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Transferability of barley retrotransposon primers to analyze genetic structure in Iranian *Hypericum perforatum* L. populations

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Abstract: Transferability of barley retrotransposon primers was investigated to analyze population structure in St. John's wort (*Hypericum perforatum* L.) based on inter-retrotransposon amplified polymorphism (IRAP). Seven long terminal repeat (LTR) retrotransposon primers derived from the barley genome were used to detect genetic polymorphism in eight Iranian populations and three cultivars (Helos, New Stem, and Topaz) of *H. perforatum* based on IRAP analysis. Nine possible LTR primers/primer combinations successfully amplified fragments from the *H. perforatum* genome. In total, 311 bands of 100–3000 base pairs were amplified, of which 244 were polymorphic. The number of polymorphic fragments ranged from 10 (Nikita/5′LTR-2) to 57 (3′LTR), with an average of 27.11. Principal coordinate analysis (PCoA) could clearly differentiate samples of wild populations and cultivars. Based on analysis of molecular variance (AMOVA), among populations variance explained 58% of total molecular variation. This study demonstrates that IRAP markers can be utilized not only to determine the relationships of *Hypericum* populations and cultivars, but also as a tool for selection of suitable populations for breeding programs.

Key words: Genetic diversity, *Hypericum perforatum*, IRAP markers, transferability

1. Introduction

Medicinal plants are significant to urban and rural populations in many countries and regions. Most medicinal plant species are harvested from the wild, and the extent of their use has led to genetic erosion or even extinction of some species (Larsen and Olsen, 2007; Vandebroek and Balick, 2012). Conservation of medicinal plants is an important part of biodiversity conservation (Kate and Laird, 1999). In some developing countries such as Iran, there are no reliable data available on the economy of wild medicinal plants, which may become extinct within a few years (Mahdei, 2005). It is important to assess the value of these plants as important biological resources and to document the intellectual property rights (Domyati et al., 2011).

St. John's wort (*Hypericum perforatum* L.) is a perennial herb indigenous to Europe that has been introduced in many regions due to its medicinal value. It produces pharmaceutically important metabolites with antidepressive, anticancer, and antiviral activities (Kubin et al., 2005; Barcaccia et al., 2006). In recent years, interest in *H. perforatum* as a natural antidepressant in phytotherapy has grown rapidly. In Iran, this medicinal plant is mostly collected from populations growing in the wild. To meet the increase in demand and to prevent genetic erosion due to overharvest from wild growing plants, *H. perforatum* has to be produced by cultivation. Effective breeding strategies are required, therefore, to produce cultivars of St. John's wort that give a homogeneous quality of extracts and exhibit favorable agronomic characteristics to ensure stable quality and yield (Mártonfi et al., 1996). Genetic diversity is the raw material for the development of improved cultivars. Hence, information on genetic diversity in wild gene pools is crucial for the efficient maintenance of genetic diversity and its utilization in breeding (Canter et al., 2005).

DNA markers have a key role in the study of genetic variability and diversity and in the tracking of individuals or lines carrying particular genes or traits of interest. DNA markers can generate 'fingerprints', which are distinctive patterns of DNA fragments in individuals. The advent of the PCR was a breakthrough for molecular marker technologies, and made many fingerprinting methods possible (Kalendar et al., 2011). Few studies have attempted

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to analyze genetic diversity and population structure in *H. perforatum* using DNA markers. Halusková and Cellárová (1997) performed RFLP analysis of *H. perforatum* based on rDNA probes to characterize somaclones and their progenies. The result revealed the occurrence of sexual recombination in *H. perforatum* plants. Barcaccia et al. (2006) used RAPD, ISSR, and AFLP markers to determine levels of genetic variation within and among 15 local populations of *H. perforatum* L. collected from northern Italy, provinces of Belluno and Treviso. Molecular analyses revealed that all the ecotypes were polyclonal, not being dominated by a single genotype, and characterized by different levels of differentiation among multilocus genotypes. Percifield et al. (2007) performed AFLP analysis of 56 *Hypericum* accessions, representing 11 species, to gain a better understanding of diversity within *Hypericum* species, especially within cultivated accessions of *H. perforatum*, and to establish a molecular methodology that will provide breeders and regulators with a simple, affordable, and accurate tool with which to identify purported *H. perforatum* material.

Retrotransposons are ubiquitous in the plant kingdom and are amongst the most variable of all genomic components. They are present in high copy numbers and are highly heterogeneous in plant genomes, and can be transmitted both vertically and horizontally across generations and between different plant species, respectively (Du et al., 2013). Retrotransposon-based molecular markers have a number of advantages over other molecular markers, such as their abundance and dispersion throughout almost the entire length of all host chromosomes. They also present high information content and transferability across species and genera, which ensure more reliable methods for DNA fingerprinting. Popularization of these markers has been hindered because the development of retrotransposon-based marker systems for a new plant species requires the isolation, cloning, sequencing, and characterization of the retrotransposon sequence, as a prerequisite to obtaining the species-specific retrotransposon primers. Investigations about the transferability of retrotransposon primers developed from one plant species onto others would be of great value to enable the low-cost and highly efficient development of retrotransposon-based molecular markers in plant species with very little or no information on retrotransposon sequences. For those plant species with abundantly available public data of retrotransposon sequences or retrotransposon-based marker systems, transferable primers from other plant species would be useful to further increase the number of markers (Kalendar et al., 2011; Du et al., 2013).

Inter-retrotransposon amplified polymorphism (IRAP) analysis is a whole-genome approach that has broad applicability in determining genetic variability within and among plant populations (Kalendar et al., 1999; Alavi-Kia et al., 2008) and relationships among cultivars (Kalendar et al., 2006; Branco et al., 2007; Smýkal et al., 2011). A virtue of IRAP is its experimental simplicity compared with AFLP. All that is needed is simple PCR followed by electrophoresis to resolve the PCR products. IRAP can be carried out with a single primer matching either the 5′ or 3′ end of the LTR but oriented away from the LTR itself, or with two primers.

The aims of present study were to: i) investigate the transferability of retrotransposon primers derived from barley, a monocot, to *H. perforatum*, an evolutionary distant eudicot, and ii) describe patterns of genetic variation and distribution within and among wild and commercially cultivated accessions of *H. perforatum*, using IRAP molecular markers.

2. Materials and methods

2.1. Plant materials

Eight wild populations of *H. perforatum* were collected from various regions of northwest Iran including Arasbaran, Ardabil, Malekan, and Seywan (East Azarbaijan Province) (Table 1; Figure 1). The cultivars Topaz (Seidler-Lozykowska and Dabrowska, 1996), New Stem, and Helos (bred for tolerance to anthracnose disease) were also included in the study. In total, 110 individuals were sampled for molecular analysis.

2.2. DNA extraction and PCR amplification

From each population and cultivar, 10 field-grown plants were sampled and DNA was isolated from 1.0–1.5 g of fresh leaves following the procedure of Saghai Maroof et al. (1984). DNA quality and quantity were checked using 0.8% agarose gel and a spectrophotometer.

IRAP analysis was performed using seven LTR primers designed based on barley (*Hordeum vulgare*) *Nikita*, *Sukkula*, and *BARE-1* retrotransposons (Kalendar et al., 1999; Manninen et al., 2000; Boyko et al., 2002). The primer sequences, retrotransposon source, and orientation are shown in Table 2. Genomic DNA samples were diluted with sterile deionized water to 25 ng/μL. The IRAP amplification was performed in a 10 μL reaction mixture containing 50 ng of DNA, 7 μL of Master Mix (containing 1 μL of 1X PCR buffer, 0.4 μL of $MgCl_2$ 50 mM, 0.1 μL of dNTPs 250 mM, 0.1 μL of *Taq* DNA polymerase 5 U/μL, and 5.40 μL ddH_2O), and 1 pmol of each primer. The annealing temperature was optimized using gradient PCR. The amplification program consisted of 94 °C for 4 min, 30 cycles of 94 °C for 60 s, annealing at the temperature specified in Table 3 for 60 s, and 72 °C for 2 min, with a final extension at 72 °C for 7 min. PCR products were analyzed by electrophoresis on 4% ultrathin (0.2 mm) nondenature polyacrylamide gel and detected by ethidium bromide staining, using a Gel-Scan 3000 electrophoresis system

Table 1. Information on the collection sites of the eight populations of *Hypericum perforatum* included in the analysis.

No.	Population	Altitude (m)	Latitude	Longitude
1	Arasbaran-Janjal	1811	38°51′N	46°51′E
2	Arasbaran-Balan	2046	38°52′N	46°49′N
3	Arasbaran-Alibolaghi	1248	38°51′N	46°40′N
4	Arasbaran-Alhort	1661	38°55′N	46°50′N
5	Arasbaran-Mazgar	1880	38°54′N	46°51′E
6	Seywan	1679	38°21′N	45°46′E
7	Ardabil	1379	38°14′N	48°17′E
8	Malekan	1296	37°08′N	46°06′E

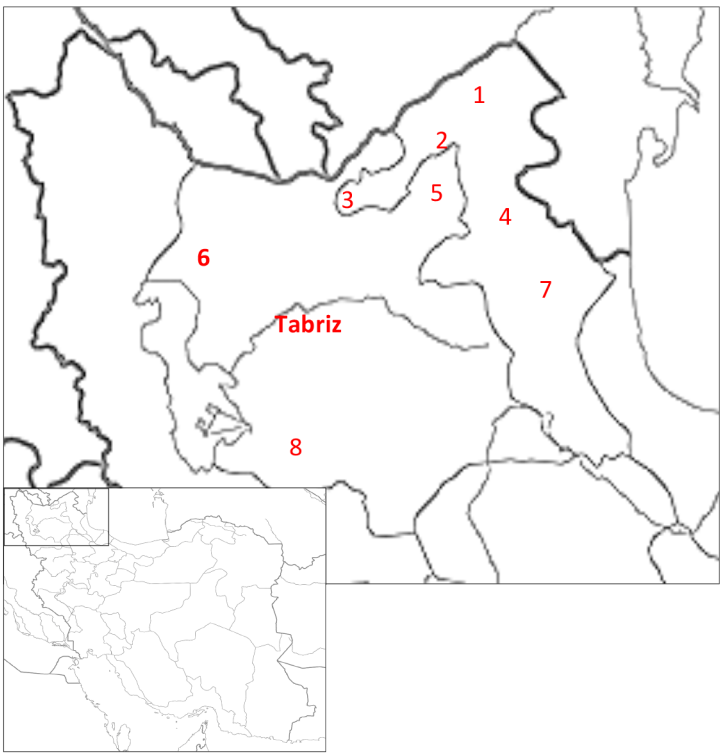

Figure 1. Information on the *Hypericum perforatum* populations. Map of the collection sites of the eight populations of *Hypericum perforatum* from northwest Iran. Numbers refer to the collection sites listed in Table 1.

(Corbett, Sydney, Australia). Thermo Scientific GeneRuler 100 bp Plus DNA Ladder 100 to 3000 bp was used in order to determine the lengths of the DNA fragments.

2.3. Data analyses

Each IRAP band was treated as a single locus (presence/absence) and scored using 1Dscan EX 3.1 software (Meyer Instruments, Houston, TX, USA). The software was set up to score only sharp and prominent bands. We calculated the Shannon index (I), defined for multilocus markers as $I = -\Sigma p_i log p_i$, and unbiased Nei's genetic diversity index (HE), $H_E = (1 - 1/n)\Sigma p_i^2$, where p_i is the frequency of the ith fragment in the 148 samples (Nei, 1973). Polymorphic information content (PIC) was calculated for each marker using the following formula: $PIC = 1 - f_i(1 - f_i)$, where f_i is the frequency of the ith marker fragment when present and $1 - f_i$ is the frequency of the ith marker fragment when

Table 2. Primer name, retrotransposon type, position, and sequences.

Name and orientation	Element origin in barley	Position	Sequence
Nikita →	Nikita	1-22	CGCATTTGTTCAAGCCTAAACC
Sukkula →	Sukkula	4301-4326	GATAGGGTCGCATCTTGGGCGTGAC
LTR6149 →	*BARE-1*	1993-2012	CTCGCTCGCCCACTACATCAACCGCGTTTATT
3′LTR →	*BARE-1*	2112-2138	TGTTTCCCATGCGACGTTCCCCAACA
LTR6150 ←	*BARE-1*	418-439	CTGGTTCGGCCCATGTCTATGTATCCACACATGTA
5′LTR1 ←	*BARE-1*	1-26	TTGCCTCTAGGGCATATTTCCAACA
5′LTR2 ←	*BARE-1*	314-338	ATCATTCCCTCTAGGGCATAATTC

absent (Roldain-Ruiz et al., 2000). The marker index (M_I) was also calculated as $M_I = PIC \times$ EMR, where EMR is "the effective multiplex ratio (E) defined as the product of the total number of loci/fragments per primer (n) and the fraction of polymorphic loci/fragments (β) ($E=n.\beta$)" (Powell et al., 1996). Principal coordinate analysis (PCoA) based on binary genetic distance was performed using GenAlEx version 6.2 software (Peakall and Smouse, 2006). To describe the population relationship, cluster analysis was carried out based on a neighbor-joining algorithm and pairwise unbiased Nei's genetic distances (Nei, 1972) using GenAlEx version 6.2 software (Peakall and Smouse, 2006). Bootstrapping based on 1000 replicates was used to assess the statistical support of internal branches in the dendrogram (Mohammadi and Prasanna, 2003) using phyclust, an R package (Chen, 2010). The analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was used to partition the total genetic variation to among- and within-population variance components and to calculate the genetic variation in each population.

3. Results

3.1. IRAP polymorphism

Nine out of 28 possible seven LTR primers and their combinations yielded multiple DNA fragments from genomic DNA of all local populations and foreign cultivars with a high polymorphism level among individuals (Table 3). An example of IRAP banding patterns related to some

Table 3. Annealing temperature (T_a), total number of markers (TM), number of polymorphic markers (PM), percentage of polymorphic markers (PM%), marker index (MI), and polymorphic information content (PIC) for primer/primer combinations in IRAP analysis.

Primer combination	T_a	TM	PM	PM%	MI	PIC
3′LTR	60	73	57	78.08	12.01	0.27
5′-LTR-1	65	30	27	90.00	8.01	0.33
5′-LTR-2	60	53	35	66.03	6.24	0.27
Nikita	60	16	13	81.25	4.22	0.40
Sukkula	60	39	31	79.48	7.88	0.32
3′LTR/5′LTR2	60	27	18	66.66	4.44	0.37
3′LTR/Nikita	65	20	13	65.00	2.70	0.32
5′LTR-2/Nikita	60	13	10	76.92	2.60	0.34
3′LTR/Sukkula	70	40	40	100.00	13.20	0.33
Total	-	311	244	-	-	-
Mean	-	34.55	27.11	78.15	6.81	0.33

of the Iranian populations is shown in Figure 1. Analyzing PCR amplified DNA fragments with nine LTR primers/combinations in 110 *H. perforatum* genotypes, we found a total of 244 polymorphic bands (78.15%), ranging from 100 to 3000 bp. Maximum number of markers was obtained using 3′LTR primer (73), whereas only 16 fragments were amplified using Nikita primer in the studied sample of genotypes. The number of polymorphic fragments in the total *H. perforatum* sample ranged from 10 (Nikita/5′LTR-2) to 57 (3′LTR), with an average of 27.11. The proportion of polymorphic markers (PMs) in the total sample varied from 66.03 (5′LTR-2) to 100 (3′LTR/Sukkula), with an average of 78.15% (Table 3).

PIC values ranged between 0.27 (3LTR and 5′LTR-2) and 0.40 (Nikita), with an average of 0.32. The primers/primer combinations with minimum and maximum number of bands showed the highest and lowest PIC values, respectively. Moreover, Nikita/5′LTR-2 with 2.6 and 3′LTR/Sukkula with 13.2 showed the highest and lowest values of M_I, respectively, with an average of 6.81 in the whole sample.

3.2. Genetic diversity

The binary data matrix obtained from IRAP amplification profiles revealed conspicuous genetic variability both within and among the eight wild populations and three cultivars. In total, 244 polymorphism fragments were amplified with LTR primers and varied from 78 in *Topaz* to 107 markers in *Ardabil*. All the studied populations or cultivars had private markers except *Arasbaran-Balan* and *Arasbaran-Alibolaghi*. Eight private markers were found in *Topaz*, seven in *Helos*, five in *Arasbaran-Alibolaghi*, four in *Arasbaran-Alhord* and *Arasbaran-Mazgar*, two in *Nwe Stem* and *Arasbaran-Janjal*, and one in *Seywan* and *Malekan* (Table 3).

3.3. Genetic relationships

The relationship among foreign cultivars and wild Iranian populations of *H. perforatum* as revealed by PCoA based on all scored polymorphic markers is presented in Figure 2. The first three coordinates explained 85.85% of the total molecular variation. The first coordinate, which explained 42.87% of the variation, discriminated foreign cultivars from most of the local populations except *Arasbaran-Mazgar*, *Arasbaran-Alhord*, and *Malekan*. The second coordinate, which explained 33.08% of the variation, clearly discriminated *Topaz* from the other two cultivars, as well as *Ardabil* and *Arasbaran-Balan* populations from other wild populations. Among the cultivars, *Topaz* and *Helos* showed high similarity compared with *New Stem*. In the case of wild populations, *Ardabil*, *Arasbaran-Balan*, and *Arasbaran-Janjal* populations were closely grouped.

Genetic relationships among *H. perforatum* populations and cultivars were ascertained based on the neighbor-joining algorithm. The dendrogram clearly separated cultivars and wild populations (Figure 3). Congruent with

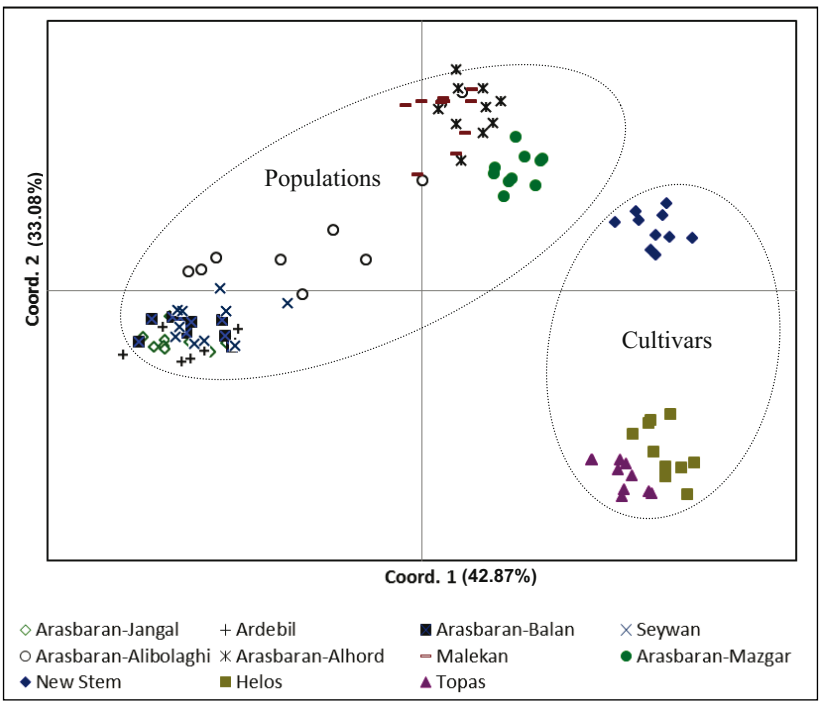

Figure 2. Relationship among foreign cultivars and Iranian's wild populations of *Hypericum perforatum* according to bidimensional PCoA projections.

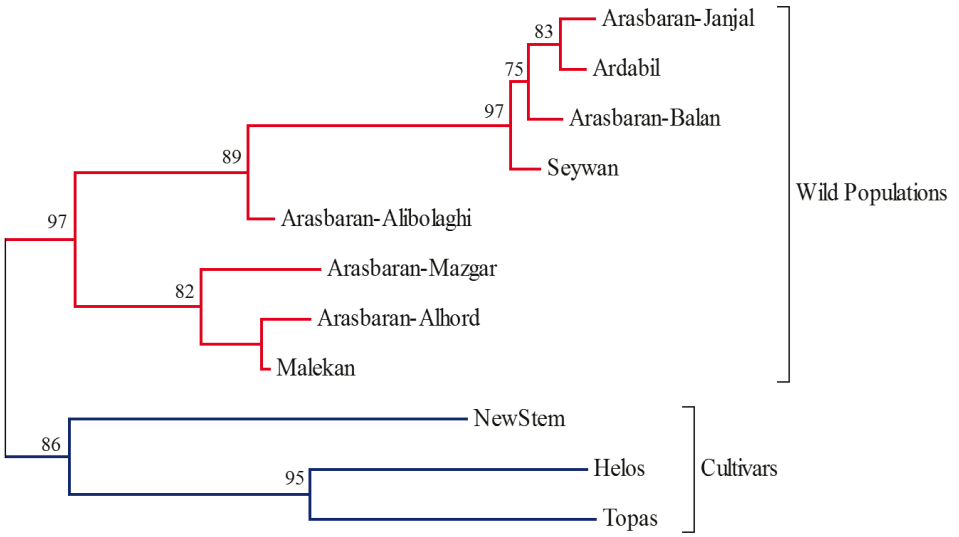

Figure 3. Neighbor-joining cluster analysis based on Nei's genetic distance coefficients showing the genetic relationships among 11 *Hypericun perforatum* wild populations and cultivars based on IRAP analysis.

the PCoA, the *Helos* and *Topaz* cultivars tightly clustered and were closely associated with *New Stem*. In addition, the dendrogram showed that wild populations segregated into two clusters, independently of the geographic origin of the samples (Figures 1 and 3).

Assuming Hardy–Weinberg equilibrium, the average Nei's genetic diversity (H_E) was estimated to be 0.129 within wild populations and 0.077 at the cultivar level. Shannon's index (I) ranged from 0.09 to 0.24, with an average of 0.17 in wild populations and 0.10 in the cultivars, respectively (Table 4). Among the eight wild populations, Arasbaran-Mazgar and Malekan exhibited the highest and lowest levels of genetic variability with PM of 135 and 87, *HE* of 0.185 and 0.09, and I of 0.24 and 0.13, respectively, while among the cultivars New Stem and Topaz possessed the highest and lowest genetic diversity with PM of 82 and 78, *HE* of 0.08 and 0.07, and *I* of 0.11 and 0.09, respectively (Table 4).

Table 4. Number of polymorphic markers (PM), private markers, mean of heterozygosity, Shannon's information index, and Nei's gene diversity in the studied populations and accessions of *H. perforatum*.

Population/cultivar	PM	Private markers	Shannon's index	Nei's index	Within-population variance
Arasbaran-Janjal	104	2	0.17	0.13	15.85
Arasbaran-Balan	95	0	0.16	0.12	15.68
Arasbaran-Alibolagh	95	0	0.17	0.13	24.89
Arasbaran-Alhord	93	1	0.16	0.13	15.52
Arasbaran-Mazgar	135	5	0.24	0.18	13.70
Seyvan	105	4	0.15	0.11	15.13
Ardabil	107	1	0.19	0.14	16.44
Malekan	87	4	0.13	0.09	17.37
New Stem	88	2	0.11	0.08	10.34
Helos	82	7	0.09	0.07	8.69
Topaz	78	8	0.10	0.08	8.33
Mean in wild populations	102.62	2.14	0.17	0.12	16.82
Mean in cultivars	82.67	5.67	0.10	0.08	9.12
Total mean	97.18	3.20	0.15	0.11	14.72

The total gene diversity (H_T) over the 11 *H. perforatum* populations and cultivars was 0.22. Average within-population diversity (H_S) and the coefficient of differentiation among population diversity (G_{ST}) were 0.099 and 0.39, respectively. AMOVA revealed significant within and among *H. perforatum* populations variance. As with F_{ST}, in the AMOVA the level of genetic variation among populations was higher than that within populations. AMOVA revealed that 58% of the total molecular variance is attributable to among-population genetic diversity. Among the studied populations, the maximum genetic distance was observed between *Malekan* and *Topaz*, whereas *Arasbaran-Alhord* and *Malekan* populations showed the lowest genetic distance.

The analysis of single plant DNAs showed high levels of similarity within all *H. perforatum* cultivars and wild populations as revealed by within-population variance (Table 4). The mean within-population diversity in wild populations and cultivars was 16.82 and 9.12, respectively. The within-population variance in all the studied populations and cultivars ranged from 8.33 (*Topaz*) to 24.89 (*Arasbaran-Alibolagh*), with an average of 14.72. Among the wild populations, *Arasabran-Mazgar* (13.70) showed the higher homogeneity.

4. Discussion

Hypericum perforatum deserves attention not only for its pharmaceutically important metabolites but also for its remarkable evolutionary and adaptive capacities. This species has evolved into an extremely successful widespread weed worldwide as well as in Iran (Riazi et al., 2011). A major characteristic of this success appears to be reproductive versatility coupled with genomic plasticity (Mayo and Langridge, 2003). To improve *H. perforatum* and produce homogeneous plant products, a good knowledge about population structure and diversity is required.

The analysis of genetic diversity and relatedness between or within different species and populations is a central task in genetics (Tanksley and McCouch, 1997). Molecular markers have been used to investigate the genetic diversity, population structure, and reproductive biology of *H. perforatum* (Arnholdt-Schmitt, 2000; Halušková and Košuth, 2003; Barcaccia et al., 2006; Percifield et al., 2007). However, due to the lack of a specific marker system for these plants, most of the studies used marker systems such as RAPD and ISSR. In the present work, we took advantage of the ubiquity and abundance of LTR retrotransposons in plant genomes and their role in genomic diversification to develop and apply retrotransposon markers based on the IRAP method for the first time to *H. perforatum*. Retrotransposon based markers are a powerful molecular tool, but these markers are not readily available, due to the difficulty in obtaining species-specific retrotransposon primers for *H. perforatum*. Therefore, the present study investigated the transferability of retrotransposon primers derived from barley to analyze genetic diversity and population structure in wild populations and cultivars of *H. perforatum*. The primers generated specific amplification patterns showing the universal applicability of this marker type. Five out of seven primers designed based on barley retrotransposon families produced multiple bands in *H. perforatum* genomic DNA when used individually in IRAP.

Transferability of barley retrotransposon primers across other plants has been reported in several studies. Teo et al. (2005) successfully used the same retrotransposon primers for the identification and characterization of banana cultivars and classification of *Musa* genome constitutions. Alavi-Kia et al. (2008) used seven long terminal repeat (LTR)-retrotransposon primers derived from barley for analysis of genetic diversity and phylogenetic relationships in Iranian species of the genus *Crocus*. Fifteen out of 28 possible LTR primer combinations successfully amplified fragments from the *Crocus* genome. In lemon balm (*Melissa officinalis* L.), the same seven primers designed based on LTRs of barley retrotransposons and their combinations were successfully used to amplify DNA fragments from wild Iranian populations as well as two populations from Germany and Japan based on the IRAP technique (Ghaffariyan et al., 2011). In our study, five out of seven barley retrotransposon primers individually or in combination amplified DNA fragment from the *H. perforatum* genome (78.15% polymorphism) (Table 3). However, the same primers produced higher levels of polymorphism in *Musa* (Teo et al., 2005), *Crocus* (Alavi-Kia et al., 2008), and *Melissa* (Ghaffariyan et al., 2011). Amplification based on primer combinations indicated the integration of retrotransposons in tail-to-tail, head-to-head, and head-to-tail orientations in *H. perforatum*. Retrotransposon markers from other sources have been applied in various plant species (Kalendar et al., 1999; Manninen et al., 2000; Baumel et al., 2002; Boyko et al., 2002; Branco et al., 2007; Du et al., 2013).

High levels of genetic differentiation along with low estimates of genetic similarities were observed especially between wild populations and cultivars. It is also evident from both neighbor-joining clustering and PCoA that the cultivars and wild population of *H. perforatum* were only partially separated. However, there are multiple instances where populations from different geographical regions were more closely associated. Additionally, the contribution of 58% of the total amount of molecular genetic diversity observed by among-population difference is indicative of low levels of gene flow between populations. High among-population variation was previously reported in *Hypericum* species by Percifield et al. (2007), Pilepić et al. (2008), and Farooq et al. (2014). High differentiation

among populations is mostly coupled with limited gene flow among them. The low gene flow and the high differentiation among populations has been explained mainly by founder events such as time since colonization (Jacquemyn et al., 2004), number of initial founders in populations, and their reproductive and dispersal potentials (Coleman and Abbott, 2003). Meirmans and van Tienderen (2004) documented that the clonal diversity within a population reflects the sexual genetic pool from which the clones originated, the frequency of clonal origin, and the somatic mutations that subsequently accumulate in established clones. The diversity arising from the clonal origin is directly dependent on reproductive mode, and hence varying degrees of apomixis between *H. perforatum* landraces may have a significant impact on the diversity of local populations.

In conclusion, the results of this study confirmed the transferability of retrotransposon primers derived from plant species for genome analysis in distantly related lineages. This is the first report on the genetic profile of *H. perforatum* originating from Iran. The level of genetic diversity revealed in this study indicated the potential of *H. perforatum* germplasm for exploitation in breeding strategies. Knowledge generated on the genetic diversity and population structure of *H. perforatum* is of prime interest and will facilitate the selection of suitable populations for future breeding programs of this plant. In addition, this information could be utilized in the development of hybrid genotypes adapted to different environments.

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