of the species.

In conclusion, *Dionysia revoluta*, the widespread species of its genus, is morphologically and genetically variable. The genetic variation is however not completely congruent with neither geography nor morphology. It is suggested that the morphological diversity in *D. revoluta* could be related to the phenotypic plasticity. Despite the fact that the adaptation to diverse conditions during the time causes the physical and behavioral changes and cause gradual changes in different characters, the relative frequency of alleles in populations of *D. revoluta* is highly affected by the occurrence of other species in sympatry. Regarding the morphological and molecular evidence presented before, it can be concluded that hybridization would result in high genetic diversity within populations of *D. revulota*.

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REFERENCES

Abdmeishani S, Shah Nejad Bushehri A. 1998. Supplemental Breeding Plants, Volume 1, University of Tehran Press, Tehran.

Arens P, Coops H, Jansen J, Vosman B. 1998. Molecular genetic analysis of black poplar (*Populus nigra* L.) along Dutch rivers, Mol. Ecol. 7: 11-18.

Beismann H, Barker JHA, Karp A, Speck T. 1997. AFLP analysis sheds light on distribution of two Salix species and their hybrid along a natural gradient. Mol Ecol 6: 989-993.

Borjian A, Deylami E, Dousti AF. 2014. *Dionysia assadii* sp. nov. (Primulaceae: sect. Dionysiopsis) from southern Iran. Nordic J 32 (6): 717-722.

Chapman JR, Nakagawa S, Coltman DW et al. 2009. A quantitative review of heterozygosity-fitness correlations in animal populations. Mol Ecol 18: 2746-2765.

Chase MW, Hills HG. 1991. Silica gel: an ideal desiccant for preserving field-collected leaves for use in molecular studies. Taxon 40: 215-220.

Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Evanno G., Regnaut S., Goudet J. 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. Mol Ecol 14: 2611-2620.

Franks SJ, Weber JJ, and Aitken SN. 2014. Evolutionary and plastic responses to climate change in terrestrial plant populations. Evol Appl 7 (1): 123-139.

Jamzad Z. 1996. The genus *Dionysia* (Primulaceae) in Iran. Iran J Bot 7: 15-30.

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, van de Wiel C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E, Malcevschi A, Marmiroli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazquez A, Karp A. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol Breed 3: 381-390.

Li C. 1976. Larg Random Mating Population. First Course in Genetics. Boxwood Press, Pacific Grove, CA.

Lidén M. 2007. The genus *Dionysia* (Primulaceae), a synopsis and five new species, Willdenowia 37 (1): 37-61.

Mast AR, Kelso S, Richards AJ, Lang DJ, Feller DMS, Conti E. 2001. Phylogenetic relationships in *Primula* L. and related genera (Primulaceae) based on noncoding chloroplast. Intl J Pl Sci 162: 1381-1400.

May RM. 1994. Biological diversity: differences between land and sea. Phil Trans R Soc London B: Biol Sci 343: 105-111.

Meirmans PG. 2012. AMOVA-Based Clustering of Population Genetic Data. J Heredity 103 (5): 744-750.

Melchior H. 1943. Entwicklungsgeschichte der Primulaceen: Gattung Dionysia. Mitt. Thür. Bot Vereins 50: 156-174.

Merila J and Hendry AP. 2014. Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. Evol Appl 7 (1): 1-14.

Porras-Hurtado L, Ruiz Y, Santos C, Phillips C, Carracedo A, Lareu MV. 2013. An overview of Structure: applications, parameter settings and supporting software. Front Genet 4: 1-13.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945-959.

Scalone R, Albach DC. 2012. Degradation of sexual reproduction in *Veronica filiformis* after introduction to Europe. BMC Evol Biol 12: 233, DOI: 10.1186/1471-2148-12-233.

Trift I, Lidén M. 2004. Phylogeny and biogeography of *Dionysia* (Primulaceae). Intl J Plant Sci 165 (5): 845-860.

Vaneechoutte M. 1996. DNA fingerprinting techniques for microorganisms. A proposal for classification and nomenclature. Mol Biotechnol 6: 115-142.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acid Res 23: 4407-4414.

Wendelbo P. 1961. Studies in Primulaceae I. A monograph of the genus Dionysia. Aarbok Univ. Bergen, Mat.-Naturvitensk. Ser. 1961 (3).

Westberg E, Kadereit JW. 2014. Genetic evidence for divergent selection on *Oenanthe conioides* and *Oe. aquatica* (Apiaceae), a candidate case for sympatric speciation. Biol J Linn Soc 113: 50-56.

Winfield MO, Arnold GM, Cooper F et al. 1998. A study of genetic diversity in Populus nigra subsp. betulifolia in the Upper Severn area of the UK using AFLP markers,.Mol Ecol 7: 3-10.

Younesi S, Mehregan I, Assadi M, Nejadsattari T, Liden M. 2016. *Dionysia robusta* (Primulaceae), a new species from W Iran. Willdenowia 46 (1): 105-112.