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Genetic and morphological diversity in *Cousinia cylindracea* (Asteraceae) populations: Identification of gene pools

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Abstract. *Minaeifar AA, Sheidai M, Attar F. 2015. Genetic and morphological diversity in* Cousinia cylindracea *(Asteraceae) populations: Identification of gene pools. Biodiversitas 16: 288-294. Cousinia is* one of the largest genera in the Asteraceae family. It contains 600 to 700 species distributed in Southwest and Central Asia. In Iran with 270 species it is the largest genus after *Astragalus*,. *Cousinia* probably is unique in the degree of diversification of all its parts and high numbers of species in restricted area. In this investigation 90 plant specimens of 10 geographical populations of *Cousinia cylindracea* Boiss. were studied from morphological and genetic (ISSR) points of view. Both intra and inter-population morphological and genetic variability was observed in the studied populations. ANOVA and CVA tests revealed significant morphological difference among these populations. Similarly, AMOVA tests revealed significant molecular difference among geographical populations. Mantel test produced significant positive correlation between genetic distance and geographical distance of the studied populations. Networking, STRUCTURE analysis and population assignment test revealed low degree of gene flow among these populations. The results identified two different gene pools of *C. Cylindracea* in Iran, supporting Rechinger suggestion that *C. cylindracea* might have two varieties in Iran.

Key words: Asteraceae; *Cousinia*; genetic variability; gene pools; ISSR; morphological diversity

INTRODUCTION

Cousinia Cass. is one of the largest genera in the family Asteraceae and the largest genus in tribe Cardueae (Frodin 2004). It contains 600 to 700 species in Southwest and Central Asia. The distribution area of *Cousinia* is nearly identical with the Irano-Turanian region (Knapp 1987). In Iran, after *Astragalus*, *Cousinia* is the largest genus with over than 270 species and 43 sections. *Cousinia* species are distributed in mountainous parts of Iran. Some of the *Cousinia* species have medicinal values, and used as diuretic, antiseptic and antibacterial (Joudi et al. 2011).

Cousinia cylindracea Boiss., is distributed in more than 10 provinces of Iran from Alborz and Zagros ranges (Zar et al. 2012). It is a perennial herb, with decurrent stem leaves, and small head bearing a few florets (Rechinger 1979). Cytological study on *C. cylindracea* shows chromosome number of 2n=2x=26 (Djavadi 2012).

One of the main concerns of mankind is the conservation of biodiversity at present time. Increasing human population and more extensive use of natural resources and space, cause damage to plant biodiversity. To conserve biological diversity, we must first understand the distribution of organisms and how and why these organisms are geographically distributed as they are (Sigrist and Carvalho 2008). Population genetic investigation is one of the main steps for understanding the population genetic structure and fragmentation, inter-population gene flow and diversification (Sheidai et al. 2014).

Habitat fragmentation generally is expected to reduce genetic diversity and to increase inter-population genetic divergence by restricting gene flow among fragmented populations, increasing inbreeding and increasing random genetic drift within populations (Hou and Lou 2011). An examination of the genetic diversity among populations within a species is crucial for a better understanding of evolutionary processes and the nature of the species.

Cousinia cylindracea is a species that grow in different geographical regions of Iran and forms several local populations. Therefore, the present study was performed to identify the population genetic structure, gene flow and morphological diversity of *C. cylindracea* populations and to identify probable gene pools of this species in the country. This information can be used in conservation program of this plant species.

Molecular markers that have been used in plants' investigations in general, consist almost exclusively of markers deemed to be neutral or nearly neutral. These data have been used to study the speciation process, genetic diversity analysis as well as populations' genetic structure (Sheidai et al. 2014). We used ISSR (Inter simple sequence repeats) molecular markers for genetic diversity analysis, as these molecular markers are reproducible, easy to work, cheap and also provide useful data for evolutionary and population genetic studies (Sheidai et al. 2014).

MATERIAL AND METHODS

Plant materials

Extensive field visits and collections were undertaken during 2013-2014 from the North-West to South- West of Iran and several geographical populations were identified for *Cousinia* species including *C. cylindracea* (Figure 1). Many plant specimens were randomly collected from 10 geographical populations (Table 1). We used 90 randomly collected plant specimens for genetic and morphological studies. The fresh leaves were used for DNA extraction. The voucher specimens were deposited in Herbarium of Shahid Beheshti University (HSBU) and Central Herbarium of Tehran University (TUH).

Morphometry

Forty-seven morphological characters were studied (38 quantitative and 9 qualitative characters) such as: the basal, apical and stem leaves length and width, length and width and number of head, number and length of florets, seeds length and width, bracts shape and etc. (Table 2).

ISSR assay

DNA was extracted from the fresh leaves that were randomly collected from 9 plants in each population and dried in silica gel powder. The genomic DNA was extracted using CTAB-activated charcoal protocol (Murray and Thompson 1980). 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 1% Agarose gels.

Ten ISSR primers; (AGC)5GT, (CA)7GT, (AGC)5GG, (GA)9A, (GA)9C, UBC 807, UBC810, UBC 811, UBC 823 and UBC 834 commercialized by UBC (the University of British Columbia) were used. PCR reactions were performed in a 25µL volume containing 10 mM Tris- HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP ; 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of Taq DNA polymerase. Amplification reactions were performed in Techne thermocycler (Germany) with the following program: PCR was carried out with an initial denaturing step (94°C/5 min), followed by 45 cycles of 94°C/30 s, 52-58°C/30 s, 72°C/1 min, and a final incubation at 72°C for 10 min. the amplification products were visualized by running on 1.5% Agarose gel, followed by Ethidium bromide staining. The fragments size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany). The experiment was replicated 3 times and constant ISSR bands were used for further analyses.

Data analyses

Morphological studies

The analysis of variance (ANOVA) test was performed to show significant morphological difference among the studied populations. For grouping of the plant specimens, UPGMA (Unweighted paired group with arithmetic average) and CVA (Canonical variate analysis) were used. Morphological data were standardized (mean = 0, variance = 1) for these analyses (Podani 2000). Principal components analysis (PCA) was performed to identify the most variable morphological characters among the studied populations.

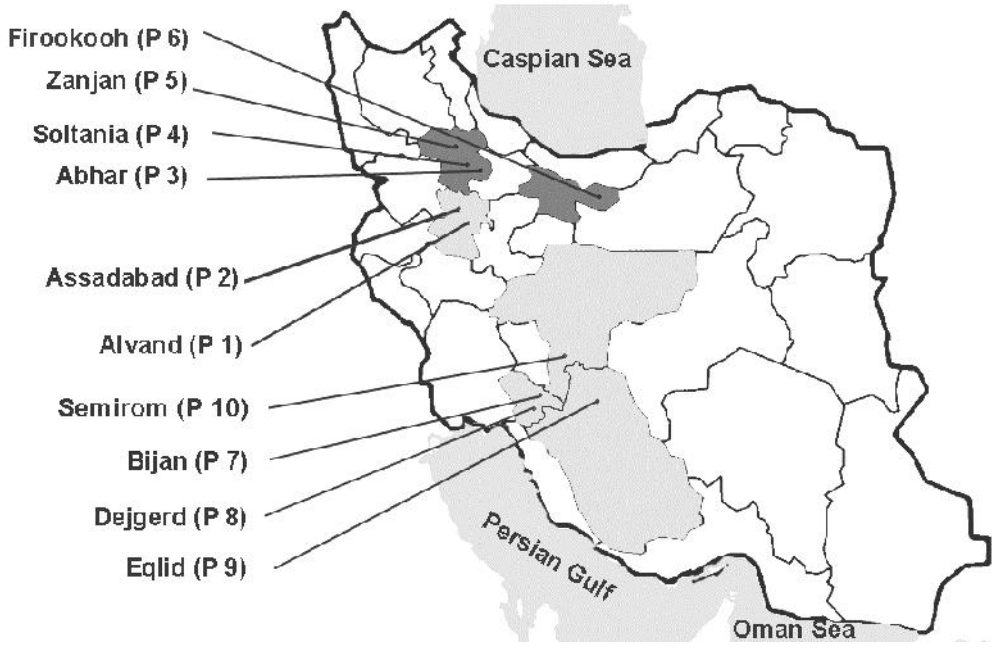

Figure 1. Distribution map of the studied *Cousinia cylindracea* populations in Iran

Table 1. *Cousinia cylindracea* populations and their localities

Pop.	Locality	Altitude (m)	Longitude	Latitude	Temperature (°C)	Rainfall (mm)
1	Iran: Hamadan, Alvand	2038	48° 26' 51"	34° 48' 07"	10	350
2	Iran: Hamadan, Asadabad Pass.	2150	48° 11' 45"	34° 49' 55"	9	400
3	Iran: Zanjan, Abhar	2145	48° 58' 57"	36° 07' 11"	10	350
4	Iran: Zanjan, Soltania	1975	48° 43' 03"	36° 22' 39"	9	350
5	Iran: Zanjan, Zanjan	1850	48° 22' 06"	36° 26' 30"	9	350
6	Iran: Tehran, Firoozkooh	2152	52° 32' 13"	35° 41' 06"	8	400
7	Iran: Kohgiluyeh and Boyer-Ahmad, Bijan pass.	2950	51° 30' 45"	30° 52' 21"	9	800
8	Iran: Kohgiluyeh and Boyer-Ahmad, Dejgerd	2335	51° 55' 49"	30° 39' 21"	10	700
9	Iran: Fars, Eqlid	2410	52° 36' 29"	30° 53' 34"	12	350
10	Iran: Isfahan, Semirom	2250	51° 48' 02"	31° 45' 53"	11	400

Table 2. Morphological characters in the studied populations

R	Characters	R	Characters
1	Stem length (mm)	25	Head width (mm)
2	Basal leaves length (mm)	26	Head length/width Ratio
3	Basal leaves width (mm)	27	Involucral bracts number
4	Basal leaves length/Width Ratio	28	Bracts seriate number
5	Basal leaves petiole length (mm)	29	Florets number
6	Basal leaves terminal spin length (mm)	30	Florets length (mm)
7	Basal leaves marginal spins length (mm)	31	Corolla limb length (mm)
8	Basal leaves teeth number	32	Corolla tube length (mm)
9	Stem leaves number	33	Limb length / tube length ratio
10	Stem leaves length (mm)	34	Receptacle bristles length (mm)
11	Stem leaves width (mm)	35	Seed number
12	Stem leaves length/width ratio	36	Seed length (mm)
13	Stem leaves terminal spin length (mm)	37	Seed width (mm)
14	Stem leaves marginal spins length (mm)	38	Seed length / Width Ratio
15	Stem leaves teeth number	39	Stem indumentum (0: Glabrous; 1: Low density; 2: Medium density; 3: High density)
16	Uppermost leaves number	40	Basal leaves form (0: Dentate; 1: Pinnatifid; 2: Pinnatisect)
17	Uppermost leaves length (mm)	41	Basal leaves indumentum (0: Glabrous; 1: Low density; 2: Medium density; 3: High density)
18	Uppermost leaves width (mm)	42	Stem leaves form (0: Dentate; 1: Pinnatifid; 2: Pinnatisect)
19	Uppermost leaves length/widthr	43	Stem leaves indumentum (0: Glabrous; 1: Low density; 2: Medium density; 3: High density)
20	Uppermost leaves terminal spin length (mm)	44	Uppermost leaves form (0: Dentate; 1: Pinnatifid; 2: Pinnatisect)
21	Uppermost leaves marginal spins length (mm)	45	Uppermost leaves indumentum (0: Glabrous; 1: Low density; 2: Medium density; 3: High density)
22	Uppermost leaves teeth number	46	Head indumentum (0: Glabrous; 1: Low density; 2: Medium density; 3: High density)
23	Head number	47	Bract shape (0: Spreading; 1: Curved)
24	Head length (mm)		

Molecular analyses

Genetic diversity and population differentiation. ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). Genetic diversity parameters were determined for dominant molecular markers in each population. These parameters were Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Freeland et al. 2011). Nei's genetic distance was determined among the studied populations and used for clustering. Significant genetic differences among the studied populations were determined by: 1-AMOVA (Analysis of molecular variance) test (with 1000 permutations) for dominant molecular markers as implemented in GenAlex 6.4 (Peakall and Smouse 2006), 2- Nei's Gst analysis of dominant markers as implemented in GenoDive ver.2 (2013) (Meirmans and Van Tienderen 2004). Furthermore, populations' genetic differentiation was studied by G'st_est = standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008).

In order to overcome potential problems caused by the dominance of ISSR markers, a Bayesian program, Hickory (ver. 1.0) was used to estimate parameters related to genetic structure (Theta B value) (Tero et al. 2003).

Grouping of the populations. For grouping of the plant specimens, we used Neighbor Joining (NJ) clustering and Neighbor Net method of networking after 100 times bootstrapping (Huson and Bryant 2006). The Mantel test was performed to check correlation between geographical distance and genetic distance of the studied populations (Podani 2000). PAST ver. 2.17 (Hamer et al. 2012), DARwin ver. 5 (Cirad 2012) and SplitsTree4 V4.13.1 (Huson and Bryant 2013) programs were used for these analyses.

Population genetic structure. The genetic structure of geographical populations was studied by two methods; First we carried out structure analysis (Pritchard et al., 2000), for dominant markers (Falush et al. 2007). Second, we performed K-Means clustering as done in GenoDive ver. 2. (2013). Model-based clustering was performed by STRUCTURE software ver. 2.3 (Pritchard et al. 2000). The Markov chain Monte Carlo simulation was run 20 times for each value of K (2-10) for 20 iterations after a burn-in period of 10^5. Evanno test was carried out to identify the proper number of K (Evanno et al. 2005). We used two summary statistics to present K-Means clustering; 1- pseudo-F (Calinski and Harabasz 1974) and 2- Bayesian Information Criterion (Schwarz 1978).

Gene flow. The occurrence of gene flow among populations was checked by different methods. First we performed indirect Nm analysis of POPGENE ver. 2 for ISSR loci studied according to the following formulae:

$$Nm = 0.5 (1 - Gst)/Gst.$$

Then we used STRUCTURE plot based on admixture model. Finally, the population' assignment test was performed by using maximum likelihood method as implemented in GenoDive ver.2 (2013) (Meirmans and Van Tienderen 2004). Frichot et al. (2013) latent factor mixed models (LFMM) was used to check if ISSR markers show correlation with environmental features of the studied populations. The analysis was done by LFMM program Version: 1.2 (2013).

RESULTS AND DISCUSSION

Morphometry

ANOVA test revealed significant difference in quantitative morphological characters among the studied populations ($P < 0.05$). Moreover, CVA plot (Figure 2) separated the studied populations based on all morphological characters including both quantitative and qualitative characters, supporting ANOVA result. In general, two major clusters were formed in UPGMA tree (Figure not given), populations 1, 2, 7, 8, 9 and 10 showed morphological similarity and were placed in the first major cluster, while populations 3, 4, 5 and 6 formed the second major cluster. PCA plot supported the grouping made by UPGMA tree and also revealed some degree of intra-population morphological variability. Therefore, combination of UPGMA and PCA plot indicated morphological divergence among the studied populations.

Genetic diversity analysis

AMOVA test revealed significant genetic difference among the studied populations ($P = 0.01$). It also revealed that 58% of total genetic variability occurred among populations while, 42% occurred within populations. These results indicated the presence of high level of genetic variability both within and among *C. cylindracea* populations. This conclusion was supported by Gst and Hickory analyses. The Gst value obtained among populations after 999 permutations was 0.557 ($P = 0.001$) and Theta B value = 0.40, which is significant. Population genetic differentiation was shown by high values obtained for Hedrick's standardized fixation index after 999 permutation (G'st=0.66, P=0.001) and Jost's differentiation index (D-est = 0.232, P = 0.001).

Populations grouping based on genetic data

The Population grouping based on ISSR data by NJ tree and Neighbor-Net diagram produced similar results. Therefore, Neighbor-Net diagram (Figure 3) is presented and discussed here. In general two major clusters were formed. Neighbor Net diagram revealed closer genetic affinity between populations No. 3-6, and also between population No. 1, 2, 7-10.

Population's genetic structure and gene flow

K-Means clustering and Evanno test performed on STRUCTURE analysis produced the best number of gentic groups as k=2. This genetic grouping is in agreement with NJ tree result, presented before. Therefore, two gene pools are identified for *C. cylindracea* in the country. STRUCTURE plot (Figure 4) revealed close genetic affinity between populations No. 3-6 (the first gene pool) and also between population numbers 1, 2, 7-10 (the second gene pool). This is in agreement with NeighborNet diagram. STRUCTURE plot also indicated very low degree of genetic admixture between the two gene pools. Moreover, mean Nm value of 0.40 was obtained for the studied populations that showed low value of gene flow, supporting STRUCTURE plot result. LFMM analysis revealed that many of the studied ISSR loci were significantly ($P<0.05$) correlated with the environmental features studied. These may have adaptive values and being used by plants of either gene pools for local adaptation. Among these loci, some had low Nm value (<0.50) such as ISSR loci 3, 5, 6, 7, 11, 12, 14, 16, 17, 24, 31-35, 48, and 50-52. However, some other loci had high Nm value of >1.00 or even much higher. For example, ISSR loci 22, 23, 27, 49, and 54.

Discussion

Genetic diversity is an important factor for adaptation of plants to the environmental changes they encounter. In general those populations that have high level of genetic variability may have better chance of survival compared to

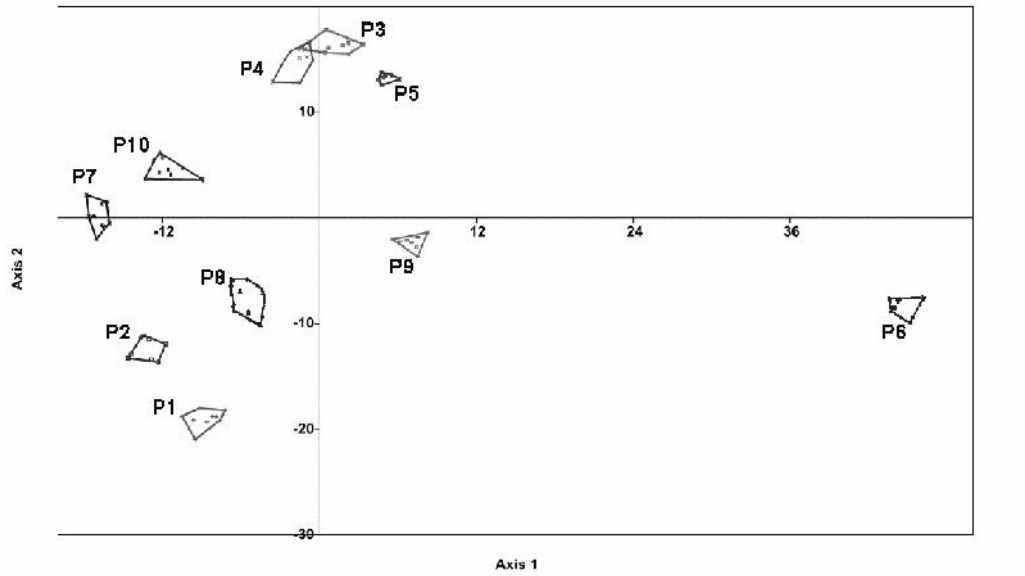

Figure 2. CVA plot of populations based on morphological characters. numbers indicate the plant specimens (Numbers 1-10) studied from each geographical population

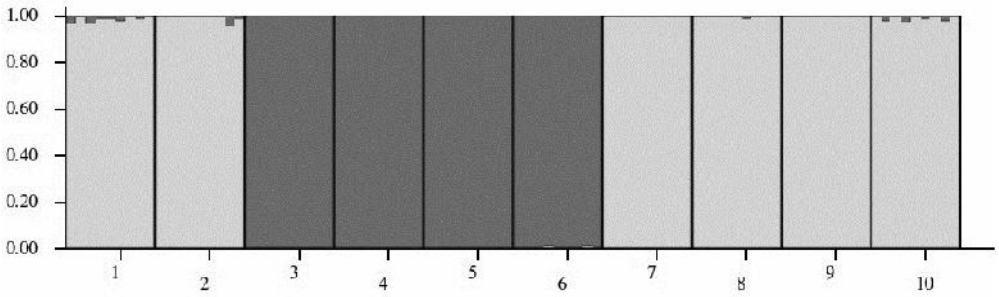

Figure 4. STRUCTURE plot of in *Cousinia cylindracea* populations studied

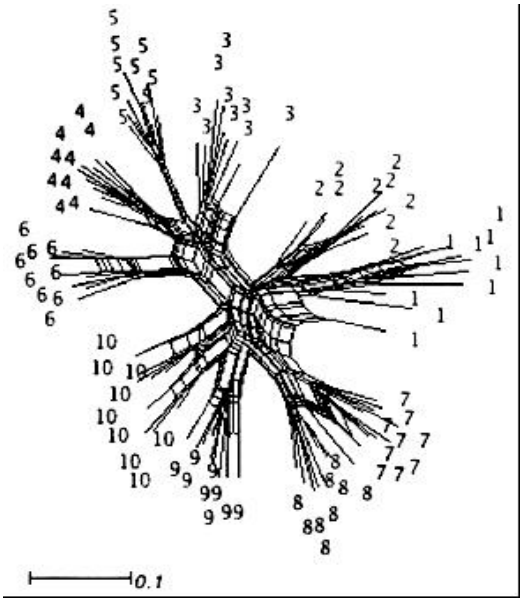

Figure 3. Neighbor Net diagram of ISSR data

the ones with lower degree of genetic diversity (Sheidai et al. 2012, 2013).

The present study revealed a high level of within population and among population genetic variability in *C. cylindracea.* The occurrence of high within population genetic diversity has been reported in other plant species and outcrossing nature of these species has been suggested to be the reason for that (Bodo-Slotta et al. 2010). The same may holds true for *C. cylindracea* populations.

An examination of the genetic diversity among populations within a species is crucial for a better understanding of evolutionary processes and the nature of the species. AMOVA and Hickory tests revealed significant genetic difference among the studied populations. Moreover, the low value of Nm (0.40) obtained for the studied populations showed low value of gene flow. Due to limited gene flow among populations, genetic differentiation increases. In situations with complete absence or very limited amount of gene flow, genetic drift is a strong evolutionary force and brings about high degree of within populations genetic homogeneity.

This may lead to adaptation to local habitats (Hou and Lou 2011). In fact, many plant species grow within a range of different habitats and have developed adaptive strategies suited to their particular habitat (Schneller and Liebt 2007).

STRUCTURE analysis revealed population genetic fragmentation in *C. cylindracea* and identified the presence of two gene pools for this species in Iran. Among-population differentiation in phenotypic traits and allelic variation is expected to occur as a consequence of genetic fragmentation, isolation, drift, founder effects and local selection (Jolivet and Bernasconi 2007). In fact the populations of the two gene pools in *C. cylindracea* differed in their morphological characters too. The populations 3-6 are different from other population in some characters such as: bract shape, stem leaves, uppermost leaves, head indumentums and head number. This finding is in agreement with Rechinger suggestion, that the *C. cylindracea* may contain two different varieties (Rechinger 1979). Therefore, genetic fragmentation followed by local adaptation might be the reason for the presence of two different taxonomic forms (varieties) in *C. cylindracea*. In many studies different ecotypes were identified and formed due to among populations genetic differentiation followed by population morphological divergence (Sheidai et al. 2012, 2013).

It is interesting to mention that populations 1 and 2 in close-by geographical locations (Figure 1), but they showed completely different genetic make up and belonged to two different gene pools. It seems they are genetically isolated by ranges of mountains such as: Kharagan mountain, Cheragi mountain, Ozonbolag mountain. The presence of strong isolation by distance and genetic differentiation of the studied populations was evidenced by significant Mantel test and population differentiation indices presented before. The populations growing on mountains not only are differentiated on the mountains along vertical axes, but genetic changes can also occur along horizontal axes. For instance, ridges may provide geographical barriers to gene flow between populations on their opposite sides, so genetic differentiation may occur across ridges (Taberlet et al. 1998).

Assessments of levels of within- and among-population genetic variation have been used to prioritize populations for conservation efforts (Petit et al. 1998) with, all else being equal, more weight given to those exhibiting higher levels of within-population variation, and to those that are more genetically divergent from others. These populations may have increased likelihood of persistence over less variable population and hence the ability of a population to contribute demographically to the species through time, and have increased adaptability in the face of future environmental changes. LFMM result showed that along with genetic drift, low degree of gene flow and migration, adaptive loci also helped populations to diverge and adapt these plants to their local condition. Therefore we have a new taxonomic group bellow the species level that can be considered as a new variety for *C. cylindracea* based on morphological and genetic data. We shall publish details of this new variety in future publication.

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