Original Article

The relationship analysis of taxonomical, phylogeographycal, variation and genetical structure between *Veronica anagallis-aquatica* L. populations in Iran

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Abstract

In this research the geography, genetic diversity and genetic structure of *Veronica anagalis-aquatica* L. populations (water speedwell) were studied in Iran. Water speedwell (*V.a.a)* are perennial plants and in Iran three subspecies are found in moist habitats, such as river margins. The three subspecies have the same general appearance, and it has been a challenging subject to study long-term demographic differentiation and recognition of their potential diversity. Here, we studied migration routes and aim to solve taxonomic problems of this species in Iran. Samples collected from the north, northwest and center of Iran were analysed for phylogeographic and phylogenetic studies. The use of modern molecular tools, along with morphological and eco-ecological studies, was essential for population surveys. For this purpose, we studied relationships among the individuals and population structure using the ISSR marker. Extracting DNA of 96 individuals from 16 different populations in Iran and calculating the genetic distance demonstrated that the total genetic variation related to the difference in ISSR segments was mainly (80%) intra-population variation, 17% variation between populations and 3% diversity among the regions. Genetic analysis also showed that there were two major genotypic groups. This study showed that the results of molecular analyses better predict the position and relationship between species than other classical taxonomic methods. Further studies with the help of other molecular markers, as well as the inclusion of more taxa in the study, are necessary and appear promising.

Keywords: Phylogeography, Population genetics, *Veronica anagallis-aquatica* L., ISSR

Introduction

In this research, the phylogeography and genetic composition of the populations of *Veronica anagalis-aquatica* L., Sp. Pl. 12(1753) in Iran has been studied.

Phylogeography the science of studying the geographical distribution of genetic groups of individuals in a species has gained large interest with the rise of DNA sequence analysis [1, 2]. In this study, ISSR markers were used, because ISSR analysis has the proven potential for phylogeographic analysis [3]. *Veronica anagallis-aquatica* belong to the genoms veronica with 500 species in the world and a major center of distribution in the temperate regions of the Northern Hemisphere and New Zealand. Of these 54 annual or perennial species occur in Iran, among them 18 species that are endemic [4, 5]. Phylogenetic affinities of veronica have been adequately supported by molecular data and is generally consistent with data on the number of chromosomes, morphology, life cycle and phytochemistry [6-8]. Accordingly *Veronica anagallis-aquatica* L. belongs to *V.*Sect. *Beccabunga* it is tetraploid with 2n=36 and has a base chromosome number is x=9. This plant was recognized as a high-cadmium adsorbent in the study of the comparison of cadmium absorbed from saline water in hydroponic system by two *V.a.a* [9]. It has been used in traditional Chinese medicine, as an herbal remedy for the treatment of influenza, pulmonary hemorrhage, throat and laryngeal and hernia infections. However, so far, no phylogenetic or phylogeographical study on *V.a.a* populations from Iran was conducted. To have an accurate understanding of among- and within-population relationships and solving taxonomic problems *of V.a.a* population with the help of molecular markers is critically necessary. Specifically, it is necessary to use modern molecular tools along with morphological studies because morphological evidence and traditional studies in Veronica have been inefficient. This species is a perennial rarely annual plant with a height of 10 to 150 centimeters, flowering and fruiting from early March to summer, and is an

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Iranian Turanian plant occurring in wet areas, riversides, swamps, and rivers margins [4, 10]. It is almost cosmopolitan, occurring natively in Europe, Turkey, Palestine, Lebanon, Syria, Iraq, the Caucasus, Afghanistan, Central Asia and Pakistan. In the Flora Iranica [11], the main regions distribution of the species are North, Northwest, Center and West of Iran. There are three more taxa commonly separated from *V.a.a* as either species or subspecies, which are *V.a.a* subsp. michauxii, subsp.oxycarpa, and subsp. Lysimachioides [11]. The first one is characterized by a glandular-villous indumentum on stem and leaves. Specimens from this taxon consistently clustered close in molecular and morphometric analyses by Ellmouni et al (2017) [12]. *V.a.a* subsp. *oxycarpa* is considered transitional between subsp. *anagallis-aquatica* and subsp. *lysimachioides* and this is also highlighted in the morphometric analysis [12], which grouped individuals either with subsp. *michauxii* or subsp. *lysimachioides*. According to Fischer (1981), it is characterized by ovate to elliptic leaves and apically tapering capsules [11]. *Veronica anagallis-aquatica* subsp. *lysimachioides* is characterized by glabrous surface, uniformly sessile leaves, dense inflorescence, and capsules with roundish-obtuse apex. Öztürk and Fischer (1981) mentioned that this taxon presents considerable problems in its delimitation from its closest relatives, especially from subsp [13]. *oxycarpa*, which is insufficiently understood. Though quite well characterized by Schlenker (1936 a) [14], *V. lysimachioides* has been neglected by many authors (e.g., by Marchant, 1970; Elenevskij, 1969; 1978) [15-17]. Fischer (1981) has given a full description of the taxon. It is differentiated by the very dense raceme; all leaves sessile; the complete absence of glandular hairs; pedicels up to 3.5 mm; and capsules shorter than 3.5 mm, with obtuse apex. These characters are not especially characteristic and consequently specimens formed a polyphyletic group in the morphometric analysis of Ellmouni et al (2017). From a long time ago, this plant leaves have been used as raw or cooked as a uretic spicy appetizer vegetable contains vitamin C, used in the treatment of scurvy, the elimination of blood contamination, wound healing, gastric ulcer treatment and inflammation of the last finger knuckle [18]. Despite the morphological studies on Veronica species, there has not been any comprehensive review on the among- and within-population relationships of *V.a.a*. There is a need to investigate the differences, similarities and among/within-relationships of *V.a.a* species population in different regions of Iran, to study and recognize the habitat, the probable migration routes, the geographical distribution of populations, and finally, it is necessary to have a clear understanding on "what is the relationship between biological geography with genetic similarities and differences among different populations of *V.a.a* plants in Iran?"

Materials and Methods

Sampling

Using Flora Iranica [11], locations of the *V.a.a* complex were identified in their natural habitat and samples were collected during July-September 2015. The sampling distance between populations was at least 50 km. At each population, we sampled leaves of 6 individuals having at least 1 km distance from each other. Leaves were afterwards, dried by silica gel. For the morphometric studies, the complete organs of each sample (576 individuals in total, 36 individuals for each population) were picked up, dried, stored and had a herbarium code. Using Flora Iranica, dried herbariums samples were identified (Table 1).

Table 1. The sampling location of the individuals of *Veronica anagallis-aquatica* L. species.

field no.	Province	city	Locality	Altitude (m)	Latitude	Longitude	Collection Date	Herbarium code(IAUH)
VHM-1	Gilan	Masuleh	10km from kalvaz to Khalkhal	3303	37° 36.93' N	48° 29.87' E	2015/Aug/10	IAUH-15152
VHA-2	Gilan	Aghdagh	10km from Aghdagh to Masuleh	1761	37° 9.52' N	49° 2.83' E	2015/Aug/10	IAUH-15153
VHS-3	Ardabil	Sarein	Alvares ski resort	2954	38° 13.53' N	47° 55.25' E	2015/Aug/12	IAUH-15154
VHK-4	Ardabil	Khalkhal	5 km from Aznav spring to khalkhal	1853	37° 34.9' N	48° 34.35' E	2015/Aug/11	IAUH-15155
VHG-5	Ardabil	Ghotour	Ghotour	2693	38° 19.67' N	48° 50.68' E	2015/Aug/10	IAUH-15156
VHV-6	Ardabil	Meshkinshahr	Velayat forest park	1516	38° 22.15' N	47° 40.8' E	2015/Aug/12	IAUH-15157
VHAG-7	Ardabil	Angout	Ghareh aghaj	1943	38° 51.62' N	47° 45.6' E	2015/Aug/12	IAUH-15158
VHAA-8	Ardabil	Abgarm	Abgarm	1541	37° 41.73' N	48° 24.42' E	2015/Aug/12	IAUH-15159
VHR-9	Ghazvin	Razjerd village	Razjerd village	1976	36° 21.38' N	50° 11.28' E	2015/ July /30	IAUH-15160
VHGA-10	Alborz	Gachsar	Gachsar	2303	35° 47.22' N	51° 40.62' E	2015/ July /15	IAUH-15161
VHD-11	Alborz	Dizin	Dizin ski resort	3041	36° 2.43' N	51° 25.63' E	2015/Aug/15	IAUH-15162
VHMZ-12	Hamadan	Malayer	20km from Malayer toKonjehdar village	1991	34° 8.15' N	49° 0.18' E	2015/Aug/27	IAUH-15163

VHHG-13	Hamadan	Hamadan	Ganjnameh	2041	34° 46.28' N	48° 27.58' E	2015/Aug/27	IAUH-15164
VHB-14	Kurdistan	Bijar	Changiz ghaleh	1900	35° 52.35' N	47° 32.1' E	2015/Aug/27	IAUH-15165
VHBJ-15	Azerbaijan (W)	Bazargan	Bazargan	1513	39º 24.52' N	44º 21.78' E	2015/Sept/13	IAUH-15166
VHMA-16	Azerbaijan (E)	Mianeh	44 km fromAchachi village to Mianeh	1693	37° 23.75' N	47° 47.65' E	2015/Aug/12	IAUH-15167

Molecular and phylogenetic studies

DNA of dried leaves of 96 samples of *V.a.a*, was extracted using NucleoSpin Plant II extraction kits. The genome obtained from different individuals and marked and specific primers of this analysis was used for different levels of ISSR analysis. PCR products obtained from ISSR-PCR amplification with eight selected primers suitable [19, 20]. The primers used include:$(CAA)_5$-$(AGAGAG)_2$AGAGT-$(ACACAG)_2$ACACT-$(CACA)_3$GC-$(CACACA)_2$CACARG-$(ACACAC)_2$-ACACYT-(GACA)-$(AGAGAG)_2$AGAGT.

PCR was performed in a 13 µl volume containing 6.5 µl master mix, 0.5 µl primer,4.75 µl deionization H2O, 0.5 µl DMSO and 0.75 µl genomic DNA. Amplification reactions were performed in a Lab Cycler Basic thermocycler with the following program: Preheating at 94 ^{0}C for 300^{sec}– amplification in 35 cycles (denaturation at 94 ^{0}C for 40^{sec}, extension at 72 ^{0}C for 60^{sec}, annealing at 37.8 ^{0}C for 60^{sec}) – final extention at 72^{0}C for 420^{sec}.So, annealing was different for other primers: 48.10^{0}C, 470^{0}C, 42.10^{0}C. That's why ensure the presence of replicated ISSR regions of genomic DNA (PCR product), horizontal electrophoresis with 2% agarose gel was employed. PCR products with strong single bands were determined the sequence. Next, the sequence nucleotides of the ISSR fragments from individuals of the studied species were first completely analysed using Sequen Cher software 5.4.6(Gencodes corporation, 2016)[1] and the required corrections about distance or errors was by comparing forward and reverse sequences. ISSR fragments sequencing were edited using the Sequen Cher software, the alignment done in Mac Clade [21]. After the alignment and bands intensity correction, the bands that ranged from 50 to 550bp were automatically ranked by the software. To analyse population genetics using ISSR marker, totally 96 individuals of 16 *V.a.a*, populations from the regions of North, Northwest and West of the country (Tehran, Alborz, Gilan, Ardebil, ghazvin, Hamadan, Kurdistan, west & east Azerbaijan) were studied. ISSR bands for 96 individuals from 16 *V.a.a* populations obtained were coded as binary characters (presence=1, absence=0). ISSR data as a statistical data was imported into Past software Ver 3.25 [22] and Nei's genetic distance was used for clustering [23, 24]. The "ward's method" algorithm and the "Euclidean" similarity index were used to draw the similarity dendrogram and a Principal Coordinates Analysis (PCoA) was performed using PAST software Version 3.25. PCoA has been developed for analysing the structure of natural populations through molecular markers and is often used to show genetic similarity among populations [25].

Using GenALEx 6.5 software [26] the genetic distance was calculated among all individuals, afterwards, an AMOVA (Analysis of molecular variance) test was used to estimate the genetic variation among individuals and different regions. To investigate the genetic structure, Structure 2.34 software [27] was used based on Bayesian statistical methods. The method described by Evano et al (2005) was used to find the actual number of populations (K) [28]. Using the Structure 2.3.4 software, after completing 20 repetitions of the MCMC (Markov Chain Monte Carlo) process for a hypothetical population number of populations, the results were obtained.

Results

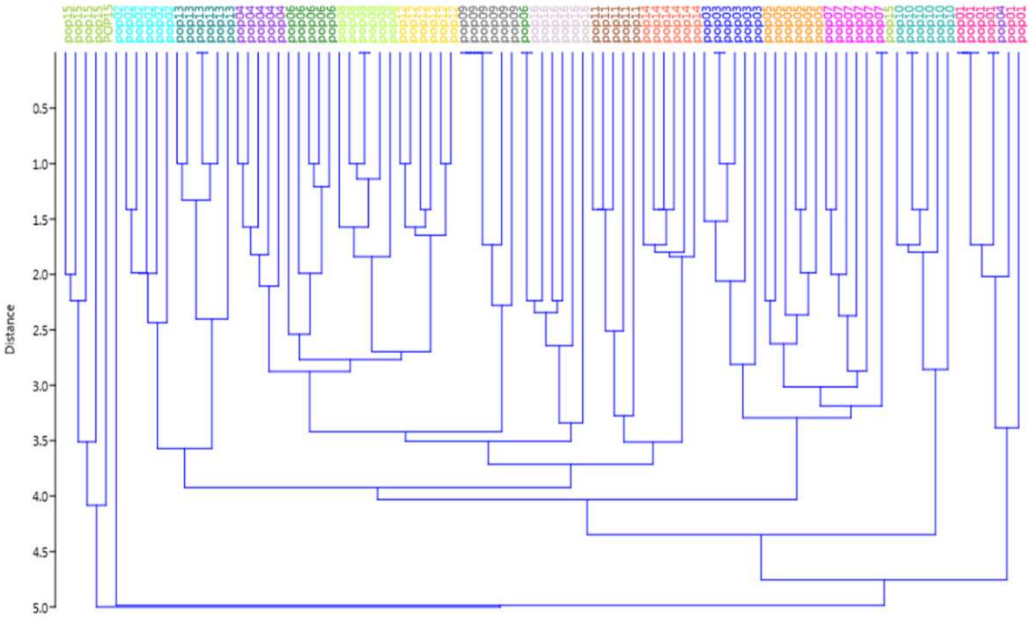
Plant materials

V.a.a. has a wide distribution due to high population diversity, but the native area is unclear and today it occurs on most continents and under many climates (Europe, East to North Africa, warm parts of Asia,…) and, of course, are naturalized in hot areas [29, 30]. This plant grew completely exposed to light (preferred light), have compatibility with wet conditions, and the type of its soil is sandy, loamy or pebble. This species appears half-hidden in areas where water velocity is low. The length and diameter of the internodes in high water velocity is less, likely to increase the plant's flexibility and its resistance to the flow of water. The root system is fibrous and either rhizomatous or stoloniferous. The blooming period occurs from late spring to late summer and lasts about 2-3 months. Leaves floating in water are similar to those in shade of the terrestrial plants [31]. Regarding the above characteristics, in all sampling sites, *V.a.a* appeared half-hidden in slow-moving streams and leaves floated in water had a lower and thicker area. There are 3 subspecies of *V.a.a* in Iran which are subsp. *michauxii*, subsp. *oxycarpa*, and subsp. *Lysimachioides* [11]. The study of morphological characters, including leaf shape, density of inflorescence, length of peduncle and pedicel, shape of bract, shape of capsule, relation between length of calyx and capsule and the other characters showed that all of populations had similar to *V.a.a* subsp. *Michauxii*, except populations Agh(2) and Gho(5). The recent populations had similar to subsp.*oxycarpa*. These results will be useful for future study of the morphological analysis of *V.a.a* species in Iran.

Similarity dendrogram for individuals

[1] www.gencodes.com. Sequencher version 5.4.6, 2016. 775 technology Drive Suite 100A Ann Arbor, MI 48108 / 734-769-7249 OR 800-497-4939.

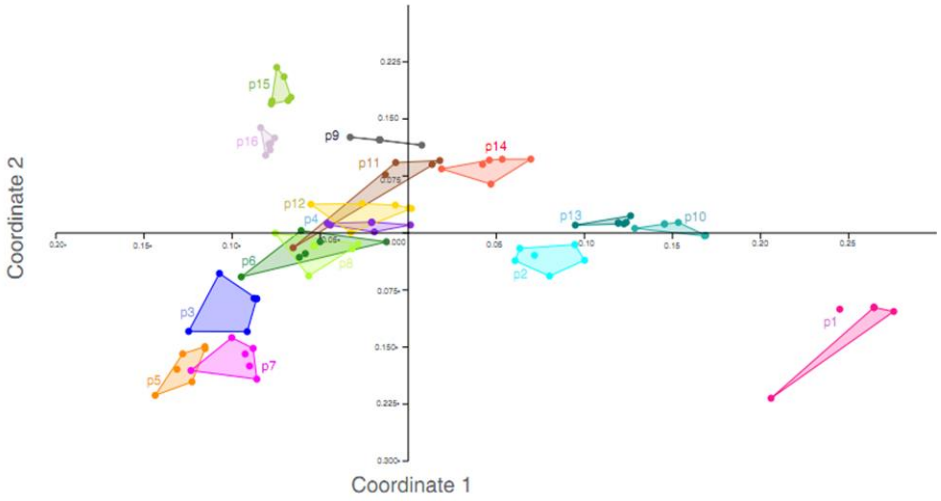
ISSR data for 96 *V.a.a* individuals is shown as similarity dendrogram in Graph 1. Genetic affinity of the studied populations was determined by NJ tree. Most of the plants in each population were placed close to each other, but some were placed intermixed with the other populations. This was true particularly for Populations Kha (4), Vel (6) and Baz (15).



Graph 1-Similarity dendrogram of 96 individuals from 16 *V.a.a* population in Iran.

Principal Coordinate Analysis

The PCoA, which is based on either population or individual genetic distance, also showed the 16 *V.a.a* population could be divided each other (Graph 2).



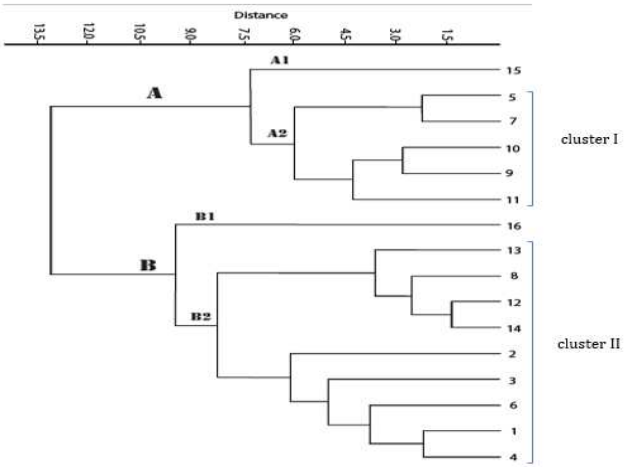
Graph 2. Graph showing the results of the principal coordinate analysis of ISSR data for 96 individuals from 16 *V.a.a* populations in Iran; based on Dice genetic distances (equivalent to Nei–Li distances) and power of C (Transformation exponent). The standard value is C=2 [32].

PCOA is another ordination method, also known as Metric Multidimensional Scaling [33]. The similarity/distance values are raised to the power of C (the "Transformation exponent") before Eigen value analysis. The standard value is C=2. Higher values (4 or 6) may decrease the "horseshoe" effect [32]. Missing data is supported by pairwise deletion. Graph

showing the results of the principal coordinate analysis of ISSR data. Multidimensional scaling plots of molecular characters, separating 16 populations from each other.

Similarity dendrogram for populations

Genetic relationship trees from whole ISSR data among 96 *V.a.a* samples (16 populations) were distributed into three groups based on Nei and Li's similarity coefficient using Neighbour- Joining (NJ) and UPGMA methods [22]. Cluster I (5 pops); Cluster II (9 pops). Sam 2 pops was not associated with any of the clusters (graph 3).



Graph 3- UPGMA dendrogram based on Nei's genetic distance 16 *V.a.a* populations in Iran

Genetic distance and AMOVA tests

AMOVA (Table 2) produced significant genetic difference among the studied populations. It also revealed that 17% of total genetic variability occurred among the studied populations while, 80% occurred within these populations and the least diversity among regions (Alborz, Gilan, Ardabil, Hamadan, Kurdistan, Azerbaijan) is 3%.

Table 2- AMOVA tests for 96 individuals of 16 V.a.a populations. Ss=Sum squares; MS=Mean squares; Df= Degree freedom.

%	Est. Var.	MS	SS	Df	source
3%	0.161	15.628	31.256	2	**Among region**
17%	1.039	11.100	144.296	13	**Among population**
80%	4.865	4.865	389.167	80	**Within population**
100%	6.065		564.719	95	**total**

Genetic Structure Computation

Using the Structure 2.3.4 software, after completing 20 repetitions of the MCMC process for hypothetical population number of populations from 1 to 5, the results were obtained (Table 3). The MCMC analysis was performed on the total data assuming a total of 750,000 rounds and removing 500,000 first rounds for K=2.

Table 3: Delta K (natural logarithm k) calculation to determine the actual number of populations in Veronica anagallis aquatica L. St.Dev=standard deviation.

Delta K	L"/STD	L"(K)	L'(K)	St-Dev. [L(K)]	Number of repetition	Ln(k)	**number(k)**
				0.1852	20	-2168.98	**1**
K=2	-0.52614	-78.99	0.325	150.1318	20	-2168.66	**2**
	0.355342	117.925	-78.665	331.8631	20	-2247.32	**3**
	-0.35266	-99.79	39.26	282.9636	20	-2208.06	**4**
	0.188953	60.53	-60.53	320.3443	20	-2268.59	**5**

The results showed that when K=2, all the individuals of the population could be distributed to corresponding groups in a high proportion. Structure plot is shown in Figure 1 (2), identified these two genetic groups (gene pools). The plot revealed that populations Sar (3), Gho (5), Baz (15) are genetically more alike and comprise the first gene pool, populations Kha (4), Mas (1) are genetically more alike and comprise the second gene pool. Individuals from other populations share allels with 2 gene pools. The populations of Raz (9), Abg (8), Mia (16), Bij (14) and Mal (12) have the maximum diversity and difference in the genetic structure of their individuals. Therefore, the highest value for gene diversity among their own individuals occurred in Hamadan (13) and Kurdistan (14).

Discussion

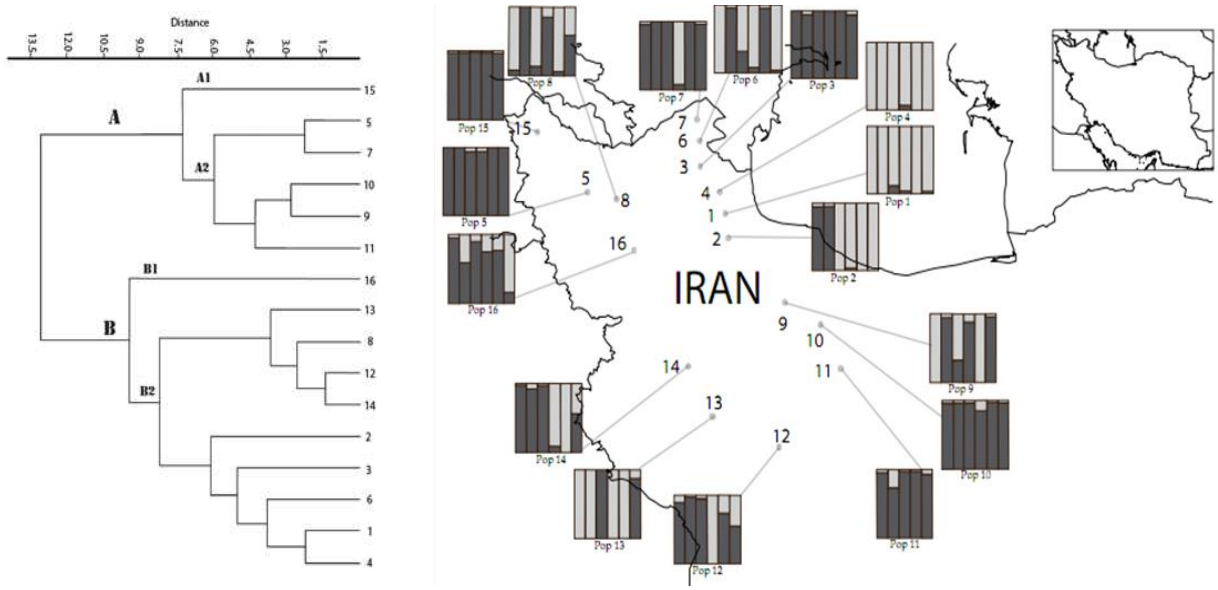
Genetic diversity among and within populations is a result of several historical events and recent evolutionary processes [34]. In the present study, gene exchange and genetic diversity was high within *V.a.a* populations in Iran. This should be due to outcrossing nature, biological productivity [35] and maybe, the genetic drift becomes active within-populations [36, 37]. So, gene flow has an important impact on population differentiation and population fragmentation [38]. In such case, gene flow introduces new genes to the local populations and

increases their genetic variability [37]. From the perspective of modern genetics, species with high genetic diversity will have a wider natural distribution and stronger environmental adaptability, survivability and evolutionary potential. Isolated populations tend to have low gene flow between them, and thus high population differentiation (e.g., populations Sar (3), Gho (5), Gach (10) and Baz (15)). If gene flow by mating system and seed dispersal are the main causes of population variation, the closer the geographical distance between two populations is, the smaller the genetic differentiation should be. As a result, more closely situated populations tend to be more genetically similar to one another [39-41] (e.g. Mas (1), Khal (4) and Gach (14), Diz (11)).

Seeds in every regions have dispersed by the waterways (river, spring and flood stream), wind, the movement of domestic animals and, sometimes, displacement of rock and the soil of *V.a.a* habitat as a building material. For the populations of each region of Gilan, Azerbaijan, Alborz and Ardabil, this phenomenon was frequently observed during three visits to gathering places (from 2015 to 2018).But, the gene exchange and diversity were so low among regions in Iran. Low diversity (3%) among regions can be caused by the effect of geographical distance disturbance, height and climatic differences.

The population genetic structure of *V.a.a* in Iran displayed admixture of alleles and none of them identified as a pure line. So, the populations of Raz (9), Abg (8), Mia (16), Bij (14), Mal (12), Vel (6) and Ham (13) have the maximum diversity and difference in the genetic structure of their individuals. The factors affecting the genetic structure of a species include the breeding system, distribution and gene flow, etc. [42, 43]. The species of *V.a.a* is a perennial herb, mainly reproducing sexually via flowers by insect-pollination and wind-pollination, its seeds being propagated by wind or water [18]. Long-distance propagation of pollen by wind and insects may exist and the growing environment of *V.a.a* is mainly at the riversides. The connectivity of wet areas, riversides, swamps, and river margins [4, 10] might make the seeds of *V.a.a* spread over a long distance by the water, both promoting gene exchange and enriching the gene pool in populations. This could have led to an increase in the within-population genetic diversity of species and a reduction in the genetic differentiation between populations (Figure 1 (2)).

Using an adaptive comparison, it is possible to comprise the genetic relationship dendrogram from whole ISSR data among 96 *V.a.a* samples (16 populations) obtained based on Nei and Li's similarity coefficient and population genetic structure then discuss and conclude (Figure 1).



(1) (2)

Figure 1: Adaptive comparison the genetic relationship dendrogram (1); population genetic structure (2).

The UPGMA and structure analyses both clustered the 16 *V.a.a* populations into two groups. Most of the plants in each population were placed close to each other, but some were placed intermixed with the other populations (Graph 1). Notably, many neighboring populations clustered together so tightly that they were not obviously distinguishable in the PCoA plot (e.g., populations 4, 6, 8, 12, and 14 in group A). The populations in group A is mostly with the lowest genetic diversity. The populations of the Gho (5), Gach (10), Diz (11), with the least diversity and variation in the population genetic structure are placed in this group. In group B, populations with more diverse genetic structures are get to together. The populations of Mas (1), Sar (3), Kha (4) have the lowest genetic diversity in group B and are get together in a cluster. The populations of Mes (6), Abg (8), have the highest genetic diversity among their populations and are also

get together. Neither UPGMA cluster analysis nor principal coordinate analysis revealed populations regarding the geographic differentiation between them. The authors suggested that the studied *V.a.a* populations may be characterized by adaptation to local conditions and local adaptation leads to the survival of individuals with certain genetic characteristics that ensure the best adaptation to current conditions. The existence of some level of differentiation among the studied populations might be due to different environmental effects including geographical, hydrographical connection, soil, climate, and biotic factors from different districts [44]. The relationships between the plant performance, genetic variation, population size and habitat were investigated for several species [45]. On the other hand, high genetic diversity increases population compatibility under different ecological conditions and increase to the chance of survival.

This study have clearly shown how far ISSR analysis has been successful in expressing inter species variation. This could potentially be used to improve the *V.a.a* gene pool. However, a more complete sampling in Iran would be necessary to rigorously test this hypothesis.

Conclusion

1. With citation in molecular differences, separate populations were observed in Iran.
2. There was a specific geographic pattern for the distribution of *V.a.a* populations in Iran.
3. There are genetic variations within the *V.a.a* populations in Iran.
4. Species individuals were distributed in two large genotypic groups with genetic interactions.

Suggestions

1. The phylogeography studies should be considered as the basis for demographic studies.
2. There is a need to use morphological study. So, phytochemical and stem anatomy data also should be used for morphometric studies.
3. There are possible to comprise the genetic and morphologic studies then we should discuss and conclude.
4. Genetics should be study precisely to determine the exact similarity of *V.a.a* in Iran with each of the three subspecies mentioned in Flora Iranica book. The certainty of these similarities can only be verified through genetic analysis.
5. The effect of productivity factor on the occurrence of among-/within- population diversity of the species of *V.a.a* in Iran should be studied more precisely.
6. Further sequences of this species should be included in molecular studies.
7. The findings of this study can be confirmed by studies on the chloroplast genome.
8. There is a need to use other markers such as ETS.

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