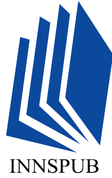

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Genetic diversity and inter-relationship of henbane populations of North-West of Iran based on IRAP and REMAP markers

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Abstract

Henbane is one of the indigenous plants of Solanaceae from Iran that considered as valuable medicinal and economic plant. In current study, genetic diversity of 96 genotypes from 10 populations including three species was assessed using IRAP and REMAP markers. The employed primers included eight complimentary sequences of LTR regions of barley genome accompanied with eleven 3'-anchored ISSR primers. In the IRAP system seven out of the 36, and in the REMAP technique, 12 out of 88 primer combinations resulted in appropriate amplifications and scorable bands. The average PICs for IRAP and REMAP markers were accounted 0.30 and 0.32, and the mean MIs were 2.59 and 2.47, respectively. Cluster analysis using REMAP markers resulted in a phylogenetic tree with three distinct clusters which somewhat confirmed with geographical distribution of species, unlike the clustering by IRAP data. ANOVA using IRAP and REMAP data showed the higher variation within populations comparing with among populations. However, the acceptable diversity was observed within and among populations based on both marker systems.

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Introduction

Eighteen species of henbane (*Hyoscyamus* spp.) have been recorded worldwide which seven species are exclusively belong to Iran indicating the richness of henbane germplasm in this country. Table 1 shows the distribution of henbane species in Iran (Khatamsaz *et al.*, 1998; Ghahraman and Khatamsaz, 2015).

Table 1. Distribution of henbane species in Iran.

Species	Distribution location
H. arachnoideus	Tehran, Zanjan, Mazandaran, Hamedan and northwest of Iran
H. insanus	Khuzestan
H. kotschyanus	Chahar Mahal Bakhtiari
H. kurdicus	Ardebil, Urmia, Tabriz and Kurdestan
H. leptocalyx	Kermanshah
H. malekianus	Baluchestan
H. niger	Ardebil, Urmia, Tabriz and Tehran
H. orthocarpus	Khuzestan
H. pusillus	Ardebil, Urmia, Tabriz and Yazd
H. reticulatus	Ardebil, Urmia, Tabriz, Mazandaran and Khorasan
H. scoparia	Qom
H. senecionis	Kohgiloyeh and Boyerahmad
H. squarrousus	Kerman, Mazandaran and Gilan
H. tenuicaulis	Lorestan and Khuzestan
H. turcomanicus	Khorasan and Gorgan

Henbane adventively grows in many parts of Iran and is one of the Iranian indigenous plants of solanaceae that possesses high medicinal and economical value due to containing of the three types of alkaloids hyosyamine, atropine and scopolamine (Aljibouri *et al.*, 2012; Li *et al.*, 2006). The effect of this alkaloids on sedation the central nervous system, relief of Parkinson clinical symptoms, modulation of aging tremors, and using them up as hypnotic, antispasmodics, general anesthetic and pupil opener agents have been approved in medical sciences (Ghorbanpour *et al.*, 2013; Moradi *et al.*, 2012; Kiasalari *et al.*, 2011; Sengupta *et al.*, 2011; Begum *et al.*, 2010; Pokorny *et al.*, 2010). The economic and medicinal importance and also significant distribution and localization of henbane in Iran (Khatamsaz *et al.*, 1998) necessitates the implementation of domestication and breeding programs on this plant. The first step of this challenges is determining of genetic diversity amounts and the genetic relationships among different species and ecotypes of henbane. Genetic variation among and within populations of plant species is one of the main issues studied by breeders and geneticists to select high yielding and resistant varieties (Gepts, 2006).

Assessing the Genetic diversity of henbane species has been tried using morphological markers (Mirzadeh *et al.*, 2013; Khosrow Mehr *et al.*, 2012; Abou-lsba *et al.*, 2007), seed storage proteins (Mahfouz *et al.*, 2011; Sheidai *et al.*, 2000), isozymes (Monatasser-Kouhsari, *et al.* 2006; Sharifi *et al.*, 2006), RAPD markers (Yousefi, 2009) and ISSR marker (Najad Habib Wash *et al.*, 2012). Morphological evaluation of henbane germplasm is very complicated and difficult, often associated with errors (Khatamsaz and Zangirian, 1998).

DNA-based markers have eliminated many problems related to morphological and biochemical markers by creating a vast number of markers and eliminating the effects of environmental factors (Naghavi *et al.*, 2004; Li *et al.*, 2008). Among the DNA molecular markers, IRAPs and REMAPs, the two RTN (retrotransposon) and PCR based markers, are very convenient for assessing genetic diversity and phylogeny due to their very high polymorphism, optimal reproducibility, technical convenience, whole genome distribution and also due to special characteristics of retrotransposons such as high frequency, high activity in most genomes, insertion stability and linear insertion enhancement in the genome, (Branco *et al.*, 2007; Docking *et al.*, 2006; Guo *et al.*, 2006; Kumar and Hirochika, 2001;

Kalendar *et al.*, 2000). These markers have previously been used to determine variation among Solanaceae species (Mohsenzadeh *et al.*, 2012; Tao *et al.*,2009; Chen *et al.*, 2007; Demirel *et al.*, 2018), but there is no report about using of them on henbane, so far. Therefore, this study was conducted to evaluate genetic diversity, and also to determination of genetic relationships of henbane populations by means of IRAP and REMAP markers. Efficiency of the markers derived from LTRs of barley genome for assessing genetic diversity and relationships among henbane populations was discussed, too.

Materials and methods

Plant samples were collected at mid-summer until mid-autumn. Henbane species were identified at Herbarium section of Shahed University Tehran based on Flora Iranica descriptors (Table 2). Plant materials of this study consisted of 10 population of henbane from North West of Iran (table 3). Young leaves of 10 accession of each population were sampled at natural habitats of plants. The samples were transferred to lab into liquid nitrogen and maintained at -80°C until DNA extraction.

Table 2. Morphological identification of Hyoscyamus seeds studied(Khatamaz, 1998; Sadat Mirzadeh Vaghafi, 2011).

Species	Seed shape	Fluffy or Lint less	Size(mm)
H. niger	Has small and deep cavities and congested and tubular walls, umbilical inclined base, dark seed color	Lintless	1.5×2
H. pusillus	Compressed houses with very thick walls, with deep troughs in the middle of the seed that extend up to the navel, very thick from head to end, with distinctive navel.	Fluffy in the navel	1.3×2
H. reticulatus	The elongated and clear cavities with a thin, deep, tubular wall, with a trough that runs from head to end, thicken from head to end, Rough surface and tuber, distinct navel	With scattered lint	1.5×1

Table 3. Geographical information of studied henbane (*Hyoscyamus spp.*) populations.

Longitude	Latitude	Above mean sea level (m)	Location	Province	Number of genotypes	Species
483001	382484	1350	Ardebil	Ardebil	10	*H. reticulatus*
477543	390428	1843	Khalkhal		10	*H. reticulatus*
476777	383981	1400	Meshkinshahr		10	*H. pusillus*
483373	376868	1700	Givi		10	*H. niger*
462885	380792	1348	Tabriz	East Azarbaijan	10	*H. pusillus*
455930	382130	1660	Shabestar		10	*H. niger*
458521	374625	1468	Azarshahr		10	*H. reticulatus*
473480	375770	1650	Sarab		10	*H. pusillus*
400412	382839	1341	Ahar		10	*H. pusillus*
472220	382420	1800	Heris		10	*H. niger*

DNA extraction and molecular markers

Genomic DNA was extracted according to Sagai-Maroof *et al.* (1984) and Probeski *et al.* (1997) from each individual. DNA samples were evaluated by spectrophotometer using optical absorption ratios and wavelength of 280/260, 230/260 and 320nm respectively for protein, polysaccharide and polyphenol contaminants, and suspended particles. Presence of fractioned DNA and RNA contamination was tested by 0.8% agarose gel electrophoresis. Finally, DNA samples were diluted to 25ng/µl.

PCR for IRAP and REMAP techniques was performed according to Klendar and Schulman (2006) with minor modifications to optimize the reactions. PCR was accomplished in a 10 µl reaction mixture containing 10ng genomic DNA, 5 µl of "2X *Taq* Master Mix RED" (Amplicon Inc., containing 150 mM Tris-HCl pH 8.5, 40mM $(NH4)_2SO_4$, 3mM $MgCl_2$, 0.2% Tween 20, 0.4mM dNTPs, 0.05U/µl *Taq* DNA polymerase, inert red dye and stabilizer), and 2.5pmol of each primer. The amplification was performed at 94°C for 4 min for initial denaturation, followed by 36 cycles of 94°C for 1 min, 55 s at respective primer annealing temperature and 2 min at 72°C with a final

extension for 8 min at 72°C. In the IRAP technique eight primers whit 25-35 nucleotide length complementary to LTRs of barley retrotransposons (table 4) were employed in singular and pair combination stats. Total of eight retrotransposon primers paired with 11 ISSR primers (table 3) were used to create REMAP markers. (Alavi-kia *et al.*, 2008; Teo *et al.*, 2005; Kalendar *et al.*, 1999).

Table 4. Characteristics of the retrotransposon primers used in this study.

Sequence 5′→3′	Name and direction of primer
GATAGGGTCGCATCTTGGGCGTGAC	Sukkula→
CGCATTTGTTCAAGCCTAAACC	Nikita→
CTCGCTCGCCCACTACATCAACCGCG TTTATT	LTR6149→
TGTTTCCCATGCGACGTTCCCCAACA	3′LTR→
CTGGTTCGGCCCATGTCTATGTATCC ACACACATGTA	LTR6150←
TTGCCTCTAGGGCATATTTCCAACA	5′LTR1←
ATCATTCCCTCTAGGGCATAATTC	5′LTR2←
GGAATTCATAGCATGGATAATAAACG ATTATC	LTR7286→

Visualization of amplified fragments was accomplished by 1.8% agarose gel electrophoresis with voltage of 120V for two hours using 1X TBE buffer and a Bio-Rad UV gel documentation system. For determination of fragments size, a standard molecular weight marker (100-3000bp) were loaded at each run.

Table 5. Specifications of ISSR primers used in REMAP.

Junction temperature	Melting point	Sequence 5′→3′	Primer name	Number
53	49.2	(AC)8T	ISSR1	825
53	53.3	(AC)8C	ISSR2	826
52	54.9	(AC)8G	ISSR3	827
ø	56.1	(TG)8G	ISSR4	830
53	48	(AG)8yT	ISSR5	834
49	50.3	(AG)8yC	ISSR6	835
ø	48	(GA)8yT	ISSR7	840
53	50.3	(GA)8yC	ISSR8	841
ø	50.3	(CT)8rG	ISSR9	845
49	50.3	(CA)8rC	ISSR10	847
49	50.3	(CA)8rG	ISSR11	848

ø. For nonproliferation, r represents A or G and Y represents C or T

Statistical analysis

Clear and distinguishable amplified fragments were scored in the binary system (1 for presence and 0 for absence of the bands). Polymorphic information content (PIC; Botstein *et al.*, 1980) and marker index MI (Powell *et al.*, 1996) were estimated as indices of markers capability. The distance matrix was calculated among the genotypes according to Huff *et al.* (1993) and were subsequently used for Analysis of molecular variance (AMOVA; Exkoffier, 1992) and construction of dendrograms. AMOVA was performed to segregation of within group molecular variance from between group using GenAlEx 6.4 software (Peakall and Smouse, 2006). Dendrograms based on minimum evolution algorithm using MEGA4 (Tamura *et al.*, 2007) were separately organized for IRAP and REMAP markers to dissociate the genotypes and populations. Principal coordinate analysis (PCoA) was performed to validate relationship patterns using GenAlEx 6.4 software (Peakall and Smouse, 2006).

Results and discussion

DNA quality and quantity

Data derived from spectrophotometry indicated average amount of 1.75 and 0.005 for 260/280 and 320 absorption ratio, respectively. Clear non-stop and non-smear bands of extracted DNAs were observed on 0.8% agarose gel for all samples. These results indicated that DNA was purified and free from RNA and polysaccharide contamination. It seems that using of high density of CTAB and NaCl in DNA extraction protocol was effective in removal of polysaccharides. The high density of PVP resulted in elimination of polyphenols, too (Probeski *et al.*, 1997).

Polymorphism and efficiency of IRAP markers

Out of 36 possible combinations derived from eight barley LTR primers, only seven combinations had appropriate and scorable amplification that indicates lower association of barley LTR-RTNs in *Hyoscyamus* genome. While other researchers' results have shown the high efficiency of barley LTR retrotransposons. For example, Researchers used *Bare1*-based IRAP markers in different species of *Hordeum*, *Triticum*, and *Agilops* and reported high level of polymorphic indicating high association of

Bare1 retrotransposon in these genomes (Kalendar *et al.*, 2010). High polymorphism in wheat was also identified using *Bare1* retrotransposon markers (Carvalho *et al.*, 2010).
Similar results were obtained in *Agilops* using markers based on the *Bare1* retrotransposon, too (Saeidi *et al.*, 2008). In a study using IRAPs and REMAPS based on barley LTR retrotransposons, high polymorphism and separating efficiency was observed in lemon balm genotypes (Ghafarian varjo *et al.*, 2010). Alavi-kia *et al.* (2008) Observed high polymorphism and diversity within and among *Crocus* species using barley LTR-RTNs.

The successful primer combinations produced 59 markers in total ranging from 200 to 3000bp which all were polymorph (fig. 2). The average number of IRAPs per each primer combination was 3.675 with rang between 1.5 for the combination of LTR7286 and Nikita primers and 5.18 for the LTR6149- LTR6149 combination. In another study by Branco *et al.* (2007) on rice using IRAP and REMAP markers, this value was estimated equal to 9.16. Alavi-kia *et al.* (2008) in *Crocus* genus using barley retrotransposon primers reported 4.29 and 5.14 markers for wild and agronomic species, respectively. The low value of mean number of IRAPs per primer may be due to the low number of barley LTR-RTNs associated in *Hyoscyamus* genome.

Table 6. Marker index (MI) and Polymorphism Information Compounds (PIC) of IRAPs.

Primer compound	MI	PIC
3′LTR- Nikita	2.033	0.290
3′LTR-	2.835	0.315
3′LTR-LTR6150	3.560	0.356
LTR7286- Nikita	1.073	0 .268
LTR6149	4.344	0.434
5′LTR2-5′LTR1	2.644	0 .240
Sukkula-Nikita	1.665	0 .208
Mean	2.59	0.30

Maximum and minimum PIC values were calculated 0.434 for 6149LTR - LTR 6149 and 0.208 for Sukkula-Nikita primer combinations (table 5). The polymorphic information content of IRAPs is evaluated approximately high with mean of 0.30 comparing with some other studies (Abdollahi *et al.*, 2015; Smykal, 2006; Alavi-kia *et al.*, 2008).

Mean MI was 2.59 with the range of 1.073-4.344 for LTR7286-Nikita and LTR6149, respectively (table 6). In contrast with other reports (Ghaffarian Varjo *et al.*, 2010; Smykel *et al.*, 2006; Alavi-kia *et al.*, 2008), the low values of MI in this study referred to deficient number of markers produced by IRAP primers.

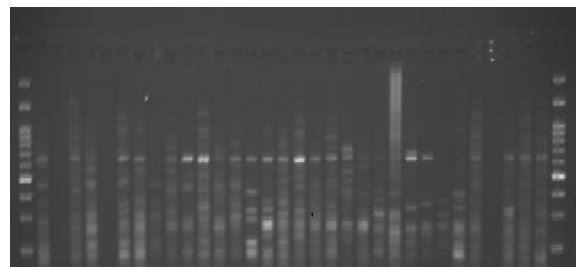

Fig. 2. Polymorphism of IRAP markers derived from the combination of 3′LTR primers in Henbane.

Polymorphism and efficiency of REMAP markers
Twelve combinations with appropriate amplification and totally 92 polymorph markers were result of implementation of PCR by the 88 possible combinations of 11 ISSR and eight LTR primers in REMAP technique. The number of markers per primer combination was 3.636 in average and ranged from 2.10 for the 3′LTR-ISSR834 to 5.69 for 5′LTR2-ISSR841 combinations. Kalender *et al.* (1999) reported this value equal to 17.4 and Branco *et al.* (2007) reported it as 3.6 in genus *Hordeum*. Alavi-kia *et al.* (2008) using barley LTR-RTN primers in the genus *Crocus* reported 6.85 and 6.41 markers per primer for wild and agronomic species, respectively. Relatively low number of REMAPs per primer in this study could be depended on deficient assembly of LTR-RTNs around the microsatellites because of totally poor association of barley LTR-RTNs into the genome of *Hyoscyamus* genus.

PIC values were ranged from 0.276 (3′LTR-ISSR841) to 0.405 (3′LTR-ISSR834) with mean of 0.32 for REMAP markers. Mean MI values were equal to 2.47 with range of 1.217 (LTR7286-ISSR835) to 3.46 (LTR-ISSR834). Ghaffarian varjo (2010) reported mean MI and PIC values for REMAPs in lemon balm equal to

0.28 and 11.72, respectively. Alavi-kia *et al.* (2008) reported the mentioned amounts in *Crocus* equal to 0.18 and 10.19, respectively. Accordingly, it was observed a moderately good PIC values and low MIs indicating that REMAPs had a good polymorphic ability but not proper number of markers for creating the favorite values of MI in this study.

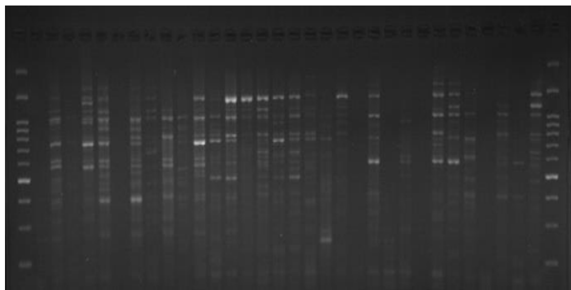

Fig. 3. Polymorphism of REMAP Markers from the ISSR835-LTR7286 Primer Combination in Henbane.

Table 7. Marker index (MI) and Polymorphism Information Compounds(PIC) of REMAP.

Primer compound	MI	PIC
5′LTR2-ISSR835	3.329	0 .369
5′LTR2-ISSR841	3.264	0 .326
3′LTR-ISSR827	1.881	0.313
3′LTR-ISSR834	1.217	0 .405
3′LTR-ISSR847	1.464	0 .366
3′LTR-ISSR841	2.229	0.276
5′LTR1-ISSR826	2.863	0.278
5′LTR1-ISSR841	2.295	0.286
LTR7286-ISSR825	3.388	0.286
LTR7286-ISSR840	1.817	0.376
LTR7286-ISSR847	3.460	0.302
LTR7286-ISSR835	3.460	0.346
Mean	2.47	0.32

Analysis of molecular variance

Results AMOVA based on 59 IRAP and 92 REMAP markers showed that in both analyzes the ratio of among population to total variance (ϕpt) was significant at 0.01 level. In both marker systems, within population variances was estimated more than among population variances, and could be explained that both IRAP and REMAP markers showed significant genetic variation within and among henbane species. The total variance estimated by REMAP markers is approximately 1.5 times more than of IRAPs which could be due to the greater number of REMAP markers. Accordingly, the within group variance was estimated more than among group in many studies via AMOVA (Tabatabai *et al.*, 2018; Tabrizivand Taheri *et al.*, 2017; Fathi *et al.*, 2014; Gholamzadeh Khoi *et al.*, 2012).

Table 8-1. Results of AMOVA based on IRAP markers.

Sources of variation	df	SS	MS	Est Var	ϕpt	P
Among poulations	9	178.217	19. 802	1.129	0.122	0.001
Within populations	86	772.867	8.987	8.987		
Total	95	951.083		10.115		

Table 8-2. Results of AMOVA based on REMAP markers.

Sources of variation	df	SS	MS	Est Var	ϕpt	P
Among poulations	9	342.106	38.012	2.562	0.160	0.001
Within populations	86	1157.663	13.461	13.461		
Total	95	1499.740		16.023		

Relationships among Hyocyamus populations based on IRAPs

Dendrogram of 96 henbane genotypes was drown by means of IRAP data based on distance matrix (Huff et. al., 1993) through Minimum Evolution method (fig. 4). The dendrogram was not included any arguable information about segregation of genotypes based on their species and or their geographical locations indicating existence of the high similarities of RTN insertion sites among the all genotypes.

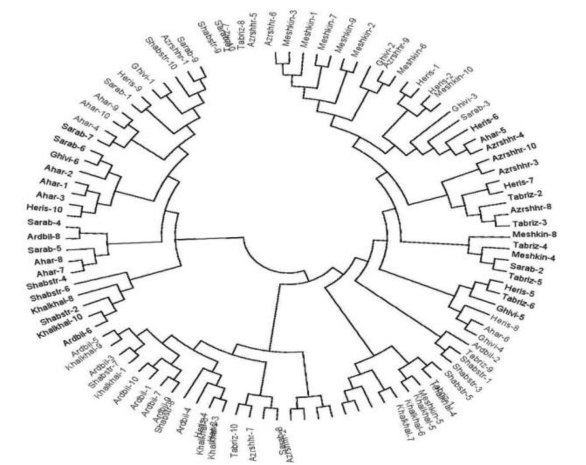

Fig. 4. Dendrogram derived from IRAP markers using minimum evolution algorithm.

PCoA based on IRAP markers

In PCoA, the first three principal coordinates explained the 25.28% of total variance (table 9). AS in IRAP technique, fragment amplification occurs between two RTNs sufficiently close together (Kalendar and Schulamn, 2006), it could be expressed that RTN insertion sites and consequently IRAP markers were not accumulated in a particular part of the henbane genome. The results of decomposition into two first coordinates confirm the findings of cluster analysis about lack of proper segregation among species and geographical locations.

Table 9. Percentage of variance explained by the first three coordinates based on IRAP and REMAP markers in Henbane.

REMAP markers	IRAP markers	Component
7.55	10.99	First
6.49	7.65	Second
4.70	6.64	Third

Relationships among Hyoscyamus populations based on REMAPs

Dendrogram based on REMAP data was constructed by the method same as IRAPs indicated three distinct clusters of all studied genotypes (fig. 6).

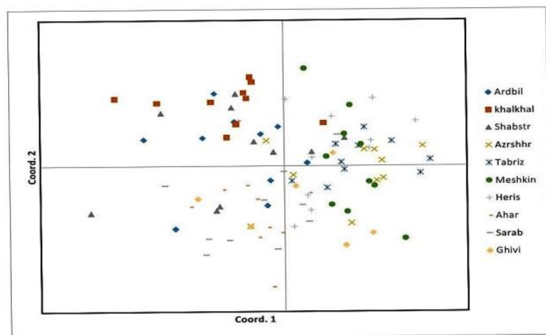

Fig. 5. Biblot derived from the two first coordinates based on IRAP markers.

Within the first group the number of 9, 2, 10, 8, and 10 genotypes were located that respectively belonged to Shabestar, Givi, Ardebil, Azarshahr and Khakhal populations. These 39 genotypes included 11 genotypes of *H. niger* and 28 genotypes of *H. reticulatus*. Species *H. pusillus* did not associated in this group. Group 2 consisted of 29 genotypes which 10, 10, 5 and 4 genotypes of them related to Ahar, Sarab, Heris and Givi populations, respectively. On the other hand, 20 genotypes of *H. pusillus* and 9 genotypes of *H. niger*, independent from *H. reticulatus* organized the group number 2. The number of 10 genotypes of Meshkinshahr, 10 of Tabriz, 5 of Heris, 2 of Azarshahr and only one genotypes of Shabestar populations associated in constructing the group number 3. Otherwise, group 3 consisted of 20 *H. pusillus* accessions, 6 *H. niger* accessions and only two *H. reticulatus* accessions. Totally, it could be explained that REMAP data were not capable in segregating of species *H. niger* from two others indicating existence of many similarities in microsatellites and RTN insertion sites. However, except *H. niger*, REMAPs could separate species *H. reticulatus* from *H. pusillus* whereas *H. reticulatus* was allocated into group 1 and *H. pusillus* into groups 2 and 3.

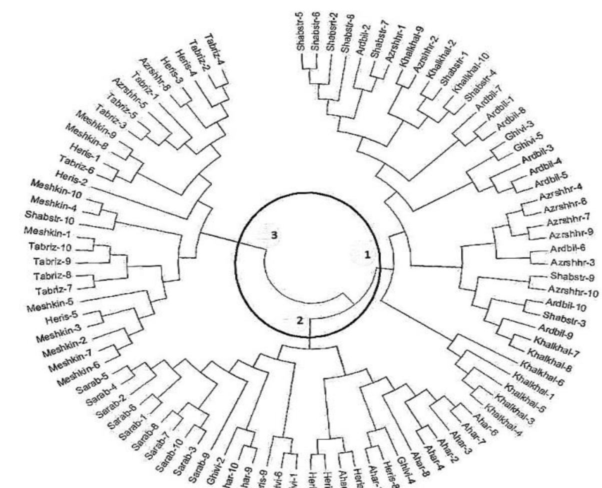

Fig. 6. Dendrograms derived from REMAP markers using minimum evolution algorithm.

PCoA based on REMAP markers

Principal coordinate analysis showed that the first three components determined 18.74% of the total variance. This indicates that the amplifiable distances between microsatellites and RTNs are almost independently distributed on whole chromosomes of *Hyoscyamus* genome. The biplot derived from two

first coordinates (fig. 7) could not match with cluster analysis information (fig. 6). It is because of the low ratio of variance determined by the two first vectors to total variance 14.4%, (table 9) that prepare insufficient data for grouping the genotypes.

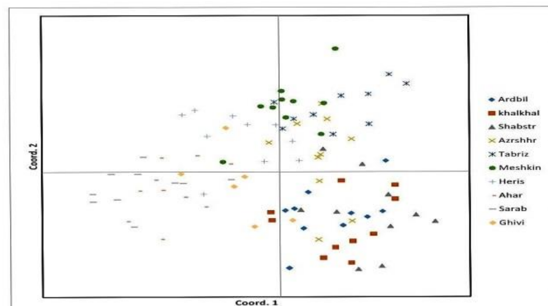

Fig. 7. Biblot derived from the two first coordinates based on REMAP markers.

Conclusion

According to acquired results it could be explain that barley LTR-RTNs had an almost weak association in *Hyoscyamus* genome comparing with some other studied genera (Alavi-Kia *et al*., 2008; Ghaffarian-Varjo *et al*., 2010). However, an acceptable genetic diversity was found among and within population by IRAP and REMAP markers. Here, REMAPs were more efficient than IRAPs in estimating genetic diversity and separating the groups of henbane considering association of microsatellites in generation of REMAPs. The information resulted from REMAP markers could separate the geographical groups and different species of henbane ignoring some exceptions. Finally, it is claimable that there are a vast genetic diversity about some henbane species in North-West of Iran. This documentation accompanied with economic and medicinal value of henbane could determine the justification and importance of organization of domestication and breeding of this plant.

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