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Assessment of relationships among and within *Helichrysum* Mill. (*Asteraceae*) species by using ISSR markers and morphological traits

Narjes Azizi[1], Masoud Sheidai[2], Valiollah Mozaffarian[3], Mitra Arman[4] & Zahra Noormohammadi[5]

Key words: *Helichrysum,* ISSR Marker, Morphology, Population, Iran.

Ključne besede: *Helichrysum,* ISSR markerji, morfologija, populacija, Iran.

Abstract

This study conducted to determine relationship among and within Iranian *Helichrysum* species (Asteraceae). In this study based on ISSR markers, the highest percentage of ISSR loci polymorphism (54.7%) occurred in *H. armenium*. The highest gene diversity over loci (1.224), Shannon's Information Index (0.224%) and Expected Heterozygosity (0.142%) occurred in *H. armenium* (0.18) and the lowest of these parameters (0%) were observed in *H. araxinum*, *H. graveolens*, *H. persicum* and *H. psychrophilum*. The highest genetic similarity occurred between *H. armenium* and *H. rubicundum* (0.989), while the lowest was between *H. polyphyllum* and *H. graveolens (0.213)*. The analysis of molecular variance (AMOVA), showed significant genetic variation among (24%) and within (76%) species. In morphological analysis traits such as indumentum, resting bud, achene length, achenial papillae, dimension of receptacle and form and apex of phyllaries were main diagnostic features. Results obtained from the morphological cluster were greatly consistent with the molecular data, to elucidating taxonomic relationships, as well as both attributed the higher diversity in *H. armenium* and *H. rubicundum* in comparison with other species and also indicated that *H. persicum* is a member of *H. oocephalum* species. Totally we confirmed the presence of 18 species in Iran.

Izvleček

V raziskavi smo obravnavali taksonomske razlike med vrstami in znotraj vrst rodu *Helichrysum* (Asteraceae) v Iranu. V raziskavi na podlagi ISSR markerjev smo pokazali, da je bil največji delež ISSR polimorfnih lokusov (54,7%) v populacijah *H. armenium*. Največja genska raznolikost lokusov (1,224), Shannonov indeks (0,224%) in pričakovana heterozigotnost (0,142%) je bila pri vrsti *H. armenium* (0,18) in najmanjši (0%) pri vrstah *H. araxinum*, *H. graveolens*, *H. persicum* in *H. psychrophilum*. Največjo genetsko podobnost smo opazili med vrstama *H. armenium* in *H. rubicundum* (0,989), medtem ko je bila najmanjša med vrstama *H. polyphyllum* in *H. graveolens* (0,213). Analiza molekularne variance (AMOVA) je pokazala značilno genetsko variabilnost med (24%) vrstami in znotraj (76%) vrst. V morfološki analizi tega rodu so bili najpomembnejši diagnostični znaki dlakavost, speči brsti, dolžina rožke, papile na rožki, velikost socvetišča, oblika in vrh ovojkovih listov. Rezultati klastrske analize morfoloških znakov so bili v skladu z molekularnimi analizami. Obe analizi sta pokazali veliko raznolikost vrst *H. armenium* in *H. rubicundum* v primerjavi z ostalimi in da vrsta *H. persicum* v bistvu pripada vrsti *H. oocephalum*. Skupaj smo v Iranu potrdili 18 vrst iz rodu *Helichrysum*.

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1 Forest and Rangeland Department, Khorasan Razavi Agricultural and Natural Resources Research and Education Center. AREEO. Mashhad, Iran. E-mail: n.azizi@areeo.ac.ir

2 Faculty of Biologia, Shahid Beheshti University, Tehran, Iran.

3 Research institute of Forests and Rangelands. AREEO, Tehran, Iran.

4 Department of biology, Payamenoor University, P. O. Box 19395-3697, Tehran, Iran.

5 Department of Biology School of Science, Tehran, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Introduction

Helichrysum Mill (Gnaphalieae) is one of the largest genera in Asteraceae with about 600 species in the world (Anderberg 1991, Bayer et al. 2007). It includes a great diversity of life forms, from annual plants to trees, although most of the species are sub shrubs or shrubs. The genus is characterized as having homogamous or heterogamous capitula, generally with hermaphrodite florets outnumbering female florets; phyllaries with a fenestrated stereome; a flat, generally smooth or toothed receptacle; cypselae glabrous or covered with duplex or twin hairs; and a pappus that is monomorphic and usually uniseriate, consists of several scabrid to plumose bristles, and is free at the base (Hilliard & Burtt 1981). Even though the highest biodiversity of *Helichrysum* is in the African continent and Madagascar (Anderberg 1991), but also ~45 of them distributed in the Mediterranean, European, western Asian, and central Asian, that are morphologically characterized as having the following traits: homogamous or heterogamous capitula, with hermaphroditic flowers outnumbering the pistillate ones; phyllaries with a fenestrated stereome; smooth or alveolate receptacle; cypselae glabrous or with duplex hairs; monomorphic uniseriate pappus, consisting of several free scabrid bristles, with patent cilia at the base.

According to Flora Iranica (Georgiadou et al. 1980) 19 species are distributed in Iran, that seven species of them are endemic.

Helichrysum is a large and taxonomically difficult genus (Galbany et al. 2009). Because of extreme morphological variation, plant identification is often obscured by high inter and intra -population variation (Puglia et al. 2016). Thus evaluation of variation within taxa and populations is necessary, and it is one of the main aims of the present study.

In this genus the relationships and infrageneric classification have remained largely unresolved. Morphological approach provided essential tools for estimating the plasticity of the species within this genus (Galbany-Casal et al. 2006a, Salmeri et al. 2014). Some studies concerning molecular markers have provided insights into phylogenetic relationships, by using nrDNA ITS and chloroplast psbA-trnH sequences and DNA ndh F sequence variation (Galbany et al. 2004, 2009, 2014, Smissen et al. 2011) and amplified fragment length polymorphism (W. Sabetta 2006, Scialabba et al. 2008, Galbany et al. 2012, 2014). Other type of DNA markers which are used for genetic analysis in *Helichrysum* species include, express sequence tag-polymerase chain reaction (ETS-PCR) that is used to evaluate phylogenetic relationships in *Helichrysum* and related genera (Galbany et al. 2014).

In the present study, we used ISSR marker that has not been reported with *Helichrysum* except in three *Helichrysum* species in Iran and seven species in Sicilian *Helichrysum* (Azizi et al. 2014, Taban et al. 2015, Puglia et al. 2016). The main aims of this study were (1) to investigate the genetic relationships within Iranian *Helichrysum* using molecular data, (2) to identify diagnostic morphological traits which are useful for the classification of genera, and (3) to assess the relationships among and within *Helichrysum* species.

Material methods

Plant material

In this work, samples of 101 populations of 19 species of *Helichrysum* genus were studied. In morphological section, 87 populations contain: 1 population (2 individuals) of *H. araxinum* Takht. ex Kirpt., 18 populations (49 individuals) of *H. armenium* DC., 3 populations (8 individuals) of *H. artemisioides* Boiss & Hausskn., 2 populations (7 individuals) of *H. aucheri* Boiss., 3 populations (8 individuals) of *H. davisianum* Rech. f., 3 populations (8 individuals) of *H. glanduliferum* Schultz-Bip., 4 populations (10 individuals) of *H. globiferum* Boiss., 3 populations (6 individuals) of *H. graveolens* (M. B.) Sweet., 5 populations (23 individuals) of *H. leucocephalum* Boiss.,5 populations (14 individuals) of *H. makranicum* (Rech. F. & Esfand.) Rech. f., 3 populations (9 individuals) of *H. oligocephalum* DC., 7 populations (18 individuals) of *H. oocephalum* Boiss., 3 populations (8 individuals) of *H. pallasii* (Spreng.) Ledeb.,1 populations (2 individuals) of *H. persicum* Ghahremani & Noori, 3 populations (8 individuals) of *H. plicatum* DC., 1 populations (3 individuals) of *H. polyphyllulm* Ledeb., 5 populations (13 individuals) of *H. pseudoplicatum* Nab., 3 populations (9 individuals) of *H. psychrophilum* Boiss., 14 populations (44 individuals) of *H. rubicundum* (C. Koch.) Bornm.

The localities in which these were collected are detailed in Table 1. 66 populations were collected by us from different parts of Iran between 2010–2012 and are kept in the Herbarium Shahid Beheshti University (HSBU) and 34 populations are based on specimens from Research institute of forests and rangelands (TARI) herbarium.

Morphometric analysis

Based on material and literature data, 36 morphological traits were investigated for 249 individuals of 87 populations. Of the traits incorporated in the analysis,

28 were quantitative and 8 were qualitative (Table 3), totally only eight of them corresponded to vegetative features and the remaining traits being concerned with the synflorescence, capitula or florets. Indumentum, involucral bracts, florets and pappus were examined under a Zeiss Stemi DV4 binocular stereoscopic microscope. For the quantitative traits, the mean of measurements per specimen was used in the analyses. As well as these traits were scored at population level for the joint populations both used in molecular and morphological analyses, (the mean of three measurements per specimen was used in the analyses). Measurements were taken with a precision of 0.1 mm. The quantitative morphological traits were divided in to discrete groups and along with qualitative traits were coded as binary or multistate traits.

UPGMA (Unweighted Paired Group using Average mean), NJ, WPGMA clustering methods with 100 times bootstrapping as well as principal components analysis (PCA), principal coordinate analysis (PCoA) and multidimensional scaling (MDS) were performed to group the plants specimens based on morphological traits. The Euclidean distance was used for clustering methods. Cophenetic correlation was determined to check the fit of dendrograms to the original distance matrix (Podani 2000). Data analyses were performed by using PAST ver. 2.17 (Hamer et al. 2012).

ISSR Analyses

A total, 64 populations of 18 species (Table 1) were used for DNA extraction by using CTAB-activated charcoal protocol (Križman et al. 2006). We used 2–3 randomly collected plant specimens for any population. ISSR assay DNA was extracted from silica-gel dried leaves collected in the field or from herbarium material.

The extraction procedure was based on activated charcoal and Polyvenyl Pyrrolidone (PVP) for binding of polyphenolics during extraction and on mild extraction and precipitation conditions. This promoted high-molecular weight DNA isolation without interfering contaminants. Quality of extracted DNA was examined by running on 0.8% agarose gel.

The ISSR bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). Genetic diversity parameters were determined in each species. These parameters were percentage of allelic polymorphism, allele diversity (Weising 2005), Nei's gene diversity (*H*), Shannon information index (*I*) (Weising 2005, Freeland et al. 2011), number of effective alleles and percentage of polymorphism. The genetic divergence of the studied populations was checked by principal coordinate analysis provided and after 999 permutations.

Model-based clustering as performed by STRUCTURE software ver. 2.3 (Pritchard et al. 2000), was carried out to group the studied populations based on genetic affinity. This program was also used to reveal the genetic admixture of the studied populations.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as performed in GenAlex 6.4 (Meirmans & Van Tienderen, 2004), was used to show the molecular difference among the studied populations.

Results

Morphological comparison

In order to define relationship among 19 *Helichrysum* species based on comparison of morphological features, 36 morphological traits were evaluated. Based on the Euclidian distance, a taxonomic distance matrix of those measured quantitative and qualitative traits using WPGMA method was calculated (not shown), and employed for dendrogram construction. The individuals collected here were grouped into twelve clusters with single to multiple species at a Euclidean distance of 0.96. The first cluster consisted of *H. makranicum* separated from all other species with brown wish pigmentation of tip of middle involucral bracts, corolla lobes recurved, lack the basal leaf and resting bud. The second cluster consisted of the populations of *H. psychrophilum* and *H. pallasii.* Third cluster included populations of *H. artemisioides* and *H. davisianum* differ from other species in having traits as achene length, branches stem and the shape of basal and middle leaf. *H. polyphyllum, H. pseudoplicatum* and *H. plicatum* specimens with common morphological trait as phyllaries whit longitudinal plicate and presence of buddy vertical prop separated from other species placed in forth cluster. Fifth cluster included the *H. graveolens* populations with a close relationship to fourth cluster. Sixth cluster composed of *H. leucocephalum* that differs from other species in having recurved to behind involucral bracts. Seventh cluster composed of individuals of two populations of *H. rubicundum*. Eighth cluster composed of two subclusters: Oshnavieh population of *H. glanduliferum* based on yellow multi cellular achenial papillae as the diagnostic trait – comparison to other populations – with an individual of Miab population of *H. globiferum* species formed first subcluster and second subcluster that divided into two subclusters: populations of *H. oligocephalum* with two individuals of Miab population of *H. globiferum* placed in first subcluster, and *H. araxinum* with populations of *H. oligocephalum*, *H. armenium* and *H. glanduliferum* together formed second subcluster. In continues, Azerbaijan and Razan populations of *H. oligocephalum* with a close

relationship to *H. armenium* of Semirom formed ninth cluster. The Makoo population – except to one individual – and an individual of Kaleibar population of *H. rubicundum* with close relationship with Gasemlou and Naghadeh populations of *H. aucheri* along with Marand population of *H. armenium* are placed together in tenth cluster. Eleventh cluster was complex with 4 species that were consisted of: *H. oocephalum* and *H. persicum* with 34 common morphological traits – except to type and shape of lamina tip of middle involucral bracts – along populations of *H. rubicundum* and *H. armenium*. Twelfth cluster had divided into three subcluster, that *H. rubicundum* and an individual of Naghade population of *H. aucheri* plasted in first subcluster, an individual of Givi population with Kaleibar, Peygham and Tabriz populations of *H. rubicundum* plasted in second subcluster. Finally individuals and populations of *H. armenuim*, *H. globiferum* and *H. rubicundum* formed three subclusters.

Principal component analysis showed that six components explained 68.2% of the total variation which is contributed by all traits of the study (Figure 1). The first component presented 15.6% of the variation in which the stem indumentum trait was predominant in the first component and contributed most of the total variation. The second component presented 15.46% of the variation in which, attenuated resting bud and its indumentum, middle and upper stem leaf indumentum, achenial papillae, achene length and receptacle dimension had the highest loadings. The third component explained 10.6% of the total variation and middle involucral bracts indumentum was dominant.

The two multivariate approaches, UPGMA and PCoA, are used in the analysis of genetic relationships within and among the sections and subgenera of *Helichrysum* which have produced comparable results generally.

ISSR Results

The ISSR technique is particularly powerful in discovering the genetic diversity of the genus when there is little information available for genetic characterization of species genomes. A total of 1145 PCR products were generated by 10 ISSR primers, that all of them were polymorphic. Each primer received bands from 76 (CA7AT) to 247 (CA7AC) with an average of 105 bands per primer within a size range from 100 (811) to 2000 bp (CA7AT). Among ISSR primers, seven primers revealed the amplified DNA fragments that were unique to twelve *Helichrysum* species (Table 2). These ISSR primers amplifying species-specific DNA fragments would provide molecular tools for *Helichrysum* species authentication. We identified many putative species-specific markers.

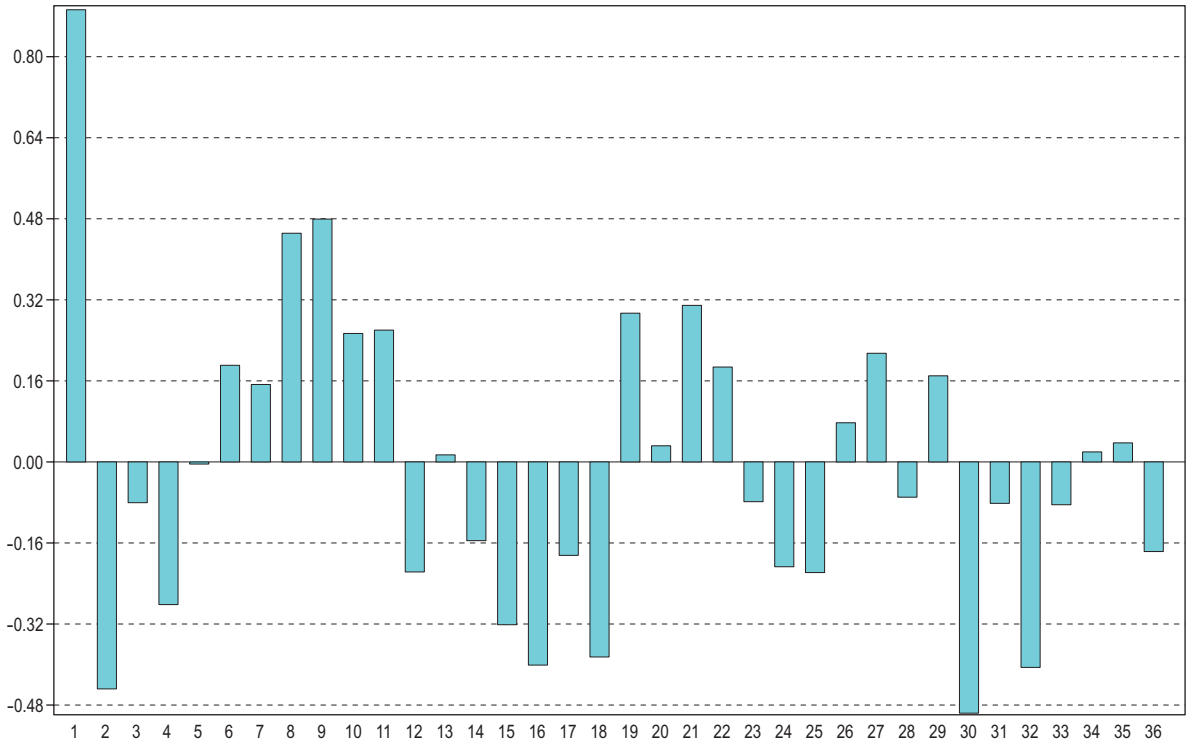

Figure 1: Contribution of the variables to component 1 (PC1).
Slika 1: Prispevek spremenljivk k prvi komponenti (PC1).

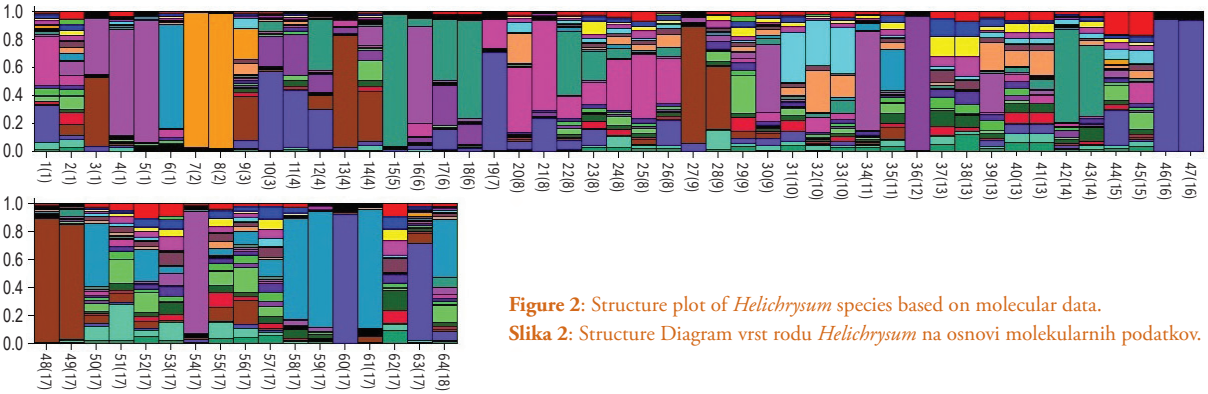

Figure 2: Structure plot of *Helichrysum* species based on molecular data.
Slika 2: Structure Diagram vrst rodu *Helichrysum* na osnovi molekularnih podatkov.

The highest gene diversity over loci (1.224), Shannon's Information Index (0.224%) and Expected Heterozygosity (0.142%) occurred in *H. armenium* (0.18) and the lowest of these parameters (0%) were observed in *H. araxinum*, *H. graveolens*, *H. persicum* and *H. psychrophilum*. The highest genetic similarity occurred between *H. armenium* and *H. rubicundum* (0.989), while the lowest was between *H. H.polyphyllum* and *H. graveolens (0.213)*. The analysis of molecular variance (AMOVA), showed significant genetic variation among (24%) and within (76%) species the studied. The admixture estimates from STRUCTURE (Figure 2) show high degrees of gene exchange among populations.

Cluster analysis based on the ISSR genotyping profiles

Genetic similarity for populations ranged from 0.80 to 0.99 (Table 2). The highest genetic similarity occurred between *H. armenium* and *H. rubicundum,* while the lowest was between *H. polyphyllum* and *H. graveolens*.

A dendrogram was constructed to infer phylogenetic relationships by using NJ method, the total number of amplified ISSR fragments was considered among 64 populations of 18 *Helichrysum* species and it was consisted of six well-supported distinct clusters (Figure 3).The first cluster was consisted of eight species: *Helichrysum pallasii* and *H. psychrophilum* were placed close together, *H. davisianum*, *H. artemisioides* and *H. makranicum*, *H. oocephalum* and *H. persicum* along an individual of 13 km Naghadeh population of *H. rubicundum* were placed in this cluster. The second cluster was mostly combined with 10 species that were sub grouped into two subclusters: first subcluster formed with five species: *H. rubicundum*, *H. armenium*, *H. oligocephalum H. plicatum*, and *H. leucocephalum* and second subcluster with five species: *H. plicatum*, *H. pseudoplicatum*, *H.graveolens, H. glandoliferum* and *H. globiferum*. The third Cluster was divided into two subclusters: The populations of *H. makranicum*, *H. artemisioides*, *H. araxinum*, *H. pseudoplicatum* and *H. polyphyllum* Formed first subcluster and populations of *H. globiferum* were placed in second subcluster.

Figure 3: NJ tree of molecular data in *Helichrysum* populations.
Slika 3: Drevo NJ molekularnih podatkov v populacijah rodu *Helichrysum*.

PCoA

The principal coordinate analysis (PCoA) was performed to specify the association between populations in more detail. The overall separation pattern of the samples in PCoA plot, were in agreement with NJ molecular dendrogram (Figure 4).

The distribution pattern of many populations such as: *H. rubicundum*, *H. armenium*, *H. globiferum* populations according to ISSR dendrogram and PCoA analysis were confirmed by hierarchical AMOVA substantially, showing that 80% of the overall variation was attributed to variation within each species, whereas only 20% was due to variation among different species.

A dendrogram based on the combined (Figure 5) ISSR marker and morphology data from 61 populations was constructed in order to generate more robust results. Totally four major clusters were formed, the first comprising the *H. glanduliferum*, populations of *H. armenium* and *H. globiferum.* Second cluster included two subgroups: one was included the populations of the *H. globiferum*, the second subgroup was included *H. plicatum* and *H. pseudopolicatum* with a close relationship with *H. Pseudoplicatum.*

The third cluster was made up from four subclusters: a population of *H. armenium* was the outer of the other subgroups, Second subcluster was included *H. armenium* and a population of *H. globiferum.* The third subcluster was formed from *H. leucocephalum* and fourth subcluster was consisted of *H. armenium* populations.

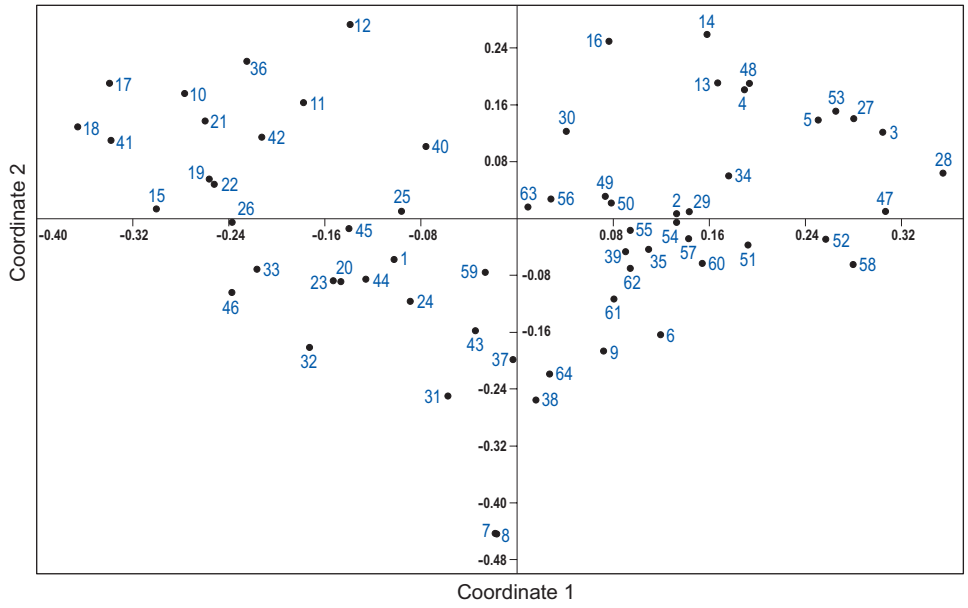

Figure 4: PCoA plot of *Helichrysum* species based on morphological data.
Slika 4: Diagram PCoA morfoloških podatkov vrst rodu *Helichrysum.*

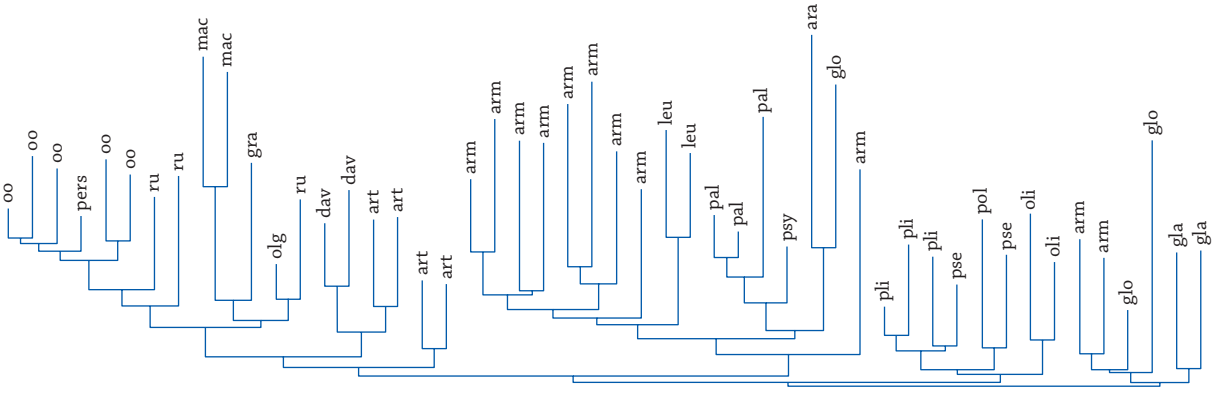

Figure 5: NJ tree based on the combined ISSR marker and morphology data.
Slika 5: Drevo NJ na osnovi kombinacije ISSR markerjev in morfoloških podatkov.

Fourth cluster was divided into five subclusters: a population of *H. armenium* with *H. araxinum* wich was placed in first subcluster. *H. artemisioides* and *H. davisianum* have formed second subcluster. A population of *H. rubicundum* and *H. globiferum* were placed in third subcluster. *H. makranicum* and *H. graveolens* have formed fourth subcluster and in continues *H. oocephalum*, *H. persicum* and two populations of *H. rubicundum* were included at fifth subcluster.

These species were separated clearly by the principal coordinate analysis and were corresponded largely to those which were obtained through combined cluster analysis.

These data demonstrated the ISSR technique was a valuable molecular method for the authentication of medicinal plants at genomic DNA level.

Discussion

In this study, we used morphological and molecular traits in order to elucidate the systematic relationships in *Helichrysum* species. Some of morphological qualitative and quantitative traits were highly reproducible and informative for species identification and classification in Iranian *Helichrysum* plants.

For example: the absence of basal leaf and resting bud and the shape of corolla lobes traits (Discriminate *H. makranicum* from the other species) is the imported have great taxonomic value which distinguishes the *H. makranicum* from the other species. As well as basal leaf width trait is very useful for distinguishing certain *H. davisianum* and *H. polyphyllum* of other spesies.

Many authors, such as Behnke (1984), Güemes et al. (1992), Servettaz et al. (1994), Bini Maleci et al. (1995) and Mráz (1998) emphasize the great value of trichomes in modern taxonomy. The two *Helichrysum* species (*H. coralloides* and *H. parvifolium*) that both possess many unique trait states were distinguished from each other in terms of the indumentum trait (Mckenzie et al. 2014).

Traits such as stem indumentums and basal and middle leaf indumentums which are observed for Iranian *Helichrysum* - except in *H. armenium* and *H. rubicundum* which this trait is very variable- and is particularly useful for distinguishing species.

Totally, the trichomes found on rest bud, stems, leaves and phyllaries all species were very helpful in distinguishing species. Also, it varies considerably in *H. rubicundum* and *H. armenium* species with ranges which often overlap among these taxa. However, it remains trait as a very useful trait for distinguishing species.

Phyllaries are always densely or laxly arranged imbricate in outer, medial and inner rows of *Helichrysum* species, that the type of imbricate is helpful trait in distinguishing species in *Helichrysum* sect. *stoachadina* and Sicilian *Helichrysum* (Galbany et al. 2011, Puglia et al. 2016). It remains as a very useful trait for distinguishing certain pairs of taxa that lack any other distinctive feature: for example, it is useful to distinguish the Sicilian specimens of *H. italicum* from the remaining specimens of *H. italicum* subsp. *italicum*. In our study shape and indumentum traits of phyllaries traits were also helpful in distinguishing species.

The Ratio of Innermost phyllaries length to middle phyllaries length trait is helpful in distinguishing species of *H.* sect. *Stoechadina* from *H.* sect. *Virginea* (Galbany-Casals et al. 2011, personal observation).

The thickness of stem trait in *H. davisianum*, *H. artemisioides* and *H. polyphyllum* is the imported taxonomic value which distinguishes them from the other species. However, stem thickness can be very variable in *H. armenium, H. rubicundum, H. aucheri* and *H. globiferum* too.

Among quantitative traits, receptacle dimension and achene length are valuable for distinguishing species from each other. The achenial papillae trait is used for distinguishing *Helichrysum* species (Galbany-Casals et al. 2011, personal observation). We observed types of achenial papillae are consisted of yellow/white duplex hair and yellow/orange multi cellular biseriate glandular hairs on the achane surfaces of *Helichrysum* species, that was important in species characterization. The yellow duplex hairs were present on the achane surfaces of *H. davisianum* and in *H. artemisioides*, were mixed with duplex hairs, while the white duplex hairs were present on the achane surfaces of other species. A population of *H. glanduliferum* has yellow/ orange multi cellular biseriate glandular hair.

Some results obtained were not in agreement with the literature. In our analyses, *H. graveolens* have attenuated resting bud, whereas in Flora Iranica (Rechinger et al. 2009), they always lack resting bud or stable resting bud.

In Flora Iranica, absence or presence of lateral stem and the number of involucre bracts are very diagnostic traits for distinguishing *H. artemisioides* of *H. davisianum.* But in our observation, both species overlap in terms of these trait. *Helichrysum artemisioides* is closely related to *H. davisianum*, they share 32 qualitative and quantitative traits, both having a similar habit, slim and branching stems, narrow basal and stem leaf, turbinate capitula with linear phyllaries and long achene with abtuse pappus hairs apical cell. However, they were different in terms of some features, since *H. artemisioides* specimens are usually glandular, with oblanceolate or oblanceolate-spathulate basal leaf and whit glandular in part of tomentose inner involucral bracts, whereas *H. davisianum* specimens are

usually lanate or tomentose glandular, with linear basal leaf, tomentose or tomentose- glandular inner involucral bracts. But since they are very similar and closely related it remains quite difficult to identify valid qualitative morphological traits in order to distinguish these species. Flora Iranica accepted the existence of two species mainly based on two traits: *H. artemisioides* compared to *H. davisianum* specimens have branched stem and sometimes with single capitula but in our observation these traits overlap. Consequently, due to these reasons, we suggested that the *H. artemisioides* at the sub specific level under *H. davisianum,* although more information is required in order to conclude. The molecular and morphological studies showed that there is a high degree of variability in *H. rubicundum* and *H. armenium*.

Results of morphological and molecular cluster analysis showed that the separation was in good agreement with their botanical classification, and inter/ intra species relationships were greatly in agreement with affinities which are suggested in Flora Iranica (Georgiadou et al. 1980). For example: *H. artemisioides* and *H. davisianum* showed close relationships together. As well as *H. pallasii* is closely related to *H. psychrophilum*. In fact, they shared many common qualitative morphological traits, both of them are having tomentose indumentum big capitula size with sub hemispherical to hemispherical shape, and densely or moderatly imbricate phyllaries. Although, *H. psychrophilum* specimens and *H. pallasii* specimens can be usually separated by papuss bristles traits, as well as *H. psychrophilum* specimens are usually shorter and they have more ratios of innermost phyllaries length / outermost phyllaries length.

H. plicatum, H. pseudoplicatum and *H. polyphyllum* specimens were gathered together in one cluster due to presence of vertical buddy prop and longitudinal plicate of phyllaries. Although according to our molecular and morphological studies, *H. plicatum* and *H. pseudoplicatum* are generally more similar. They share many common features of leaf and flowers morphology and have the same stem indumentum trait. According to Flora of Turkey (Davis et al. 1975), these species are accepted at the sub species level under *H. plicatum.* In fact, these two species are different in some traits: *H. plicatum* exhibits much variability in leaf shape, dimension and indumentums, moreover this species has slightly larger capitula than *H. pseudoplicatum* and sometimes they have heterogamous capitula, whereas *H. pseudoplicatum* has always homogamus capitula. So in agreement with Flora Iranica, we believe that these taxa are different species.

H. polyphyllum differs in some qualitative and quantitative features such as: resting bud indumentums, stem thickness, involucrral bract imbricate, receptacle dimensions, basal leaf width and number of flower per capitula. *H. polyphyllum* species has ± glabrous resting bud whereas *H. plicatum* and *H. pseudoplicatum* have tomentose or lanate resting bud, *H. polyphyllum* usually is taller with ticker stem, dense involucral bract, with wider basal leaves and larger receptacle and it has the most number of flower, although, according to Flora Iranica some overlap traits, are in disagreement with the species affinities suggested in Flora orientalis, according to molecular and morphological traits, we concluded that exact separation is clear between them.

Some results which obtained were not in agreement with the literature of Flora Iranica, for example: *H. rubicundum* with a wide and continuous distribution area, large populations is a variable taxon, particularly regarding the indumentum and the habit trait. There are ascendent or decumbent specimens with types of lanate, lanate – glandular, tomentose; tomentose in part glandular stem indumentums and, tomentose- lanate, tomentose- glandular and lanate- glandular indumentums on greenish leaves that usually have revolute margins, although leaves can be observed with flat margins. The yellow, orange, purple or red inner involucral bracts are convex oblanceolate or ovate. After studying numerous representative specimens, we believe that this variability reflects phenotypic plasticity.

Finally, while Flora Iranica describes the absence resting buddy prop as a diagnostic trait for *H. rubicundum*, on some specimens, we have observed that present them on vegetative stems. *H. rubicundum* species are founds in different regions of west, north and center parts of Iran, in a wide range of ecological conditions, while *H.aucheri* grows in small area of *H. rubicudum* niche, in western Azerbaijan. Both species are closely related together and are difficult to distinguish. In fact, they do not differ in any qualitative and quantitative trait, although these traits sometimes overlap. Flora Iranica describes these taxa and distinguishes *H. rubicundum* from *H. aucheri* as distinct species, based on several morphological traits: *H. rubicundum* having basal resting bud ovate, 6–9 mm diameter, oblanceolate basal leaf, convex and wide oblanceolate intermedial phyllaries with transverse compression, 34–36 flowers with 2.8 –3.6 mm long, achene 0.8–1.2 mm long with 22–23 obtuse pappus, and *H. aucheri* having basal resting bud elliptic or ovate, ±10–12 mm diameter, oblanceolate-spathulate basal leaf, flat and narrow lanceolate-spathulate intermedial phyllaries with truncate or rotundate apex, 43–48 flowers with 4–5 mm long, achene 1–1.5 mm long with 28–35 clavate pappus. But based on our study on numerous specimens, there are no unique traitistics for each of the species because these traitistics overlap and the separation between them is not

clear, except flowering time that *H. rubicudum* often is distinguishable due its involucres color.

Following these reasons, we combined *H. aucheri* at the sub specific level under *H. rubicundum.*

Ghahremaninejad et al. (2004) described a specimen of *Helichrysum* of Torbat Heydarieh as a new endemic species under the name *H. persicum.* They stated, *H. persicum* is closely related to *H. artemis*ioides and *H. davisianum* in terms of having obconic capitula and differs from those species interms of having shorter and unbranched flowering stem, more bulky resting bud, wider inflorescence, shorter involucre, in a lower number of florets and pale yellow to brown phyllaries than those of *H. artemisioides* and more number of puppi than those of *H. davisianum.* But according to our analysis, *H. persicum is* more closely related to *H. oocephalum* with a wide and continuous distribution area in north, northeast and east parts of Iran, it is mostly a rather variable species whereas *H. persicum* consists of a specimen which is located in a single location. In addition to our own data which was collected from field observations, we weren't able to find any specimen of this taxon in its location.

In fact, they do not differ in any qualitative and quantitative trait, and both have a similar habit, basal resting bud, lanate- glandular stem indumentum, capitula with hermaphrodite flowers whit white duplex hair on achene surface. Anyway, all traits of *H. persicum* fits within the range of *H. oocephalum* morphological variability -for example: hemispherical to sub hemispherical or (±) turbinate. Following these reasons and molecular data, we considered *H. persicum* as a specimen of *H. oocephalum.*

Conclusions

In Iran, some of *Helichrysum* taxa have a wide distribution area with a common ecological niches and form populations together, that are not clearly recognizable from each other. Thus phenotypic plasticity is an explanation for their morphological variation, also there may be a genetic component regarding the morphological differences.

A high degree of similarity was observed between morphological and molecular clusters, it were in good agreement with Flora Iranica. According to both data*: H. artemisioides*, *H. davisianum* and *H. makranicum* have close relationship. So *H. graveolens*, *H. polyphyllum*, *H. pseudoplicatum* and *H. plicutum* were placed close together, H. *psychrophilum* and *H. pallasii* confirmed a close relationship and in the following, close relationship between *H. leucocephalum*, *H. globiferum* and *H. rubicundum* was in good agreement with their botanical classification.

Although, results of morphological cluster analysis showed an unclear delimitation between specimens and populations of *H. armenium*, *H. rubicundum* and *H. globiferum*, according to molecular data, this disorder was observed in *H. armenium*, *H. rubicundum* and *H. oligocephalum*. These species mostly overlap in distribution area and analysis showed sharing gene between them – according to STRUCTURE – this may be as a result of hybridization to which is in agreement with Taban et al. (2015).

Our results showed high level of variability at both morphological and molecular levels. However, when molecular markers and quantitative traits were analyzed in the same plant material, more conclusive information was obtained.

Masoud Sheidai, https://orcid.org/0000-0003-3983-6852
Zahra Noormohammadi, https://orcid.org/0000-0003-3890-9001

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Table 1: Locality of *Helichrysum* species and populations studied.
Tabela 1: Lokacije obravnavanih populacij vrst rodu *Helichrysum*.

No.	Species	Locality	Voucher No.
1	*H. araxinum**	West- Azerbaijan Makou	HSBU2012201
2	*H. armenium**	Hamadan: 12 km Asad - Abad	TARI47446
3	*H. armenium**	West Azerbaijan: Uroumia: Mavana	HSBU2012202
4	*H. armenium**	West Azerbaijan: Uroumia: Mamakan	HSBU2012203
5	*H. armenium**	West Azerbaijan: Uroumia: Solouk Wateroff	HSBU2012204
6	*H. armenium**	West Azerbaijan: Uroumia: Silvana	HSBU2012205
7	*H. armenium**	West Azerbaijan: 15 km - Oshnavieh	HSBU20122006
8	*H. armenium**	West Azerbaijan: 25 km Khoy - Chaldoran	HSBU2012207
9	*H. armenium*	East Azerbaijan: 2 km Tabriz - Ahar	HSBU2012208
10	*H. armenium*	East Azerbaijan: Marand	HSBU2012209
11	*H. armenium*	Kordestan: Marivan: Garan Col	HSBU2012210
12	*H. armenium**	Kordestan: Sanandaj: Ariz Col	HSBU2012211
13	*H. armenium**	Kordestan: Divan-dareh, Zagheh	HSBU2012212
14	*H. armenium**	Kordestan: Abidar Park	HSBU2012213
15	*H. armenium*	Hamadan: 10 km Asad - Abad	HSBU2012214
16	*H. armenium**	Hamadan: Asad - Abad: Aladagh	HSBU2012215
17	*H. armenium*	Hamadan: Heydareh	HSBU2012216
18	*H. armenium**	Hamadan: Kaboodar - Ahang	HSBU2012217
19	*H. armenium**	Esfahan: Semirom: Deh - Vanak	TARI62142
20	*H. artemisioides**	Chaharmahal - Bakhtiari: Lordegan	TARI66101
21	*H. artemisioides*	Kohkilooyeh: Sisakht	TARI
22	*H. artemisioides**	Arak: Mahalat	TARI37836
23	*H. aucheri*	West Azerbaijan: 15 km - Oshnavieh	HSBU2012218
24	*H. aucheri*	West Azerbaijan: Near Ghasemloo	HSBU2012219
25	*H. davisianum**	Yazd: Shirkooh	HSBU2012220
26	*H. davisianum**	Yazd: Damgahan	TARI77715
27	*H. davisianum*	Espahan: Semirum	TARI31670
28	*H. glanduliferum*	West Azerbaijan - Solouk Waterfall	HSBU2012221
29	*H. glanduliferum**	West Azerbaijan: 18 km Naghadeh - Oshnavieh	HSBU2012222
30	*H. glanduliferum**	West Azerbaijan: 35 km Naghadeh - Oshnavieh	HSBU2012223
31	*H. globiferum**	West Azerbaijan: Khoy - Chaldoran	HSBU2012224

No.	Species	Locality	Voucher No.
32	*H. globiferum**	East Azerbaijan: Payam Vilage	HSBU2012225
33	*H. globiferum**	East Azerbaijan: Miab	HSBU2012226
34	*H. globiferum**	Hamadan: Razan	HSBU2012227
35	*H. graveolens*	Golestan: Golestan Park	TARI12623
36	*H. graveolens**	Mazandaran: Lapasar	TARI21658
37	*H. graveolens*	Mazandaran: Javaher - Deh	TARI56835
38	*H. leucocephalum**	Fars: Abadeh - Tashk	HSBU2012228
39	*H. leucocephalum**	Fars: Arsanjan Bonab Park	HSBU2012229
40	*H. leucocephalum*	Fars: Neyriz, Palangan	HSBU2012230
41	*H. leucocephalum*	Fars: Morghak Valley	HSBU2012231
42	*H. leucocephalum*	Yazd: Damgahan	HSBU2012232
43	*H.makranicum**	Hormozgan: Bashagard, Angooran Vilage	TARI44366
44	*H.makranicum*	Hormozgan: Senderk, Darreh- Pahn	TARI39250
45	*H.makranicum**	Hormozgan: Senderk - Arakin	TARI44458
46	*H.makranicum*	Hormozgan: Bashagard	TARI 44431
47	*H.makranicum*	Hormozgan: Jakdan - Senderk	TARI 44371
48	*H. oligocephalum**	Kermanshah: Shahoo amount	HSBU2012233
49	*H. oligocephalum**	Tehran: Darakeh	HSBU2012234
50	*H. oligocephalum*	Alborz: Vardich	HSBU2012235
51	*H. oocephalum**	Khorasan: Esfarayen, Shah - Jahan	TARI48649
52	*H. oocephalum**	Khorasan: Kashmar - Neyshaboor	TARI35577
53	*H. oocephalum**	Khorasan: Neyshaboor, Boozhan	TARI48982
54	*H. oocephalum**	Khorasan: Mashad, Dizbad	TARI48907
55	*H. oocephalum**	Hormozgan: Senderk- Arakin	TARI44273
56	*H. oocephalum*	Hormozgan: Senderk	TARI44484
57	*H. oocephalum*	Hormozgan: Ghotbabad	TARI49923
58	*H. pallasii**	East Azerbaijan: Haris	TARI469
59	*H. pallasii**	West Azerbaijan: Miab	HSBU2012236
60	*H. pallasii**	Tehran: Shemshak	TARI49026
61	*H. persicum**	Khorasan: Robat sefid	TARI21312
62	*H. plicatum**	Alborz: Damavand	HSBU201237
63	*H. plicatum**	East Azerbaijan: Goli - Deragh	TARI34974
64	*H. plicatum**	West Azerbaijan: Uroumiah: Solouk	HSBU2012238
65	*H. polyphyllum**	West Azerbaijan: Uroumiah: Solouk	HSBU2012239
66	*H. pseudoplicatum**	East Azerbaijan: Mishoo - Dagh	TARI29836
67	*H. pseudoplicatum*	West Azerbaijan: Uroumiah: Mavana	HSBU2012240
68	*H. pseudoplicatum**	West Azerbaijan: Uroumiah: Razhan	HSBU2012241
69	*H. pseudoplicatum*	Semnan: Shali - Hikoo	TARI40583
70	*H. pseudoplicatum*	Kordestan: Chehel - Cheshmeh	TARI75306
71	*H. psychrophilum*	Alborz: Dmavand - Tar Lake	TARI49279
72	*H. psychrophilum*	West Azerbaijan: Mavana	TAR69894
73	*H. psychrophilum**	Mazandaran: Ramsar	TARI51214
74	*H. rubicundum*	East Azerbaijan: Aras River	HSBU2012242
75	*H. rubicundum*	East Azerbaijan: 15 km Tabriz	HSBU2012243
76	*H. rubicundum**	East Azerbaijan: 22 km Tabriz- Ahar	HSBU2012244
77	*H. rubicundum*	East Azerbaijan: 25 km toTabriz,	HSBU2012245
78	*H. rubicundum*	West Azerbaijan: Salmas	HSBU2012246
79	*H. rubicundum*	East Azerbaijan: 2 km Tabriz - Ahar	HSBU2012248
80	*H. rubicundum**	East Azerbaijan: Poldasht	HSBU2012249
81	*H. rubicundum*	Alborz: Damavand	HSBU2012250

No.	Species	Locality	Voucher No.
82	*H. rubicundum*	Zanjan: Soltanieh	HSBU2012251
83	*H. rubicundum*	Ardabil: Lahrood- ghotoor soo	HSBU2012252
84	*H. rubicundum**	West Azerbaijan: Kaleibar - Rend	HSBU2012253
85	*H. rubicundum*	East Azerbaijan: Kaleibar - Aras	HSBU2012254
86	*H. rubicundum*	43 km Ardabil - Givi	HSBU2012255
87	*H. rubicundum*	24 km Ardabil - Givi	HSBU2012256
88	*H. armenium*•	East Azerbaijan: Marand: Mishoodagh	HSBU2012258
89	*H. armenium*•	East Azerbaijan: East of Mishoodagh	HSBU2012259
90	*H. armenium*•	West Azerbaijan: Marand: Kiamaki - Dagh	HSBU2012260
91	*H. globiferum*•	West Azerbaijan: Marand: Kiamaki - Dagh	HSBU2012268
92	*H.makranicum*•	Sistan and Balouchestan: Jazmoorian	TARI22922
93	*H. oligocephalum*•	West Azerbaijan: Urumiah: Khalil - Kooh	HSBU2012269
94	*H. oligocephalum*•	Kordestan: Marivan - Sanandaj	HSBU2012261
95	*H. plicatum*•	West Azerbaijan: Ghasemloo Valley	HSBU2012257
96	*H. polyphyllum*•	West Azerbaijan: Urumiah: Silvana	HSBU2012264
97	*H. oocephalum*•	Hormozgan: Senderk - Darrepahn	TARI44485
98	*H. oocephalum*•	Khorasan: Mashhad: Shah - Taghi	HSBU2012263
99	*H. rubicundum*•	West Azerbaijan: Oshnavieh	HSBU2012265
100	*H. rubicundum*•	West Azerbaijan: 13 km Naghadeh - Oshnavieh	HSBU2012266
101	*H. rubicundum*•	West Azerbaijan: Urumiah: Silvana	HSBU2012267

Note: populations 1 to 87: populations included only in the morphological analyses, *: populations included in the morphological and molecular analyses, •: populations included only in the molecular analyses

Table 2: Genetic diversity parameters in *Helichrysum* species.
Tabela 2: Dejavniki genetske raznolikosti pri vrstah rodu *Helichrysum*.

Species	Na	Ne	I	He	UHe	P%
H.araxinum	0.021	1.000	0.000	0.000	0.000	0.00%
H. armenium	1.082	1.224	0.224	0.142	0.148	54.11%
H. artemisioides	0.356	1.107	0.091	0.062	0.083	15.07%
H. davisianum	0.233	1.063	0.054	0.037	0.049	8.90%
H. glandoliferum	0.342	1.107	0.091	0.062	0.083	15.07%
H. globiferum	0.918	1.208	0.212	0.135	0.149	45.89%
H. graveolens	0.110	1.000	0.000	0.000	0.000	0.00%
H. Leucocephalum	0.363	1.107	0.091	0.062	0.083	15.07%
H. makranicum	0.603	1.177	0.158	0.106	0.127	28.77%
H. oligocephalum	0.658	1.162	0.159	0.103	0.117	32.19%
H. oocephalum	0.699	1.104	0.123	0.073	0.078	34.25%
H. pallasii	0.247	1.065	0.059	0.039	0.047	10.96%
H. persicum	0.020	1.000	0.000	0.000	0.000	0.00%
H. plicatum	0.322	1.082	0.079	0.052	0.062	15.07%
H. polyphyllum	0.096	1.000	0.000	0.000	0.000	0.00%
H. pseudoplicatum	0.301	1.102	0.087	0.060	0.079	14.38%
H. psychrophilum	0.034	1.000	0.000	0.000	0.000	0.00%
H. rubicundum	0.726	1.147	0.154	0.096	0.105	36.30%

Abbreviations: Na = No. of different alleles, Ne = No. of effective alleles = $1/(p^2+q^2)$, I = Shannon's information index =–(p Ln (p)+ qLn (q)), He = Expected heterozygosity = 2pq, and %P = Percentage of polymorphism

Table 3: Traits for *Helichrysum* species.
Tabela 3: Znaki za vrste rodu *Helichrysum*.

01	Stem indumentum: 1- tomentose; tomentose in part glandular; 2- tomentose- glandular; 3- glandular; 4- lanate; 5- lanate- glandular
02	Stem form: 1- procumbent; 2: erect approximately erect
03	Thickness of stem: 1- thin; 2- medium; 3- thick
04	Leaf density: 1-dense; 2- moderate; 3- sparse
05	Attenuated resting bud: 1- present; 2: absent
06	Resting bud indumentums: 1- tomentose, lanate; 2- glandular; 3: ± glabrous
07	Basal leaf shape: 1- oblanceolate, oblanceolate-spathulate ; 2- liner
08	Middle stem leaves leaf indumentums: 1- tomentose, tomentose- lanate, tomentose- glandular; 2- Glandular in part lanate; 3- glandular; 4- lanat- glandular
09	Upper stem leaves indumentums: 1- tomentose, tomentose lanate, tomentose glandular; 2- glandular in part tomentose or lanate; 3- glandular
10	Inflorcense type: 1- ; 2- corymbose
11	Involucre shape: 1- turbinate or ± turbinate; 2- non turbinate
12	Heterogamy: 1- hermaphrodyt; 2- heterophrodyt; 3-hetero and hermafer
13	Involucrral bract color; 1- yellow; 2- white, cream; 3- orange, purple, red
14	Involucrral bract Imbricate: 1-dense; 2- ± moderate, 3-lax
15	Type of lamina tip of middle involucral bracts: 1- oblong- ovate; 2- linear; 3- oblong and recurved to behind
16	Middle involucral bracts indumentum: 1- tomentose- glandular or lanate- glandular, 2- ± glabrous; 3- glandular
17	Shape of lamina tip of middle involucral bracts: 1- acute; 2- obtuse
18	Shape of lamina of inner involucral bracts: 1- oblanceolate and convex;, ovate and convex; 2- linear; 3- long and recurved to behind
19	Inner involucral bracts indumentums: 1-tomentose,tomentose- glandular; 2- glandular in part tomentose; 3- glandular; 4- lanate- glandular
20	Leaf margin: 1-
21	Pappus hairs apical cell shape: 1- acute to sub acute to ; 2- obtuse to sub obtuse; 3-
22	Achenial papillae: 1- white duplex; 2- yellow/ orange duplex; 3- yellow/ orange multi cellular
23	Corolla lobes recurved: 1- erect; 2- recurved
24	Lateral stem: 1- present; 2- absent
25	Brown wish pigmentation in lamina/stereome gap of tip of Middle involucral bracts: (0) absent; (1) present.
26	Longitudinal plicate of phyllary: 1- present; 2- absent
27	Papuss bristles; 1- plumose; 2- smooth or ± smooth
28	Innermost phyllaries length / outermost phyllaries length
29	Basal leaf width (mm)
30	Achene length (mm)
31	Receptacle dimension (mm)
32	Pappus length (mm)
33	No. of flower per capitule (mm)
34	Resting bud width (mm)
35	Corolla length (mm)
36	Involucres width (mm)