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Characterization of Morphological, Phytochemical and Molecular Diversity of *Artemisia annua* Accessions in Hyrcanian Area of Iran

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ABSTRACT

***Artemisia annua* L. is an important medicinal plant used as an original source of artemisinin for treating malaria. Although there is a wide distribution of *A. annua* in Hyrcanian Areas (Mazandaran, Guilan, and Golestan Provinces in N Iran), no considerable effort has been made for diversity assessment. In this study, morphological, phytochemical, and molecular characterization of *A. annua* accessions in this area were assessed using 4 quantitative characteristics (height, fresh weight, dry weight, and trichome density), artemisinin content, and 15 ISSR primers. Using these traits, a high level of morphological, phytochemical and molecular diversity was revealed among *A. annua* accessions in the provinces (populations) of Hyrcanian Areas. At inter-population level, the highest value of artemisinin was observed in Mazandaran Province. Moreover, significant correlation between artemisinin content and trichome density was observed that could be useful for indirect selection of artemisinin yield in different accessions of *A. annua*. In addition, fifteen ISSR primers generated a total number of 222 amplified bands, consisting of 177 and 45 polymorphic and monomorphic loci across the 60 accessions, respectively. These results indicate that ISSR-PCR is a reliable tool for fingerprinting *A. annua* at the intra-population level. Our results altogether are valid contributions for gene bank management and *Artemisia annua* breeding programs.**

Keywords: *A. annua*, Artemisinin, Breeding, ISSR , UPGMA.

INTRODUCTION

Plant genetic diversity is an important advantage against possible adversaries that threaten the survival of species (Jump *et al*., 2008). Assessment of plant genetic diversity is one of the innovation activities relevant to food and agricultural research (Pazouki *et al*., 2010; Govindaraj *et al*., 2015; Humphreys, 2003; Aryakia *et al*., 2017), which can be achieved using morphological, phytochemical, and molecular assessments (Fathi *et al*., 2008; Keivani *et al*., 2010; Moghaddam *et al*., 2011; Haddadi *et al*., 2012; Ranjbar *et al*., 2014; Aryakia *et al*., 2015, Nasiri *et al*., 2016). Among plant germplasm, medicinal plants could be considered as valuable sources because of their potential for drug discovery against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain using a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques

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(Balunas and Kinghorn, 2005). From this perspective, *Artemisia annua* L. is well known as an original source of artemisinin for treating malaria, having also medical properties (reviewed by Ridder *et al*., 2008; Ghafoori *et al*., 2013; Samimizad *et al*., 2016). Accordingly, many studies were performed for evaluation of diversity in *Artemisia* (Aryakia *et al*., 2012; Ghafoori *et al*., 2013; Delabays *et al*., 2001; Singh *et al*., 2015; Nazar and Mahmood, 2011; Ranjbar *et al*., 2015) reporting a high level of artemisinin content (Mannan *et al*., 2010), morphological variation (Yang *et al*., 2010) and molecular diversity (Chen *et al*., 2014). The results of these studies could be used in developing an important database for the support of several objectives, such as genebank management, botany, evolutionary and ecological studies, or breeding programs. Although wide distribution of *A. annua* was reported from Hyrcanian area of Iran (Podlech, 1986; Naghavi *et al*., 2014), no considerable effort has been made for diversity assessment. Hyrcanian Areas of Iran, with complex topography and the maritime effect of Caspian Sea, have markedly different precipitation regimes at different parts, consisting of the rich floristic diversity revealed in a total number of 3,234 species belonging to 856 genera and 148 families of vascular plants (Akhani *et al*., 2010). Therefore, in this study, for the first time, the aim was to assess diversity of 60 *A. annua* accessions belonging to Hyrcanian Areas of 3 provinces of Iran (Mazandaran, Golestan and Guilan) using morphological, phytochemical, and molecular markers.

MATERIALS AND METHODS

Plant Materials

The seed of the sixty accessions of *A. annua* were collected from the provinces of Iran (Mazandaran, Golestan and Guilan) (Table 1 and Figure 1) and cultivated under greenhouse condition (day/night photoperiod, day/night temperature and humidity were 16/8 hours, 28/22˚C and 60±5%, respectively) in Iranian Biological Resource Center (IBRC), Karaj, Iran. Morphological characteristics and artemisinin content were assessed at the flowering stage.

Morphological Characteristics

Four quantitative characteristics of *A. annua* accessions including height (m), fresh weight (g), dry weight (g) and trichome density were assessed (Table 2). Trichome density measurement was done according to the method described by Graham *et al*. (2010). Briefly, trichomes on the abaxial surface were visualized using a Zeiss fluorescent dissecting microscope (fitted with a 470/40 nm excitation filter/ 525/50 nm emission filter). Images were recorded using Axio Vision 4.7 software (Carl Zeiss Ltd. Herts., UK) (Figure 2). Finally, trichome number was counted manually across 3×0.5 mm^2 per leaflet. Significant differences between means of provinces were determined by Duncan's multiple range tests at a level of $P< 0.05$.

Determination of Artemisinin

Plant samples and standards were quantified simultaneously by HPLC with Diode Array Detector (HPLC-DAD). For each accession, powder sample (5 g) was extracted in a Soxhlet extractor with 100 mL petroleum ether and n-Hexane (2:1) for 4 hours. The extraction solvent was removed using rotary evaporator and was reconstituted in 5 mLl of ethanol. Analysis of artemisinin was performed using an Agilent 1200 Series system (Agilent Technologies, Germany) composed of a G1379A degasser, a G1311A quaternary pump and a G1316A column oven set at 30°C, containing a Zorbax Eclipse plus C8 (250×4.6 mm, 5 µm) column with a flow rate of 1 mL min^{-1}, coupled to a G1315B Photodiode-Array Detector (PAD) set to scan from 190 to 400 nm. Analysis was

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Table 1. Geographical origins of *A. annua* accessions.

Code	IBRC no	Species	Province	Latitude	Longitude	Altitude (m) asl
1	IBRC P1000347	*Artemisia annua* L.	Guilan	36° 39' 41.7"	49° 31' 58.7"	579
2	IBRC P1000356	*Artemisia annua* L.	Guilan	36° 49' 44.3"	49° 25' 45.1"	202
3	IBRC P1000358	*Artemisia annua* L.	Guilan	37° 08' 13.1"	49° 40' 11.2"	138
4	IBRC P1000359	*Artemisia annua* L.	Guilan	37° 11' 36.9"	49° 29' 28.1"	41
5	IBRC P1000363	*Artemisia annua* L.	Guilan	37° 09' 17.1"	48° 59' 37.7"	921
6	IBRC P1000365	*Artemisia annua* L.	Guilan	37° 17' 48.0"	49° 19' 47.4"	10
7	IBRC P1000367	*Artemisia annua* L.	Guilan	37° 31' 52.3"	49° 09' 54.8"	19
8	IBRC P1000370	*Artemisia annua* L.	Guilan	37° 37' 15.5"	49° 02' 42.8"	20
9	IBRC P1000371	*Artemisia annua* L.	Guilan	37° 56' 39.7"	48° 54' 32.5"	16
10	IBRC P1000375	*Artemisia annua* L.	Guilan	38° 25' 23.7"	48° 52' 44.4"	-15
11	IBRC P1000376	*Artemisia annua* L.	Guilan	38° 09' 21.9"	48° 52' 22.1"	22
12	IBRC P1000381	*Artemisia annua* L.	Guilan	37° 24' 50.7"	49° 39' 12.4"	-10
13	IBRC P1000387	*Artemisia annua* L.	Guilan	37° 04' 33.2"	49° 52' 03.6"	188
14	IBRC P1000397	*Artemisia annua* L.	Guilan	37° 08' 51.4"	50° 12' 45.5"	-2
15	IBRC P1000402	*Artemisia annua* L.	Guilan	37° 04' 21.7"	49° 59' 52.9"	235
16	IBRC P1000008	*Artemisia annua* L.	Golestan	36° 46' 16.0"	54° 00' 50.2"	-23
17	IBRC P1000009	*Artemisia annua* L.	Golestan	36° 51' 48.0"	54° 35' 19.0"	120
18	IBRC P1000010	*Artemisia annua* L.	Golestan	36° 52' 44.2"	54° 39' 45.3"	123
19	IBRC P1000011	*Artemisia annua* L.	Golestan	36° 53' 45.9"	54° 44' 22.3"	180
20	IBRC P1000012	*Artemisia annua* L.	Golestan	36° 58' 47.4"	54° 44' 22.3"	62
21	IBRC P1000013	*Artemisia annua* L.	Golestan	37° 02' 20.9"	55° 04' 40.5"	84
22	IBRC P1000014	*Artemisia annua* L.	Golestan	37° 02' 05.5"	55° 16' 59.7"	479
23	IBRC P1000015	*Artemisia annua* L.	Golestan	37° 01' 27.1"	55° 17' 13.0"	424
24	IBRC P1000018	*Artemisia annua* L.	Golestan	36° 59' 09.8"	55° 18' 11.0"	538
25	IBRC P1000020	*Artemisia annua* L.	Golestan	37° 10' 12.0"	55° 10' 02.9"	65
26	IBRC P1000021	*Artemisia annua* L.	Golestan	37° 15' 58.4"	55° 12' 23.3"	47
27	IBRC P1000022	*Artemisia annua* L.	Golestan	37° 17' 50.4"	55° 18' 44.8"	68
28	IBRC P1000024	*Artemisia annua* L.	Golestan	37° 18' 36.7"	55° 27' 47.1"	74
29	IBRC P1000026	*Artemisia annua* L.	Golestan	37° 27' 59.3"	55° 31' 14.0"	200
30	IBRC P1000027	*Artemisia annua* L.	Golestan	37° 33' 55.7"	55° 37' 49.6"	200
31	IBRC P1000030	*Artemisia annua* L.	Golestan	37° 37' 34.9"	55° 42' 55.4"	283
32	IBRC P1000035	*Artemisia annua* L.	Golestan	37° 37' 44.8"	55° 51' 34.1"	502
33	IBRC P1000038	*Artemisia annua* L.	Golestan	37° 38' 40.1"	55° 42' 22.6"	295
34	IBRC P1000039	*Artemisia annua* L.	Golestan	37° 44' 22.7"	55° 53' 56.9"	650
35	IBRC P1000473	*Artemisia annua* L.	Mazandaran	36° 35' 04.7"	51° 46' 18.9"	-1
36	IBRC P1000474	*Artemisia annua* L.	Mazandaran	36° 34' 14.0"	51° 52' 56.1"	-6
37	IBRC P1000486	*Artemisia annua* L.	Mazandaran	36° 27' 42.3"	52° 15' 42.9"	71
38	IBRC P1000492	*Artemisia annua* L.	Mazandaran	36° 35' 47.5"	52° 40' 49.1"	1
39	IBRC P1000498	*Artemisia annua* L.	Mazandaran	36° 41' 37.0"	52° 44' 27.7"	-12
40	IBRC P1000499	*Artemisia annua* L.	Mazandaran	36° 34' 03.3"	52° 48' 38.8"	4
41	IBRC P1000501	*Artemisia annua* L.	Mazandaran	36° 30' 58.5"	52° 57' 47.0"	23
42	IBRC P1000502	*Artemisia annua* L.	Mazandaran	36° 29' 59.9"	53° 04' 48.2"	111
43	IBRC P1000503	*Artemisia annua* L.	Mazandaran	36° 23' 39.8"	53° 09' 37.1"	212
44	IBRC P1000512	*Artemisia annua* L.	Mazandaran	36° 19' 43.6"	53° 10' 40.0"	287
45	IBRC P1000518	*Artemisia annua* L.	Mazandaran	36° 10' 23.9"	53° 16' 03.7"	711
46	IBRC P1000522	*Artemisia annua* L.	Mazandaran	36° 40' 04.7"	53° 04' 31.4"	1
47	IBRC P1000523	*Artemisia annua* L.	Mazandaran	36° 46' 14.2"	53° 07' 25.9"	1
48	IBRC P1000527	*Artemisia annua* L.	Mazandaran	36° 50' 05.2"	53° 16' 15.9"	-14
49	IBRC P1000537	*Artemisia annua* L.	Mazandaran	36° 42' 25.7"	53° 38' 55.4"	33
50	IBRC P1000540	*Artemisia annua* L.	Mazandaran	36° 44' 53.1"	53° 54' 19.8"	3
51	IBRC P1000541	*Artemisia annua* L.	Mazandaran	36° 42' 03.1"	53° 48' 05.7"	315
52	IBRC P1000544	*Artemisia annua* L.	Mazandaran	36° 39' 31.7"	53° 48' 29.5"	1008
53	IBRC P1000547	*Artemisia annua* L.	Mazandaran	36° 16' 32.5"	52° 53' 18.9"	290
54	IBRC P1000548	*Artemisia annua* L.	Mazandaran	36° 11' 55.5"	52° 56' 09.1"	395
55	IBRC P1000551	*Artemisia annua* L.	Mazandaran	36° 07' 40.0"	53° 02' 57.1"	560
56	IBRC P1000572	*Artemisia annua* L.	Mazandaran	36° 04' 10.6"	52° 50' 13.8"	1785
57	IBRC P1000573	*Artemisia annua* L.	Mazandaran	36° 05' 07.9"	52° 54' 18.8"	936
58	IBRC P1000574	*Artemisia annua* L.	Mazandaran	36° 29' 54.7"	52° 46' 09.0"	16
59	IBRC P1000586	*Artemisia annua* L.	Mazandaran	36° 12' 00.4"	52° 00' 30.8"	1760
60	IBRC P1000578	*Artemisia annua* L.	Mazandaran	36° 22' 28.1"	52° 21' 07.8"	229

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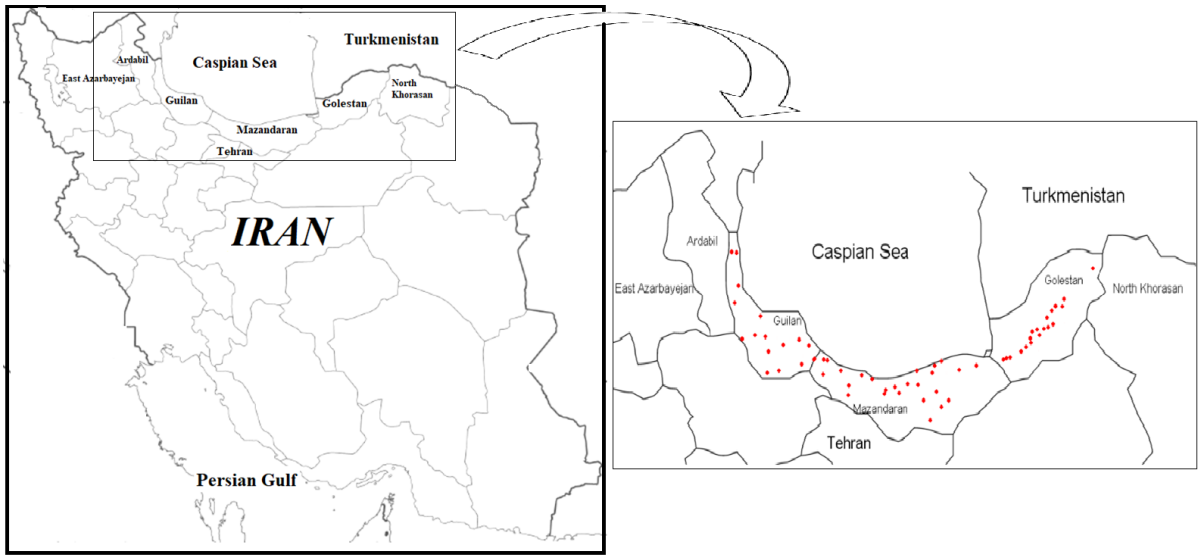

Figure 1. Collection sites of *A. annua* in three provinces.

performed using the following gradient elution: Solvent A was 0.9 mM Na_2HPO_4, 3.6 mM NaH_2PO_4 buffer (pH 7.76), and Solvent B was acetonitrile. The gradient program was initiated with 70% A, 30% B for 5 minutes, and gradually increased to 60% B and 40% A in the next 13 minutes. Each run was followed by a 10 minutes wash with 85% acetonitrile. Before injection of the next sample, the column was equilibrated with mobile phase A for 10 minutes. Before injection of each sample (20 µL), filtration was done through a 0.45 µL filter. The column temperature was set at 30°C with detection wavelength at 258 nm. Standard artemisinin with purity of 98% was purchased from Sigma Chemical Company and standard solutions of artemisinin were prepared by dissolving 1 mg of artemisinin in 10 mL ethanol.

Molecular Characteristics

Fresh leaves were collected and powdered in a pre-chilled mortar using liquid nitrogen, and the DNA was then extracted using herbal genomic DNA extraction kit (IBRC MBK0011, Iran). Fifteen ISSR primers (Table 3) that produced clear and reproducible banding patterns were used. DNA amplifications were performed in 20 µL reaction final volume containing 0.5 µL of template DNA (approximately contain 20 ng template DNA), 0.5 µL of primer (10 pmol), 9 µL PCR-grade water and 10 µL of 2X *Taq* Master Mix Red (0.2 units μl^{-1}) (Ampliqon, Denmark). The PCR amplification program was performed in a T100™ thermal cycler (Bio-Rad Company) as: 4 minutes at 94°C, followed by 35 cycles of 45 seconds at 94°C, 1 minute with varied temperatures (Table 3) as per the melting temperature of the ISSR primers used, 1 minute 30 seconds at 72°C and a final 10 minutes extension at 72°C. Amplification products were separated by electrophoresis in a 1.5% (w/v) horizontal agarose gel using 1X TAE buffer at 110V for 3 hours. After soaking in ethidium bromide solution for 15 min, ISSR banding patterns on gels were observed using gel doc system (G: BOX, Syngene, UK). The ISSR markers were scored as present (1) or absent (0) depending on fragment amplification, and this data generated the binary data matrix. GenAlEX6 was used to analyze variations at inter and intra-population level based on Analyses of Molecular Variation (AMOVA). A dendrogram was constructed using the

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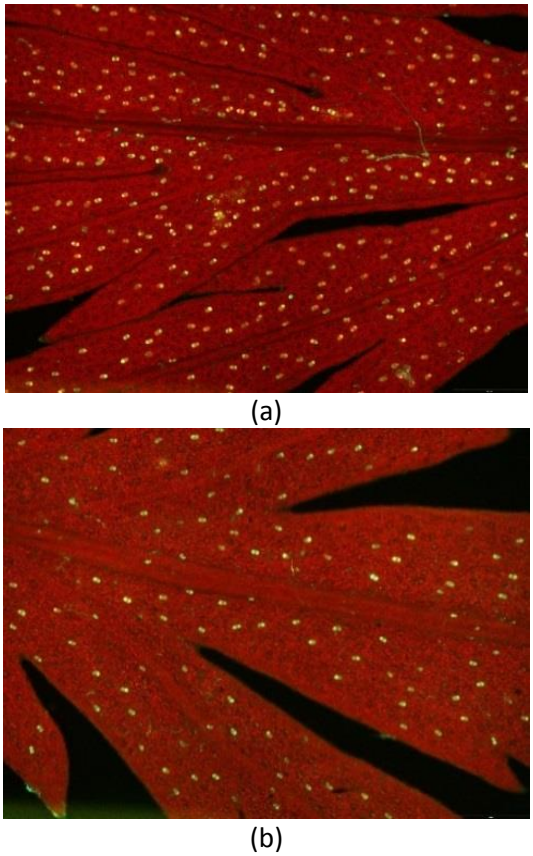

(a)

(b)

Figure 2. *A. annua* leaves showing trichomes: (a) High and (b) Low densities.

Unweighted Pair Group Method with Arithmetic average (UPGMA) algorithm to illustrate the genetic relationships among 60 *A. annua* ecotypes (Darwin ver. 6; Available through: http://darwin.cirad.fr/darwin).

RESULTS AND DISCUSSION

Based on geographical distribution, high level of morphological and phytochemical diversity was observed among *A. annua* accessions (Table 2), although artemisinin content was the only character that showed significant differences among provinces. However, maximum mean value of artemisinin (12.82±7.77), dry weight (276.08±168.46 g), and trichome number (17.59±4.51) were observed in Mazandaran Province, while the maximum fresh weight (535.67±397.59 g) and plant height (2.65±0.34 m) were observed in Guilan

Table 2. Means and standard deviations obtained for the artemisinin content and the four morphological characters assessed, in different study sites. [a]

Province	Artemisinin content ($\mu g\ g^{-1}$ DW)	Height ($m\ plant^{-1}$)	Fresh weight ($g\ plant^{-1}$)	Dry weight ($g\ plant^{-1}$)	Trichome number
Guilan	6.57 ± 4.49 (c)	**2.65 ± 0.34(a)**	**535.67 ± 397.59(a)**	247.07 ± 168.85(a)	15.36 ± 4.37(a)
Golestan	10.82 ± 7.37(ab)	2.4 ± 0.4(a)	439.89 ± 282.28(a)	241.32 ± 148.98(a)	16.5 ± 3.11(a)
Mazandaran	**12.82 ± 7.77 (a)**	2.57 ± 0.43(a)	526.46 ± 327.76(a)	**276.08 ± 168.46(a)**	**17.59 ± 4.51(a)**
Mean	10.62 ± 7.29	2.53 ± 0.4	501.35 ± 330.35	257.82 ± 160.7	16.68 ± 4.11

[a] The highest content for each character was bolded.

Table 3. List of primers, their sequence, annealing temperature, total number of bands, number of polymorphic and monomorphic loci and polymorphism percent generated by ISSR primers in 60 *A. annua* accessions.

Sequence (5′-3′)	Annealing T. (°C)	Total number of bands amplified	Number of polymorphic loci	Number of monomorphic loci	Polymorphic percentage
(GA)8T	56.6	10	9	1	90
(GA)8C	48.0	15	15	0	100
(GA)8YC	57.0	21	20	1	95.23
(GA)8YG	49.0	13	12	1	92.30
(AG)8YC	48.0	19	19	0	100
SSWN(GACA)3	41.1	16	16	0	100
HVH(TCC)5	57.0	16	9	7	65.25
BDB(TCC)5	57.0	12	6	6	50
GGGT(GGGGT)2G	57.0	19	15	4	78.94
(ACTG)4	45.0	9	4	5	44.44
(CT)8RG	48	12	12	0	100
HVH(CT)7T	50.4	16	5	11	31.25
GCW(GA)6G	57.0	14	8	6	57.14
(GA)8C, (AG)8YT	48.0	11	11	0	100
(GA)8C, (AG)8YC	48.0	19	16	3	84.21
Total		222	177	45	
Mean		14.8			

Province. In addition, the 4 morphological quantitative characteristics could not discriminate the taxonomic arrangements at inter-population level (data not shown). Previous studies showed the importance of geographical factors on morphological and phytochemical diversity (Baghalian *et al*., 2005; Ghafoori *et al*., 2013; Aryakia *et al*., 2016) which could be considered in breeding programs and evolutionary and ecological studies (Jessing *et al*., 2014; Ranjbar *et al*., 2015). These results indicate Mazandaran Province as a potent area for exploration and exploitation of the resources of *A. annua*, artemisinin and related compounds with industrial and medicinal applications.

Pearson's correlation analysis could also find significant relationships among characters. In general, morphological characters including height, fresh and dry weight were significantly correlated (Table 4). These correlations have also been reported in other species (Parker *et al*., 2010; Aryakia *et al*., 2016) which might be due to evolutionary consequences underlying within phenotypic characters (Davis, 2001; Mclellan, 2005). For example, phenotypic characters such as plant size could influence plant fecundity and flowering time (Ollerton and Lack, 1998).

Overall, significant correlation between artemisinin content and trichome density was observed (Table 2 and Figure 2), which was previously reported by Singh *et al*. (2015) as an important strategy of further increase of artemisinin yield in *A. annua*. Totally, these results might be considered in evolutionary and ecological studies (Valverde *et al*., 2001; Agren and Schemske, 1994), breeding programs (Delabays *et al*., 2001) and phylogenic studies (Spring, 2000).

Among the 20 ISSR primers tested herein, only 15 were chosen due to the success of amplification of polymorphic patterns (Table 3 and Figure 3) among 60 *A. annua* accessions. The total number of polymorphic bands was 177 and varied between 4 for the primer (ACTG)4 to 20 for the primer (GA)8YC. The average number of bands per primer was 14.8. Percentages of polymorphism per ISSR primer varied from 31.25% for HVH(CT)7T to 100% obtained for (GA)8C, which was similar to those previously reported in different *Artemisia* species (Huang *et al*., 2011; Nazar and Mahmood, 2011) and other genus (Tesfaye

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Table 4. Correlation between characters of *A. annua.*

	Artemisinin	Height	Fresh weight	Dry weight
Height	0.102			
Fresh weight	0.023	0.545**		
Dry weight	0.062	0.518**	0.978**	
Trichome no	0.269*	0.181	-0.037	0.034

*, **: Significant at 5 and 1%, respectively.

et al., 2014; Kumar *et al*., 2010). This indicates ISSR-PCR as a reliable tool for fingerprinting at the intra-population level.

Analyses of Molecular Variance (AMOVA) showed that intra-population variability was higher than inter-population variability (Table 5), which was similar to previously published reports in *Chondrus crispus* (Wang *et al*., 2008) and *Pleurochaete squarrosa* (Spagnuolo *et al*., 2007). Probably, the high intra-population diversity made them less amenable to cluster analysis (Figure 4). It might be due to the predominant sexual reproduction pattern of *A. annua* growing in Hyrcanian Areas (Mazandaran, Guilan and Golestan).

ISSR markers have been widely used in assessment of genetic relationships at intra- and inter-population level (Thul *et al*., 2012; Liu *et al*., 2013). However, there are few reports for application of ISSR markers for *Artemisia* that are mainly focused on genetic diversity assessment at intra-population level of other *Artemisia* species (Huang *et al*., 2011; Nazar and Mahmood, 2011). Our results show that the ISSR markers have robust reproducibility, and can provide a suitable alternative approach for evaluating inter-population genetic diversity.

CONCLUSIONS

Based on morphological, phytochemical, and molecular assessment of 60 *A. annua* from Iran, high level of diversity was revealed. Results showed that 4 morphological characters were not

Table 5. Hierarchical Analyses of Molecular Variance (AMOVA) for ISSR variation surveyed in 60 *A. annua* accessions.

Source	*df* [a]	*SS* [b]	*MS* [c]	Percentage of variance (%)
Inter-population	2	233.72	11.36	1
Intra-population	57	82.2041	17.29	99
Total	59	315.9241	28.65	100

[a] Degree of freedom; [b] Sum of Squares, [c] Mean of Squares.

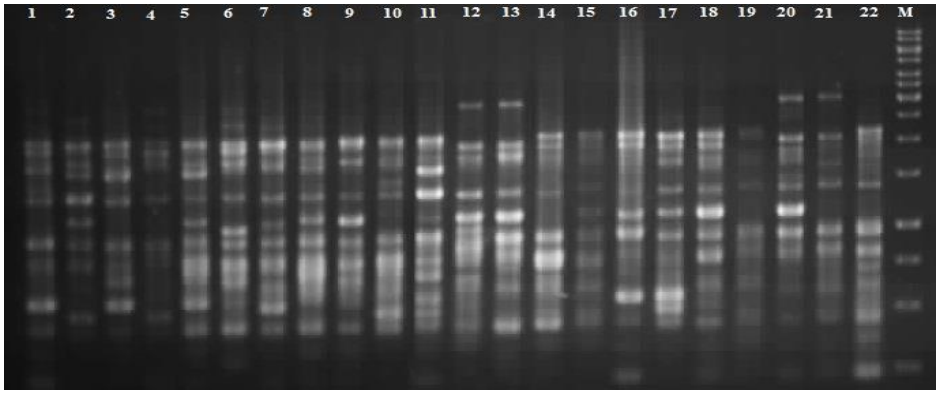

Figure 3. PCR amplification illustrates variability of ISSRs using primer (GA)8C. Golestan (1-3, 11-13), Mazandaran (5, 6, 9, 18-20, 22), and Guilan accessions (4, 7, 8, 10, 14-17, 21).

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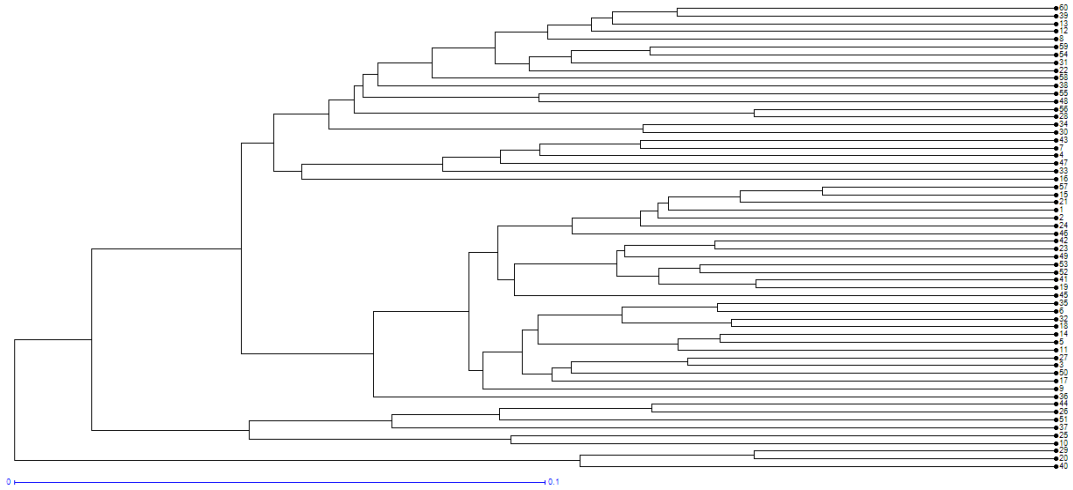

Figure 4. Dendrogram of the genetic dissimilarities among 60 *A. annua* accessions, achieved by the UPGMA method, based on 15 ISSR markers.

discriminative, but assessment of other morphological characteristics might elevate discrimination accuracy at the inter-population level (Aryakia *et al*., 2016; Naghavi and Jahansouz, 2005). Highest artemisinin content was detected in Mazandaran population and significant relationship was observed between artemisinin and trichome density, which might be considered in evolutionary and agricultural studies. Since ISSR-PCR showed as a reliable tool for fingerprinting at the population level, evaluating genetic variation/relationships of other *Artemisia* species using different ISSR primers at the level of individuals, accessions, and other taxonomic levels were recommended. Wide range of diversity observed at the intra-population level might be considered for several subjects, such as genebank management, breeding programs, and medicinal usage.

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REFERENCES

1. Agren, J. and Schemske, D. W. 1994. Evolution of Trichome Number in a Naturalized Population of *Brassica rapa*. *Am. Nat*., **143**: 1–13.
2. Akhani, H., Djamali, M., Ghorbanalizadeh, A. and Ramezani, E. 2010. Plant Biodiversity of Hyrcanian Relict Forests, N Iran: An Overview of the Flora, Vegetation, Palaeoecology and Conservation. *Pak. J. Bot.*, **42:** 231-258.
3. Aryakia, E., Karimi, H., Naghavi, M. R. and Shahzadeh Fazeli, S. A. H. 2016. Morphological Characterization of Intra- and Inter-Specific Diversity in Some Iranian Wild *Allium* Species. *Euphytica*, **211**:185-200.
4. Aryakia, E., Karimi, H., Naghavi, M. R., Yazdanfar, N. and Shahzadeh Fazeli, S. A. H. 2017. Evaluating Essential Oil Constituent of Four *Allium* Species of the Subgenus and Section *Allium*. Iran. J. Field Crop Sci., In press.
5. Aryakia, E., Naghavi, M. R., Farahmand, Z. and Shahzadeh Fazeli, S. A. H. 2015. Evaluating Allelopathic Effects of Some Plant Species in Tissue Culture Media as an Accurate Method for Selection of Tolerant Plant and Screening of Bioherbicides. *J. Agr. Sci. Tech*., **17:** 1011–1023.

[DOR: 20.1001.1.16807073.2019.21.5.13.0]

6. Aryakia, E., Ramazani, H., Ghafoori, H., Dolatyari, A., Naghavi, M. R. and Shahzadeh Fazeli, S. A. H. 2012. The Effect of Cryopreservation on Germination and Growth Indices of Some Orthodox Seeds. *Iran J. Rangel. Plant Breed. Genet. Res.* **19:** 218–230.
7. Baghalian, K., Ziai, S. A., Naghavi, M. R., Badi, H. N. and Khalighi, A. 2005. Evaluation of Allicin Content and Botanical Traits in Iranian Garlic (*Allium sativum* L.) Ecotypes. *Sci. Hortic*., **103**:155–166.
8. Balunas, M. J. and Kinghorn, A. D. 2005. Drug Discovery from Medicinal Plants. *Life Sci.*, **78:** 431 – 441.
9. Chen, D. X., Cui, G. L., Zhang, X. and Li, L. Y. 2014. Genetic Structure and Genetic Diversity of *Artemisia annua* Varieties (Strains) Populations Based on SCoT Markers. *Zhongguo Zhong Yao Za Zhi.*, **39:** 3254-3258.
10. Davis, S. L. 2001. Phenotypic and Genetic Correlations among Floral Traits in Two Species of *Thalictrum*. *J. Hered.*, **94:** 361–366.
11. Delabays, N., Simonnet, X. and Gaudin, M. 2001. The Genetics of Artemisinin Content in *Artemisia annua* L. and the Breeding of High Yielding Cultivars. *Curr. Med. Chem*., **8:** 1795-1801.
12. Fathi, A., Ghareyazi, B., Haghnazari, A., Ghaffari, M. R., Pirseyedi, S. M., Kadkhodaei, S., Naghavi, M. R. and Mardi, M. 2008. Assessment of the Genetic Diversity of Almond (*Prunus dulcis*) Using Microsatellite Markers and Morphological Traits. *Iran. J. Biotechnol.*, **6**: 98-106.
13. Ghafoori, H., Sariri, R., Naghavi, M. R., Aryakia, E., Dolatyari, A., Shahzadeh Fazeli, S. A., Ramazani, H. and Farahmand, Z. 2013. Analysis of Artemisinin Isolated from *Artemisia annua* L. by tlc and hplc. *J. Liq. Chromatogr. Relat. Technol.*, **36:** 1198–1206.
14. Govindaraj, M., Vetriventhan, M. and Srinivasan, M. 2015. Importance of Genetic Diversity Assessment in Crop Plants and Its Recent Advances: An Overview of Its Analytical Perspectives. *Genet. Res. Int.*, **1:** 1-15.
15. Graham, I., Besser, K., Blumer, S., Branigan, C .A., Czechowski, T., Elias, L., Guterman, I., Harvey, D., Isaac, P .G., Khan, A .M., Larson, T. R., Li, Y., Pawson, T., Penfield, T., Rae, A .M., Rathbone, D. A., Reid, S., Ross, J., Smallwood, M .F., Segura, V., Townsend, T., Vyas, D., Winzer, T. and Bowles, D. 2010. The Genetic Map of *Artemisia annua* L. Identifies Loci Affecting Yield of the Antimalarial Drug Artemisinin. *Sci.*, **327:** 328-331.
16. Haddadi, P., Ebrahimi, A., Langlade, N. B., Yazdi-samadi, B., Berger, M., Calmon, A., Naghavi, M. R., Vincourt, P. and Sarrafi, A. 2012. Genetic Dissection of Tocopherol and Phytosterol in Recombinant Inbred Lines of Sunflower through Quantitative Trait Locus Analysis and the Candidate Gene Approach. *Mol. Breed*., **29**: 717-729.
17. Huang, W. D., Zhao, X. Y., Zhao, X., Zhao, H. L., Wang, S. K. and Lian, J. 2011. A Combined Approach Using ISSR and ITS Analysis for the Characterization of *Artemisia halodendron* from Horqin Sandy Land, Northern China. *Biochem. Sys. Ecol.*, **39:** 346–351.
18. Humphreys, M. O. 2003. Utilization of Plant Genetic Resources in Breeding for Sustainability. *Plant Genet. Res. Character. Util*., **1:** 11–18.
19. Jessing, K. K., O. Duke, S. and Cedergreeen, N. 2014. Potential Ecological Roles of Artemisinin Produced by *Artemisia annua* L. *J. Chem. Ecol*., **40:** 100–117.
20. Jump, A. S., Marchant, R. and Penuelas, J. 2008. Environmental Change and the Option Value of Genetic Diversity. *Trends Plant Sci*., **14:** 51–58.
21. Keivani, M., Ramezanpour, S. S., Soltanloo, H., Choukan, R., Naghavi, M. R. and Ranjbar, M. 2010. Genetic Diversity Assessment of Alfalfa (*Medicago sativa* L.) Populations Using AFLP Markers. *Aust. J. Crop Sci.*, **4**: 491-497.
22. Kumar, L. S., Jena, S. N. and Nair, K. N. 2010. ISSR Polymorphism in Indian Wild Orange (*Citrus indica* Tanaka, Rutaceae) and Related Wild Species in North-East India. *Sci. Hort*., **123:** 350–359.
23. Liu, Y., Zhang, J. M., Wang, X. G., Liu, F. and Shen, Z. B. 2013. Genetic Diversity in *Vicia amoena* (Fabaceae) Germplasm Resource in China Using SRAP and ISSR Markers. *Biochem. Syst. Ecol*., **51:** 86−93.
24. Mannan, A., Ahmed, I., Arshad, W., Asim, M. F., Qureshi, R. A., Hussain, I. Mirza. B. 2010. Survey of Artemisinin Production by Diverse *Artemisia* Species in Northern Pakistan. *Malaria J*., **9**: 310.

[DOR: 20.1001.1.16807073.2019.21.5.13.0]

25. Mclellan, T. 2005. Correlated Evolution of Leaf Shape and Trichomes in *Begonia dregei* (Begoniaceae). *Am. J. Bot.*, **92:** 1616-1623.
26. Moghaddam, M., Omidbiagi, R. and Naghavi, M. R. 2011. Evaluation of Genetic Diversity among Iranian Accessions of *Ocimum* spp. Using AFLP Markers. *Biochem. Syst. Ecol.*, **39**: 619-626.
27. Naghavi, M. R., Alaeimoghadam, F. and Ghafoori, H. 2014. *Artemisia* Species from Iran as Valuable Resources for Medicinal Uses. *World Acad. Sci. Eng. Technol*., **11:** 1058-1064.
28. Naghavi, M. R. and Jahansouz, M. R. 2005. Variation in the Agronomic and Morphological Traits of Iranian Chickpea Accessions. *J. Integ. Plant Biol*., **47**: 375-379.
29. Nasiri, J., Naghavi, M. R., Alizadeh, H., Moghadam, M. R. F. 2016. Seasonal-based Temporal Changes Fluctuate Expression Patterns of TXS, DBAT, BAPT and DBTNBT Genes alongside Production of Associated Taxanes in *Taxus baccata*. *Plant Cell Rep.*, **35**: 1103-1119.
30. Nazar, N. and Mahmood, T. 2011. Morphological and Molecular Characterization of Selected *Artemisia* Species from Rawalakot, Azad Jammu and Kashmir. *Acta Physiol. Plant.*, **33:** 625-633.
31. Ollerton, J and Lack, A. 1998. Relationships between Flowering Phenology, Plant Size and Reproductive Success in *Lotus corniculatus* (Fabaceae). *Plant Ecol.*, **139:** 35-47.
32. Parker, I. M., López, I., Petersen, J. J., Anaya, N., Cubilla-Rios, L. and Potter, D. 2010. Domestication Syndrome in Caimito (*Chrysophyllum cainito* L.): Fruit and Seed Characteristics. *Econ. Bot*., **64:** 161-175.
33. Pazouki, L., Mardi, M., Shanjani, P. S., Hagidimitriou, M., Pirseyedi, S. M., Naghavi, M. R., Avanzato, D., Vendramin, E., Kafkas, S., Ghareyazie, B., Ghaffari, M. R. and Nekoui, S. M. K. 2010. Genetic Diversity and Relationships among *Pistacia* Species and Cultivars. *Conserv. Genet.*, **11**: 311-318.
34. Podlech, D. 1986. *Artemisia*. In: "*Flora Iranica*", (Ed.): Rechinger, K. H. Graz, Academische Druck Verlagsansalt, PP. 159-223.
35. Ranjbar, M., Naghavi, M. R. and Alizadeh, H. 2014. Comparative Analysis of ADS Gene Promoter in Seven *Artemisia* Species. *J. Genet*., **93:** 767-774.
36. Ranjbar, M., Naghavi, M. R., Alizadeh, H. and Soltanloo, H. 2015. Expression of Artemisinin Biosynthesis Genes in Eight *Artemisia* Species at Three Developmental Stages. *Ind. Crops Prod*., **76:** 836-843.
37. Ridder, S., Kooy, F. and Verpoorte, R. 2008. *Artemisia annua* as a Self-Reliant Treatment for Malaria in Developing Countries. *J. Ethnopharmacol*., **120:** 302-314.
38. Singh. N. D., Kumar, S. and Daniell, H. 2015. Expression of b-Glucosidase Increases Trichome Density and Artemisinin Content in Transgenic *Artemisia annua* Plants. *Plant Biotechnol. J*., **14:** 1-12.
39. Spagnuolo, V., Muscariello, L., Cozzolino, S., Castaldo Cobianchi, R. and Giordano, S. 2007. Ubiquitous Genetic Diversity in ISSR Markers between and within Populations of the Asexually Producing Moss *Pleurochaete squarrosa*. *Plant Ecol*., **188:** 91-101.
40. Spring, O. 2000. Chemotaxonomy Based on Metabolites from Glandular Trichomes. *Adv. Bot. Res*., **31:**153-174.
41. Tesfaye, K., Govers, K., Bekele, E. and Borsch, T. 2014. ISSR Fingerprinting of *Coffea arabica* throughout Ethiopia Reveals High Variability in Wild Populations and Distinguishes Them from Landraces. *Plant Syst. Evol*., **300:** 881-897.
42. Thul, S. T., Darokar, M. P., Shasany, A. K. and Khanuja, S. P. 2012. Molecular Profiling for Genetic Variability in *Capsicum* Species Based on ISSR and RAPD Markers. *Mol. Biotechnol*., **51**: 137-147.
43. Valverde, P. L., Fornoni, J. and Nunez-Farfan, J. 2001. Defensive Role of Leaf Trichomes in Resistance to Herbivorous Insects in *Datura stramonium*. *J. Evol. Biol*., **14:** 424-432.
44. Wang, X., Zhao, F., Hu, Z., T. Critchley, A., L. Morrell, S. and Duan, D. 2008. Inter-Simple Sequence Repeat (ISSR) Analysis of Genetic Variation of *Chondrus crispus* Populations from North Atlantic. *Aquat. Bot*., **88**: 154-159.
45. Yang, M., Liu, D., Shao, A., Jin, H., Wang, X., Fan, Z. and Huang, L. 2010. Diversity and Cluster Analysis on Agronomic Traits of *Artemisia annua* Germplasm Resources in Yun-Gui Plateau. *Zhongguo Zhong Yao Za Zhi.*, **35:** 3097-3102.

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توصیف تنوع مورفولوژیکی، فیتوشیمیایی و مولکولی اکسشن‌های گندواش (*Artemisia annua* L.) در مناطق هیرکانی ایران

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چکیده

گندواش (*Artemisia annua* L.) یک گیاه دارویی مهم و به عنوان منبع اصلی متابولیت آرتمیزینین برای درمان مالاریا محسوب می‌شود. علارغم پراکنش گسترده گندواش در ایران و به ویژه در مناطق هیرکانی (شامل سه استان مازندران، گیلان و گلستان) که رویشگاه اصلی این گیاه می‌باشد، تلاش قابل توجهی برای ارزیابی تنوع آن صورت نپذیرفته است. در این پژوهش برای اولین بار توصیف مورفولوژیکی، فیتوشیمیایی و مولکولی جمعیت‌های گندواش مناطق هیرکانی به ترتیب با استفاده از ۴ صفت کمی، محتوای آرتمیزینین و ۱۵ پرایمر ISSR انجام شد. سطح بالایی از تنوع مورفولوژیکی، بیوشیمیایی و مولکولی در میان جمعیت‌های گندواش مشاهده شد. در سطح بین جمعیتی، بیشترین میزان آرتمیزینین در استان مازندران مشاهده شد. علاوه بر این ارتباط معنی‌داری بین محتوای آرتمیزینین و تراکم تریکوم مشاهده شد که می‌تواند به عنوان یک روش انتخاب غیر مستقیم عملکرد آرتمیزین در جمعیت‌های متنوع گندواش استفاده شود. پانزده مارکر ISSR نیز تعداد ۲۲۲ باند دربرگیرنده ۱۷۷ لوکوس چندشکلی و ۴۵ لوکوس یک شکل را در ۶۰ اکوتیپ گندواش تولید کردند. این نتایج نشان داد که تکنیک ISSR-PCR ابزار قابل اطمینانی برای انگشت نگاری گیاه گندواش در سطح درون جمعیتی می‌باشد. در مجموع نتایج ما می‌تواند برای مدیریت بانک ژن و برنامه‌های اصلاحی گیاه گندواش مورد استفاده قرار گیرد.

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