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Research Article

Genetic structure and molecular analysis of the species of the genus *Artemisia* L. (Asteraceae) distributed in Azerbaijan

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Abstract: In this study, the phylogenetic relationships and genetic structure of 10 collected genotypes of *Artemisia szowitziana* (Besser) Grossh and *Artemisia fragrans* Willd. species from different regions of Azerbaijan were investigated using RAPD primers. A total of 94 amplicons were amplified using selected RAPD primers. Among them 3 band with OPA-02 primers and 12 band with OPW-17 primers were amplified. The highest polymorphism among the investigated RAPD markers was 100, 90.91 and 90%, which has been obtained by OPW-17, OPT-19, and OPT-20 primers, respectively. However, the calculated average value of polymorphism was as high as 72.79% based on 10 different RAPD markers. The average PIC (Polymorphism Information Content) value (0.864) has indicated the rich genetic diversity among the studied samples. The cluster analyses by using Jaccard similarity index and UPGMA method classified all *Artemisia* L. samples into 6 major groups. Principal Component Analysis (PCA) justified 74.22% of the total variance. Based on the results obtained, it has been observed that there is a wide range of diversity in the molecular level between the population of *Artemisia szowitziana* (1-2 and 4-6 samples) and *Artemisia fragrans* (3, 5, 7 and 8, 9, 10 samples) and between population of the *Artemisia* species in Azerbaijan.

1. INTRODUCTION

The genus *Artemisia* L. is one of the largest genera of the Asteraceae family. *Artemisia* has 40 or 42 species in Azerbaijan (Alesgerove & Ibadullayeva, 2011). The species are mostly spread in arid areas and dry valleys of the Azerbaijan region (Hajıyev & Malıkov,1999). While *Artemisia* is a diverse genus of plants with 500 species (Aalı *et al.*, 2014), *Artemisia* species are used medicinally throughout the world. Despite the harmful effects of some of its species (invading nurseries and farmlands; toxic and allergenic for humans), the *Artemisia* genus has a wide area of usage in different fields, including pharmaceutics, landscape architecture, and agriculture (Badr, *et al.,* 2011; Barney, *et al.,* 2003; Hayat, *et al.,* 2010).

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The generic name *'Artemisia'* is derived from 'Artemis', which refers to Diana, a Greek Goddess. The name of the Goddess 'Artemis' was given to the genus because one of the species *Artemisia vulgaris* L. was much used in medicine for women's disease in folk remedy (Takeda, 1971; Yasmin *et al.,* 2016). The plant grows wild in the temperate Himalayas. It is common in the Kashmir Valley and the Shimla and Nainital hills (Yasmin & Naeasegowda, 2016). 5 decamer primers generated 195 RAPD fragments, of which 155 fragments were polymorphic with 79.48% of polymorphism. Most of the RAPD markers studied showed different levels of genetic polymorphism. The pairwise similarity coefficient values of Nei and Li ranged from 0.50 to 0.79 for 5 species of these antimalarial medicinal plants.

Artemisia species, particularly *A. iwayomogi* Kitam., *A. capillaris* Thunb., *A. princeps* Pamp. and *A. argyi* H. Lev. & Vaniot, are important medicinal materials that are utilized in traditional Asian medicines (Murray & Thompson, 1980). The genetic variability among individuals of *Artemisia capillaris* from Terengganu and Kelantan, Malaysia was examined by using random amplified polymorphic DNA. A total of 335 (Terengganu) and 370 (Kelantan) RAPD fragments which were all polymorphic fragments (100%) with sizes ranging from 150 to 3000 bp were scored for Terengganu samples, while 124 polymorphic fragments (95%) with sizes ranging from 200 to 2500 bp were scored for Kelantan samples. Genetic distance for samples ranges from 0.0000 to 0.26000 (Terengganu) and from 0.1300 to 1.5300 (Kelantan). The similarity index ranges from 0.0000 to 0.7838 (Terengganu) and 0.1167 to 0.8758 (Kelantan) (Sayed *et al.,* 2009). RAPD markers were used to study the genetic variability in a wild medicinal plant species *Artemisia judaica*, which grows in desert areas in the south of Jordan. A total of 1073 bands were obtained, 165 of which were polymorphic. Similarly, values among the studied accessions ranged from 0.61 to 0.02 (Al-Rawashdeh, 2011). The diversity between *Artemisia monosperma* Delile and *A. judaica* species was analyzed based on the assessment of morphological, karyological, and molecular variations. The analysis of morphological variation and molecular polymorphism as revealed by RAPD confirmed the differentiation of *A. monosperma* and *A. judaica* L. as two distinct species and showed wider variations among *A. judaica* populations compared to the *A. monosperma* populations. The latter population is clearly distinguished from the other populations by RAPD profiling, supporting the recognition of some populations of *A. judaica* in South Sinai as a separate variety (Badra, *et al.,* 2012). The RAPD method efficiently discriminated various *Artemisia* herbs. In particular, the non-specific primer 329 (5-GCG AAC CTC C-3), which shows polymorphism among *Artemisia* herbs, amplified 838 bp products, which are specific only to *A. princeps* and *A. argyi*. Based on the nucleotide sequence of the primer 329 product, a Fb (5-CAT CAA CCA TGG CTT ATC CT-3) and R7 (5-GCG AAC CTC CCC ATT CCA-3) primer-set was designed to amplify a 254 bp sized SCAR (sequence characterized amplified regions) marker, through which *A. princeps* and *A. argyi* can be efficiently discriminated from other *Artemisia* herbs, in particular, *A. capillaris* and *A. iwayomogi* (Lee, *et al.,* 2006). The aim is to perform a phylogenetic analysis of the 18S-26S rDNA, the internal transcribed spacer (ITS) nucleotide sequences of 19 *Artemisia* samples collected from the Ordu Province of Türkiye. This analysis revealed two unique haplotypes within our samples, including a rare one (Haplotype-I, represented by a single sample) and a common one (Haplotype-II, represented by 18 samples) (Koloren *et al.,* 2016). cDNA of subsp. *tridentata* and subsp. *vaseyana* of *A. tridentata* Nutt. species were normalized and sequenced. Assembly of the reads resulted in 20,357 contig consensus sequences in *A. tridentata* subsp. *tridentata* and 20,250 contig consensus sequences in *A. tridentata* subsp. *vaseyana*. A total of 20,952 SNPs and 119 polymorphic SSRs were detected between the two subspecies (Prabin, *et al.,* 2011).

Yenikend Reservoir was created in 2000 to produce electricity. Vegetation around the reservoir is constantly changing due to the rise and fall of the water level. From this point of view, the places near the reservoir are considered ecologically sensitive zones. Species of the

genus *Artemisia* are generally desert and semi-desert elements and have extensive intraspecific polymorphism. The adaptation characteristics of the species of the genus to different environmental conditions, especially around the water reservoir, were manifested in their development. Although the rise and fall of the water level in the reservoir destroys the surrounding vegetation, the regeneration and sustainability of the coenoses is evident with the dominance of wormwood species. To this end, phylogenetic relationships, genome polymorphism, and similarity indices of 10 samples of *A. fragrans* and *A. szowitsiana* species collected both from the reservoir area and other regions of Azerbaijan were studied comparatively.

2. MATERIAL AND METHODS

2.1. Plant Materials and DNA Isolation

In this study 10 different samples of *Artemisia szowitziana* (Besser) Grossh. (Figure 1) and *Artemisia fragrans* (Figure 2) were collected from the territories of Shamkir, Tovuz, Goranboy, Mingachevir and Lerik regions of Azerbaijan (Figure 3). Table 1 reflects the characteristic features of the collection area of the species studied, soil type, and composition of coenosis formed with the presence of plants and GPS data.

Figure 1. *Artemisia szowitziana*

Figure 2. *Artemisia fragrans*

Table 1. Information on the collection areas of the species studied.

No	Names of samples	Collector number	Areas where samples were collected and time of collection	Information about the area	The composition of phytocoenosis
1.	*A.szowitsiana*	1a	Shamkir district 10.06.2014	N 40.874210°; E 46.044440° 120 m a.s.l. dry gray-brown soils	*A.szowitsiana + A.fragrans + Euphorbia segueriana + Plantago major + Cichorium intybus*
2.	*A. szowitsiana*	2a	Shamkir district 03.10.2014	N 40.890620°; E 46.042456° 136 m a.s.l. dry gray-brown soils	*A.szowitsiana + A.fragrans + Euphorbia segueriana + Plantago major + Cichorium intybus*
3.	*A.fragrans*	1b	Shamkir district 03.10.2014	N 40.927860° ; E 46.192541° 120 m a.s.l. dry gray-brown soils	Wormwood semi-deserts (*Artemisia fragrans +Ephemeretum*)
4.	*A. szowitsiana*	3a	Shamkir district 12.04.2015	N 40.954657°; E 46.176473° 153 m a.s.l. roadside chestnut soils	*Artemisia fragrans + A. szowitsiana + Salsola dendroides*
5.	*A.fragrans*	2b	Shamkir district 09.07.2015	N 40.919154°; E 46.227273° 128 m a.s.l. water surrounding, gray-meadow soils	*Artemisia fragrans*
6.	*A.szowitsiana*	4a	Tovuz district 01.03.2016	N 40.999568°; E 45.610382° 558 m a.s.l. dry gray-brown soils	*Artemisia fragrans + A. szowitsiana + Salsola dendroides*
7.	*A.fragrans*	3b	Tovuz district 04.05.2016	N 40.975573°; E 45.618824° 478 m a.s.l. roadside chestnut soils	*Artemisia fragrans + Ephemeretum + Salsola dendroides*
8.	*A.fragrans*	4b	Mingachevir district 21.07.2016	N 40.919872°; E 46.490358° 116 m a.s.l. waterside gray-meadow, soils	*Artemisia fragrans*
9.	*A.fragrans*	5b	Goranboy rayonu 29.09.2016	N 38.777636° ; E 48.411884° 118 m a.s.l. roadside chestnut soils	*Artemisia fragrans + Ephemeretum*
10.	*A.fragrans*	6b	Lerik district 10.06.2017	N 38.777636° ; E 48.411884° 1232 m a.s.l. roadside meadow-brown, soils	*Artemisia fragrans+Alhagi pseudoalhagi*

The genomic DNA was extracted from the green leaves of plant samples using the CTAB protocol (Murray & Thompson, 1980). The extracted DNA was dissolved in sterile ionized water and then stored at -20° C. The concentration and purity of the genomic DNA for PCR were determined by spectrophotometry (Biotech, Epoch 5, USA). The DNA samples were then diluted to 20 ng/µl. To study the genetic diversity of *Artemisia szowitziana* and *Artemisia fragrans* Willd. at molecular level from different areas and various environmental conditions of Azerbaijan, 10 different RAPD primers were selected.

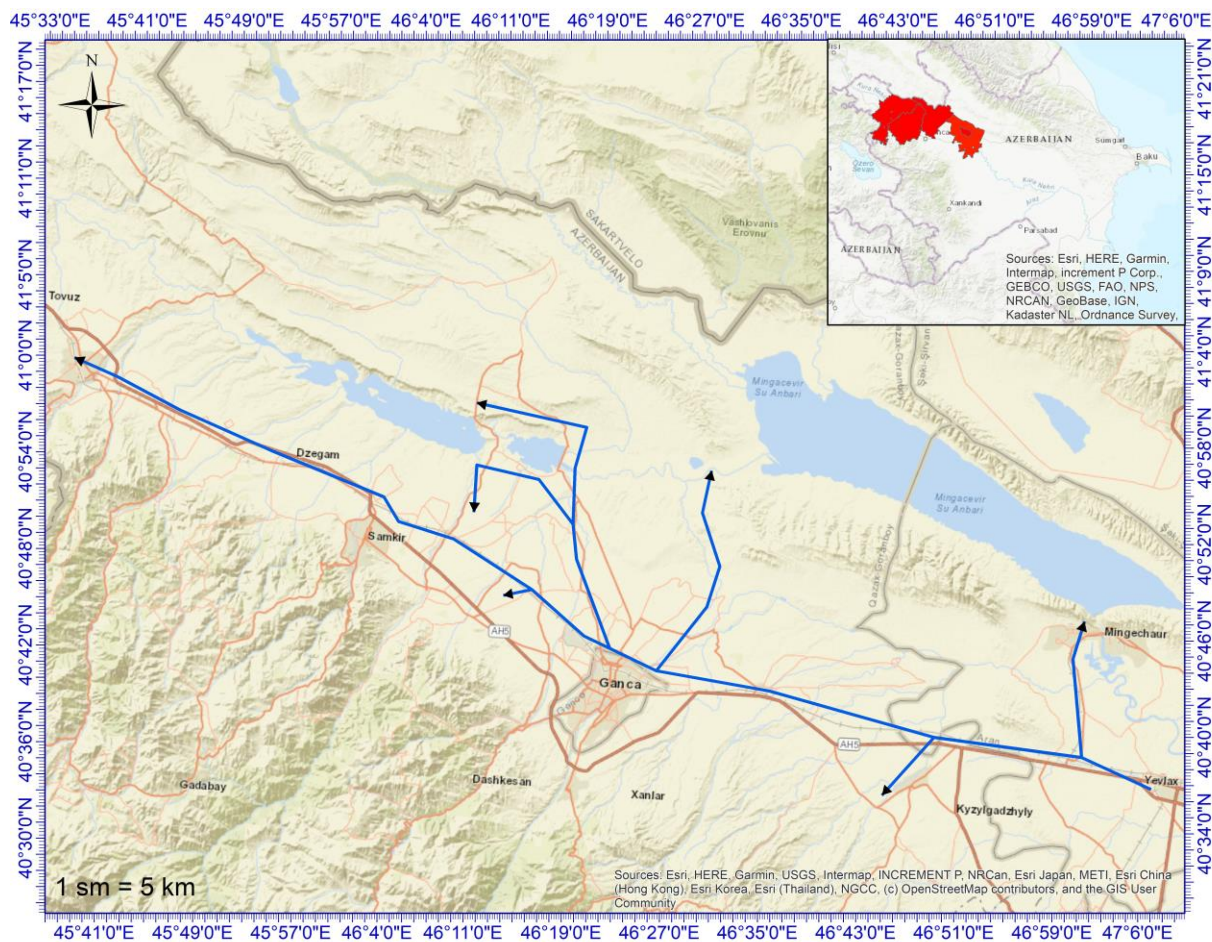

Figure 3. Dispersion map of *Artemisia* samples in different cities of Azerbaijan Republic.

2.2. PCR Amplification and Gel Electrophoresis

PCR was set up in a 25µl reaction volume containing 40 ng of genomic DNA, 1 U Taq DNA polymerase (Fermentas), 1XPCR buffer, 0.5 µM RAPD primer, and 0.2 mM of dNTP mix (Sigma). Amplification reactions were performed in a thermocycler (Applied Biosystems, 9700) using the following program: 95°C for 5 minutes followed by 30 cycles each of 94°C for 1 minute (denaturation), 36°C (varied with primer) for 1 minute (annealing), 72°C for 1 minute (extension), and finally at 72°C for 10 minutes. Amplified PCR products were separated by electrophoresis on 1% agarose gels at 100 V with 1XTAE (Tris acetate EDTA) buffer (pH-8.0). The gels were stained with ethidium bromide (0.5 mg/mL) and visualized in a gel documentation system («UVIPRO», UK). A 50 and 100-1000bp DNA ladders (Fermentas) were used as a molecular size standard. Figure 4 illustrates DNA fragments amplified with the primers studied.

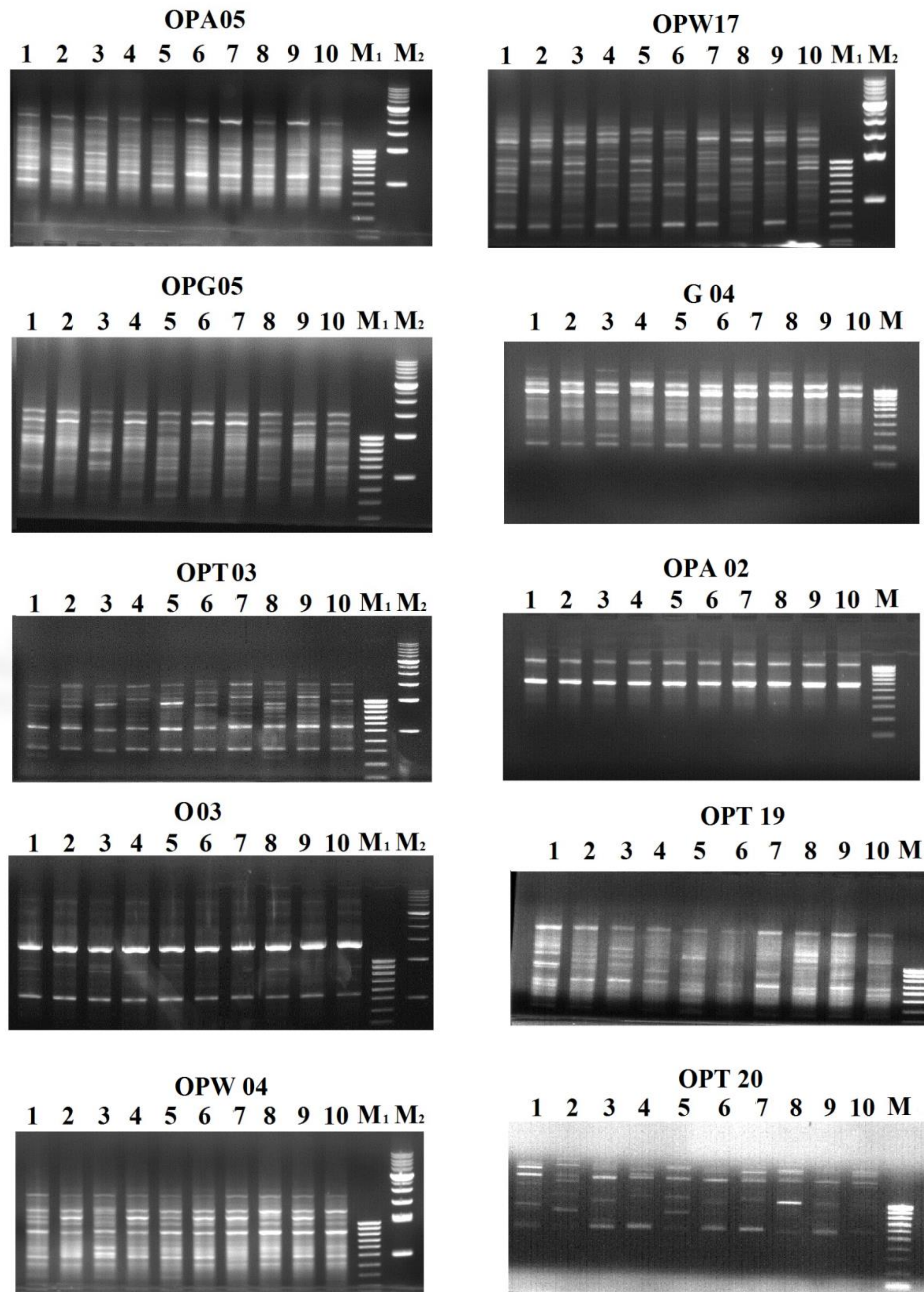

Figure 4. 1% agarose gel-analysis of DNA fragments amplified with 10 different RAPD primers on the chromosomal DNA extracted from the samples belonging to different populations of *Artemisia szowitziana* and *Artemisia fragrans* species distributed in Azerbaijan. M_1-DNA-marker (100-1000 b.p.); M_2-DNA marker (500-10000 b.p.).

1- *A. szowitziana* (dryland, Shamkir), **2-** *A. szowitziana* (dryland, Shamkir), **3-** *A. fragrans* (dryland, Shamkir), **4-** *A. szowitziana* (roadside, Shamkir), 5- *A. fragrans* (waterside, Shamkir), **6-** *A. szowitziana* (Tovuz),**7-** *A. fragrans* (Tovuz), **8-** *A. fragrans* (Mingachevir), **9-** *A. fragrans* (Goranboy), **10-** *A. fragrans* (Lerik).

2.3. Data Analysis

Banding patterns produced by ten RAPD markers were scored for the absence (0) and presence (1) of bands. Initially, by observing the banding patterns produced by different RAPD primers, a total number of bands, polymorphic bands, and percentage polymorphism were obtained. Further, the potential of these molecular markers for estimating genetic variability was assessed by measuring polymorphism information content (PIC). The PIC values were calculated using the formula PIC = 1 - ∑pi 2, where pi is the frequency of its allele.

Based on the presence (1) or absence (0) of DNA bands synthesized with each of the primers, the cluster analysis was conducted, Jaccard's genetic similarity index between the studied genotypes of *Artemisia* was determined, and the dendrogram illustrating the cluster analysis with the UPGMA (unweighted pair group with arithmetic average) method was compiled (Figure 5).

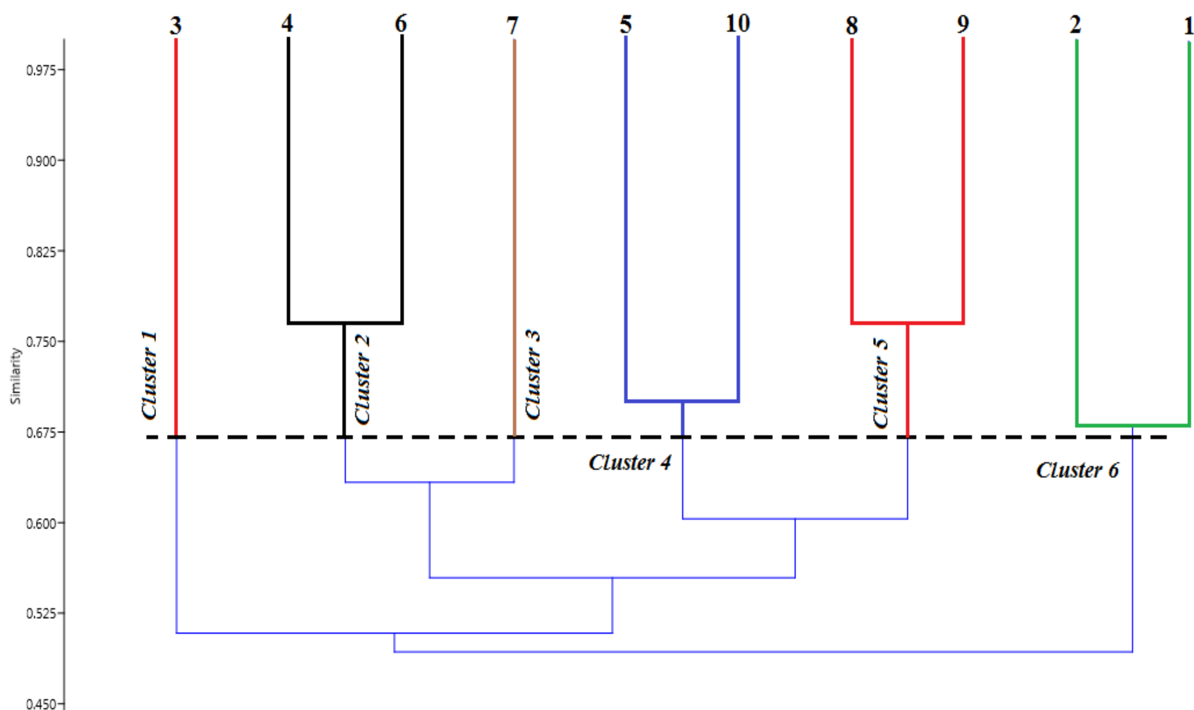

Figure 5. The dendrogram based on RAPD markers illustrating genetic relations between the samples belonging to different populations of *Artemisia szowitziana* and *Artemisia fragrans.*
1- *A. szowitziana* (dryland, Shamkir), **2-** *A. szowitziana* (dryland, Shamkir), **3-** *A. fragrans* (dryland, Shamkir), **4-** *A. szowitziana* (roadside, Shamkir), **5-** *A. fragrans* (waterside, Shamkir), **6-** *A. szowitziana* (Tovuz), **7-** *A. fragrans* (Tovuz), **8-** *A. fragrans* (Mingachevir), **9-** *A. fragrans* (Goranboy), **10-** *A. fragrans* (Lerik, Talysh).

3. RESULTS

RAPD-markers were used to study the genetic structure and phylogenetic relations of the samples belonging to *Artemisia* species characteristic of Azerbaijan at the genomic level. A total of 94 DNA spectra were amplified based on the selected RAPD-markers among the *Artemisia* samples. The number of amplicons varied from 3 to 12 which, in turn, reflects the different levels of polymorphism with molecular markers among the samples studied.

For each primer, the average number of amplified spectra was found to be 9.4. During the experiment, only 3 spectra were amplified with the OPA-02 primer, whereas 12 spectra were obtained with OPW-17. In addition, 11 spectra were amplified with the OPW-04 and OPT-20 primers.

Among the markers studied the highest level of polymorphism was registered for OPW-17, OPT-19 and OPT-20 primers (100, 90.91 and 90%, respectively) (Table 2). During the genetic diversity studies of the samples belonging to the *Artemisia annua* species based on RAPD and ISSR markers, Kumar *et al.,* (2011) found the level of polymorphism with these markers to be equal to 96.9% and 86.02%, respectively.

Table 2. List of the RAPD primers used in the present study.

№	Primers	5'→3' sequencing	№	Primers	5'→3' sequencing
1	G04	AGCGTGTCTG	6	OPT20	GACCAATGCC
2	O03	CTGTTGCTAC	7	OPW04	CAGAAGCGGA
3	OPA-02	TGCCGAGCTG	8	OPW17	CTCCTGGGTT
4	OPA-05	AGGGGTCTTG	9	OPT03	TCCACTCCTG
5	OPT19	GTCCGTATGG	10	OPG05	CTGAGACGGA

At the same time, Shafie *et al.,* (2009) reported polymorphism level to be equal to 67% with RAPD markers and to 63.16% with ISSR markers during the investigation of the phylogenetic relations of *Artemisia capillary* samples. In another study conducted by (Al-Rawashdeh, 2011), the low average polymorphism value (16.3%) was registered using RAPD markers during the genetic analysis of the samples belonging to *Artemisia judaica* species.

In our specific study, the lowest level of polymorphism was obtained with OPA-02 RAPD primer, while the average value of polymorphism calculated based on 10 different RAPD markers was found to be high and equal to 72.79%. Furthermore, in our study, high PIC (polymorphism information content) values – 0.95 and 0.9 – were observed with OPA-02 and OPT-19 markers, respectively. At the same time, PIC values were determined to be equal to 0.89 for O-03 and OPT-20 markers, and 0.88 with OPW-17, OPG-05 and OPT-03 RAPD markers. The average value of PIC equal to 0.864 obtained in the study of genetic diversity of the samples belonging to different populations of *Artemisia szowitziana* and *Artemisia fragrans* species characteristic of Azerbaijan serves as an indicator of high genetic diversity among the samples taken.

As can be observed, the cluster analysis has classified the *Artemisia* samples studied into 6 main groups. From the dendrogram it becomes evident that the first and the third clusters consist of only one sample, and in these clusters genotype 3 (cluster 1) and genotype 7 (cluster 3) are located. The location of these two samples in different clusters demonstrates that there is a substantial genetic distance between them and the other *Artemisia* samples.

During the analysis Jaccard's similarity index between genotypes 3 and 7 was determined to be equal to 0.43. The second cluster contains genotypes 4 and 6, and the genetic similarity index between these two samples is 0.76. Genotypes 5 and 10 comprise the fourth cluster, and the genetic distance index between them is 0.7. Clusters 5 and 6 contain two genotypes each as well. The fifth cluster combines genotypes 8 and 9 while the sixth cluster combines genotypes 1 and 2. In the fifth and the sixth clusters the genetic similarity index between the samples included is 0.76 and 0.68, respectively. In the study, the largest genetic distance was observed between genotypes 1 and 6. Badr et al. (2012) used cluster analysis to study the genetic diversity of Artemisia populations distributed in northern Saudi Arabia using morphological and molecular markers. They showed the classification of the samples studied into 5 clusters based on morphological traits and into 4 main groups on the basis of RAPD markers. Yasmin and Naeasegowda (2016) described the clustering of *Artemisia* samples into 4 main groups by using EST-SSR and 3 main groups using RAPD markers.

In addition to the cluster analysis, the principal components analysis is used in our study to analyze the genetic structure of *Artemisia* samples. Four components were obtained by PCA, explaining 74.22% of the total variation. The first, second, third, and fourth components accounted for 26.05, 18.78, 15.87, and 13.51%, respectively. A biplot (Figure 6) was constructed based on the two components. As can be observed, in contrast with the cluster analysis results, all the *Artemisia* samples included in the present study were divided into 4 main groups by PCA.

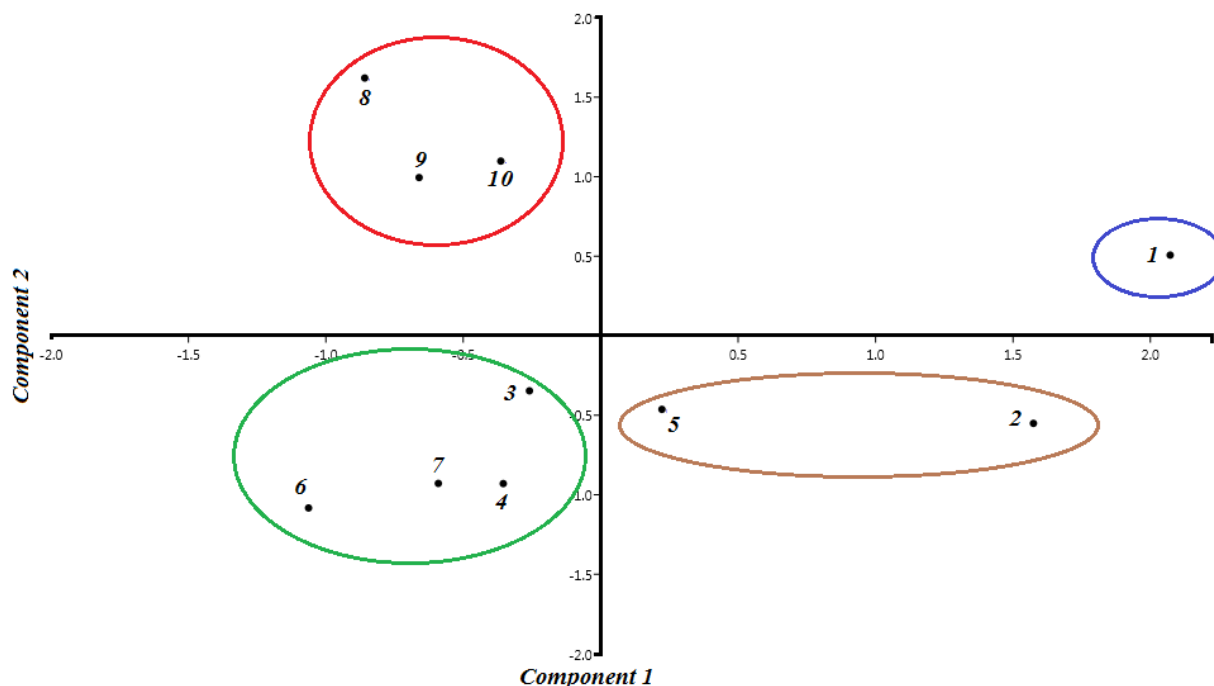

Figure 6. Biplot constructed on the basis of the two components.
1- *A. szowitziana* (dryland, Shamkir), **2-** *A. szowitziana* (dryland, Shamkir), **3-** *A. fragrans* (dryland, Shamkir), **4-** *A. szowitziana* (roadside, Shamkir), **5-** *A. fragrans* (waterside, Shamkir), **6-** *A. szowitziana* (Tovuz), **7-** *A. fragrans* (Tovuz), **8-** *A. fragrans* (Mingachevir), **9-** *A. fragrans* (Goranboy), **10-** *A. fragrans* (Lerik, Talysh).

RAPD markers are widely used genetic markers in the study of the genetic structure of different plants. The advantages of these markers are the ease of implementation, simplicity, and most importantly the absence of requirements for initial information on any part of the genome in the design of these markers. RAPD markers are universal and do not depend on the plant source. This advantage is highly important for medicinal plants, as the amount of DNA in the dried material of these plants is low, and their genome sequencing can be quite a difficult process (Shinde *et al.,* 2007). Sometimes sequence-based analysis fails to assess differences between species due to similarity in the DNA sequence of the regions amplified, but RAPD markers involve both coding and non-coding regions of the genome and are considered the most suitable ones for revealing diversity between species (Choo *et al.,* 2009).

This study shows that OPA-02, OPT-19, O-03, and OPT-20 primers, which show high levels of polymorphism compared to the other markers, can be used as suitable molecular markers for genetic diversity analysis. In addition, the cluster and principal component analysis implemented based on RAPD markers revealed high genetic diversity among samples of *Artemisia* distributed in Azerbaijan. The current study has confirmed the benefits of RAPD molecular analysis in genotype identification and assessment and differentiation of *Artemisia*

samples. Thus, the genetic data obtained in this experiment present valuable knowledge for breeders by providing new samples of medicinal plants in future breeding programs.

4. CONCLUSION

The genetic structure and polymorphism among *Artemisia* populations from different regions of the Republic of Azerbaijan were efficiently determined using molecular markers. The identification of *Artemisia* samples from Azerbaijan contributes to our knowledge of genetic relationships and the strategies required to protect natural populations and preserve genetic diversity.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Narmin Sadigova: Investigation, Resources, Original draft, Visualization. **Zarifa Suleymanova**: Investigation, Methodology, Visualization. **Javid Ojaghı:** Investigation, Methodology, Software, Visualization, Editing. **Sayyara Ibadullayeva:** Investigation, Resources, Designation of plant species. **Alamdar Mammadov:** Investigation, Methodology, Formal Analysis.

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