UDC 575.630
https://doi.org/10.2298/GENSR2102883Z
Original scientific article

STRONG GENETIC DIFFERENTIATION OF THE *Paracaryum* SPECIES (Boraginaceae) DETECTED BY INTER-SIMPLE SEQUENCE REPEATS (ISSR)

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Zhang X. and A. Shakoor (2021). *Strong genetic differentiation of the Paracaryum species (Boraginaceae) detected by inter-simple sequence repeats (ISSR).*- Genetika, Vol 53, No.2, 883-894.

Species identification is fundamentally important within the fields of biology, biogeography, ecology and conservation. The genus *Paracaryum* belongs to tribe Cynoglosseae of the family Boraginaceae is a herbaceous genus including approximately 67 species, mostly distributed in the Irano-Turanian phytogeographical region. In spite vast distribution of many *Paracaryum* species that grow in different habitats, there are not any available report on their genetic diversity, mode of divergence and patterns of dispersal. Therefore, we performed molecular (ISSR markers) of 98 accessions from 12 species of *Paracaryum* that were collected from different habitats. A set of 10 ISSR markers was used. The genetic distances were estimated based on Jaccard similarity coefficient and the descriptive statistics of populations for estimation of genetic parameters were also performed. A total of 90 polymorphic bands were obtained. The present study revealed that ISSR data can delimit the species. AMOVA and STRUCTURE analysis revealed that the species of *Paracaryum* belongs are genetically differentiated but have some degree of shared common alleles.

Keyword: ISSR, Species Identification, STRUCTURE

INTRODUTION

WIENS and PENKROT (2002), proposed to use DNA data, morphological data and character data for species delimitation while, KNOWLES and CARSTENS (2007) addressed how molecular

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data (i.e., gene trees from DNA sequence data) can be used in species delimitation. The latter authors used coalescent simulations to test the species limits and incorporated data from multiple loci. They showed the importance of population genetics in species delimitation. Similarly, MEDRANO *et al.,* (2014), applied population genetics methods to the species delimitation problem in *Narcissus* Linnaeus (1753: 289) (Amaryllidaceae J.St.-Hil. nom. cons.) by the help of amplified fragment length polymorphism (AFLP) molecular markers.

The family *Boraginaceae* s.str consists of approximately 131 genera and 2,500 species, mainly distributed in dry, cliffy and sunny habitats of Eurasia, the Mediterranean region and the western North America (BINZET and AKCIN, 2009). They are mainly annual, bi-annual or perennial herbs and shrubs, some trees and a few lianes, distributed throughout the temperate and subtropical regions of the world (RETIEF and VANWYK, 1997), with a high distribution in Iran (WILLIS, 1973). Subfamily Cynoglossoideae Weigend., is the largest subfamily having about 900 species and 50 genera. Recent molecular studies have shown that a wide range of the previously recognized tribes places into this subfamily (CHACÓN *et al*., 2016). The subtribe Cynogolossinae Dumort. (tribe Cynoglosseae W.D.J.Koch) is entirely restricted to the Old World, with a center of diversity in western Asia and the Mediterranean (CHACÓN *et al*., 2016).

Paracaryum (DC.) Boiss. belongs to tribe Cynoglosseae of the family Boraginaceae is a herbaceous genus including approximately 67 species, mostly distributed in the Irano-Turanian phytogeographical region (RIEDL, 1967; FERGUSON, 1972; MILL, 1978; NASIR, 1989).

Paracaryum is not a large genus, but from the point of view of taxonomy and nomenclature, it is very complex and includes 16 species, 12 of which occur in Iran (RIEDL, 1967). This genus is characterized by anthers included in the corolla tube, ebracteate cymes, a four-lobed ovary, an obtuse five-lobed corolla with faucal scales, and winged nutlets. In the light of recent phylogenetic analyses based on rps16 and trnL-trnF DNA sequences, the classification of *Paracaryum* is uncertain within the *Cynoglossum* L. s.l. clade, and the genus is not monophyletic (WEIGEND *et al*., 2013).

RANJBAR and NOURALLAHI (2016) were determined meiotic chromosome numbers and meiotic behavior of six populations belonging to four species growing in Iran, namely *Paracaryum modestum* (2n = 2x = 24), *Paracaryum persicum* subsp. *Macrocarpum* (2n = 2x = 24), *Paracaryum undulatum* (2n = 2x = 24) and *Paracaryum rugulosum* (2n = 2x = 24). All chromosome counts are consistent with a proposed base number of x = 12. The fatty acid compositions of the fruits of ten *Paracaryum* taxa belonging to three different subgenera were investigated for chemotaxonomic allocation using gas chromatography. Among the twenty-two analysed fatty acids, oleic, linoleic and a-linolenic acids were the major fatty acids represented (ASLI DOGRU-KOCA *et al.,* 2016). For a synthetic approach on the systematics of this family considering both phylogenetic and evolutionary aspects (SELVI *et al*., 2006), and in most researches fruit morphology has been used as the most important character.

Molecular markers are considered an important tool in order to provide data in terms of genetic polymorphisms between different individuals in a population. Among the various types of polymerase chain reaction (PCR)-based molecular markers, inter simple sequence repeat (ISSR) markers are a class of molecular markers that are based on the amplification of regions between two microsatellite sequences (ZIETKIEWICZ *et al.,* 1994; ESFANDANI-BOZCHALOYI *et al.,* 2018a, 2018b, 2018c, 2018d). These markers do not require prior knowledge of the genome of

the species. In addition, they can be assessed using relatively simple techniques, are easy to handle and require only small amounts of DNA (WANG *et al.*, 2009). We try to answer the following questions: 1) Is there infra and interspecific genetic diversity among studied species? 2) Is genetic distance among these species correlated with their geographical distance? 3) What is the genetic structure of populations and taxa?

MATERIALS AND METHODS

Plant materials

In present study, 98 plant samples were collected from 12 geographical populations belong 12 *Paracaryum* species were collected during the years 2015-2018 from their natural habitats (Table 1). Different references were used for the correct identification of species. Details of sampling sites are mentioned (Table 1). Voucher specimens are deposited in Herbarium of Azad Islamic University (HAIU).

Table 1. Voucher details of Paracaryum species in this study from Iran

No	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*Paracaryum cyclhymenium* (Boiss.) H. Riedl	Tehran, Damavand	38 ˚ 52'37"	47 ˚ 23' 92"	1144
Sp2	*Paracaryum persicum* (Boiss.) Boiss. subsp. *persicum*	Kermanshah, Islamabad	32°50′03″	51°24′28″	1990
Sp3	*Paracaryum platycalyx* Riedl	Fars, 7km from Evaj to Lar	29◦20′07″	51° 52′08″	1610
Sp4	*Paracaryum rugulosum* (DC.) Boiss.	Hamedan, 20 km S of Nahavand	38 ˚ 52'373	47 ˚ 23' 92"	2234
Sp5	*Paracaryum sintenisii* Hausskn. ex Bornm.	Azarbaiejan, Kaleiybar, Arasbaran	33° 57′12″	47° 57′32″	2500
Sp6	*Paracaryum strictum* (C. Koch) Boiss.	Azarbaiejan, Arasbaran	34 ˚ 52'373	48 ˚ 23' 92"	2200
Sp7	*Paracaryum undulatum* Boiss.	Kordestan, Sanandaj	38 ˚ 52'37	47 ˚ 23' 92"	1144
Sp8	*Paracaryum hirsutum*(DC.) Boiss.	Kermanshah, Islamabad	35°50′03″	51°24′28″	1700
Sp9	*Paracaryum tenerum*	Kordestan, Sanandaj	36°14′14″	51°18′07″	1807
Sp10	*Paracaryum bungei* (Boiss.) Khatamsaz	Ardestan, Taleghan; Bandar-Abbas;	32◦36′93″	51°27′90″	2500
Sp11	*Paracaryum salsum* (Boiss.) H.H. Hilger & D. Podlech	Tehran, Shahrud – Bastan; Turan	37°07′02″	49°44′32″	48
Sp12	*Paracaryum intermedium* (Fresen.) Hilger & Podl.	Khorassan, Kashmar-Darvaneh	28◦57′22″	51°28′31″	430

DNA extraction and ISSR assay

Fresh leaves were used randomly from 5-10 plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (DOYLE and DOYLE, 1987). The quality of extracted DNA was examined by running on 0.8% agarose gel. 10 ISSR primers; (AGC) 5GT, (CA) 7GT, (AGC) 5GG, UBC 810, (CA) 7AT, (GA) 9C, UBC 807, UBC 811, (GA) 9T and (GT) 7CA commercialized by UBC (the University of British Columbia) were used. PCR reactions were carried 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 (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications' reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 95°C, followed by 37 cycles of 1 min at 95°C; 1 min at 50-56°C and 1 min at 72°C. The reaction was completed by final extension step of 5-10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

Molecular analyses

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. A parameter like Nei's gene diversity (H), Shannon information index (I), the number of effective alleles, and percentage of polymorphism were determined (WEISING *et al.,* 2005; FREELAND *et al.,* 2011). Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (FREELAND *et al.,* 2011; HUSON and BRYANT, 2006). Mantel test checked the correlation between geographical and genetic distance of the studied populations (PODANI, 2000). These analyses were done by PAST ver. 2.17 (HAMMER *et al.,* 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (PEAKALL and SMOUSE, 2006), and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) (MEIRMANS and VAN TIENDEREN, 2004) were used to show genetic difference of the populations. Moreover, 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). The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (PRITCHARD *et al.,* 2000), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2. (2013). For STRUCTURE analysis, data were scored as dominant markers (FALUSH *et al.,* 2007). We used the admixture ancestry model under the correlated allele frequency model. A Markov chain Monte Carlo simulation was run 20 times for each value of K after a burn-in period of 10^5. The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value (EVANNO *et al.,* 2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Inforation Criterion (BIC) provide the best fit for k (MEIRMANS, 2012).

Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers the equal amount of gene flow among all populations. (ii) Population assignment test based on maximum

likelihood as performed in Genodive ver. in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5. (2012).

RESULTS

Species Identification and Genetic Diversity

All ISSR primers produced polymorphic bands. Genetic diversity parameters determined in the studied species (Table 2) revealed that *Paracaryum rugulosum* (sp4) had the highest level of genetic polymorphism (57.09%), while the lowest level of genetic polymorphism (21.75%) occurred in *Paracaryum hirsutum* (sp8). *Paracaryum rugulosum* also had the highest values for effective number of alleles (Ne = 1.27) and Shannon information index (I =0.33).

Table 2. Genetic diversity parameters in the studied Paracaryum species

SP	N	Na	Ne	I	He	UHe	%P
Paracaryum cyclhymenium	12.000	1.347	1.104	0.281	0.194	0.182	55.87%
Paracaryum persicum (Boiss.) Boiss. subsp. *persicum*	8.000	0.429	1.097	0.84	0.56	0.060	26.13%
Paracaryum platycalyx	6.000	0.258	1.029	0.23	0.16	0.020	44.38%
Paracaryum rugulosum	12.000	0.925	1.279	0.333	0.299	0.34	57.09%
Paracaryum sintenisii	11.000	0.784	1.171	0.162	0.204	0.109	36.56%
Paracaryum strictum	4.000	0.314	1.044	0.26	0.18	0.23	43.38%
Paracaryum undulatum	8.000	0.256	1.066	0.21	0.27	0.12	32.23%
Paracaryum hirsutum j	5.000	0.341	1.058	0.12	0.17	0.019	21.75%
Paracaryum tenerum	3.000	0.567	1.062	0.29	0.224	0.213	44.73%
Paracaryum bungei	14.000	0.344	1.039	0.14	0.21	0.023	43.98%
Paracaryum salsum	14.000	0.560	1.186	0.98	0.64	0.066	25.51%
Paracaryum intermedium	10.000	0.441	1.036	0.33	0.22	0.023	36.53%

(N = number of samples, Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

AMOVA test showed significant genetic difference (P = 0.01) among studied species. It revealed that 60% of total variation was among species and 40% was within species (Table 3) Moreover, genetic differentiation of these species was demonstrated by significant Nei's GST (0.56, P = 0.01) and D_est values (0.456, P = 0.01).

Different clustering and ordination methods produced similar results therefore, only PCOA plot and WARD tree are presented here (Fig. 1-2). In general, PCOA plot of the studied populations did not entirely delimit the studied species and revealed that plants in these species are intermixed. This result shows that ISSR marker canot delimit *Paracaryum* species.

Two major clusters were formed in the WARD tree (Fig. 2). The first major cluster contained two sub-clusters: *P. persicum* and *P. hirsutum* were separated from the rest of the species and join the others with a great distance and comprised the first sub-cluster. The second

sub-cluster comprised of *P. tenerum, P. cyclhymenium, P. bungei; P. salsum* and *P. intermedium.* The second major cluster also comprised two sub-clusters: three species including *P. platycalyx*; *P. undulatum P. rugulosum, P. sintenisii* and *P. strictum* were placed close to each other, while close genetic affinity between other species.

Table 3. Analysis of molecular variance (AMOVA) of the studied species

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	24	733.747	41.327	8.082	60%	
Within Pops	60	361.607	5.590	5.530	40%	60%
Total	84	1119.354		14.613	100%	

df: degree of freedom; SS: sum of squared observations; MS: mean of squared observations; EV: estimated variance; *Φ*PT: proportion of the total genetic variance among individuals within an accession, (P < 0.001).

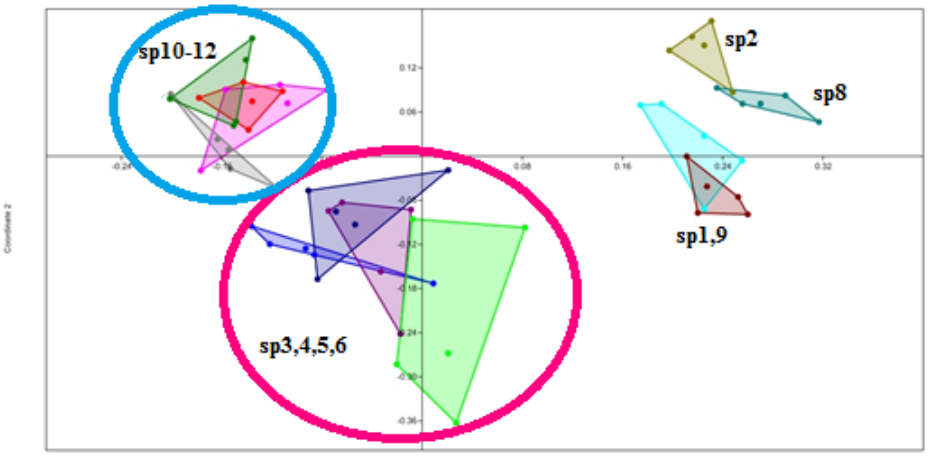

Figure 1. PCoA plots of inter simple sequence repeats data revealing species delimitation in the *Paracaryum*

This is in agreement with AMOVA and genetic diversity parameters presented before. The species are genetically well differentiated from each other. The Nm analysis by Popgene

software also produced mean Nm= 0.56, that is considered very low value of gene flow among the studied species.

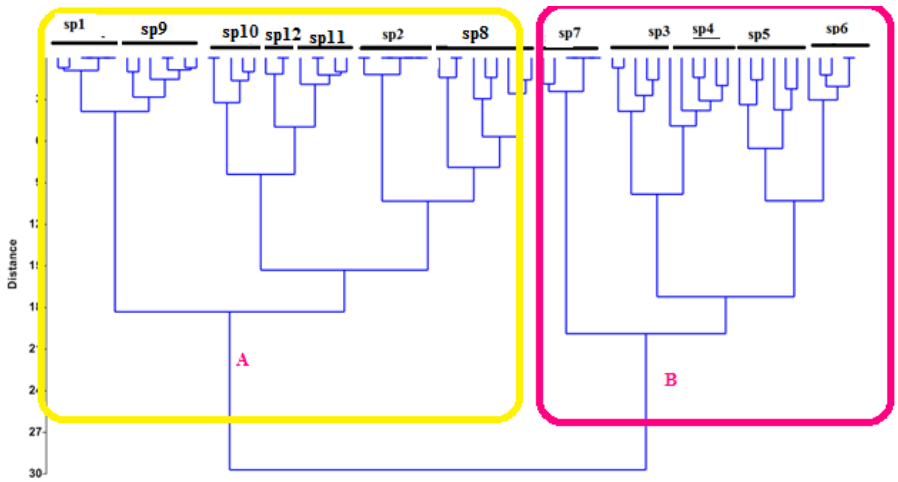

Figure 2. WARD clustering of inter simple sequence repeats data revealing species delimitation in the *Paracaryum*

Mantel test with 5000 permutations showed a significant correlation (r = 0.34, p=0.0001) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Paracaryum* species studied.

Nei's genetic identity and the genetic distance determined among the studied species (Table is not included). The results showed that the highest degree of genetic similarity (0.87) occurred between *P. rugulosum* and *P. sintenisii*. The lowest degree of genetic similarity occurred between *P. platycalyx* and *P. undulatum* (0.53).

The Species Genetic Structure

We performed STRUCTURE analysis followed by the Evanno test to identify the optimal number of genetic groups. We used the admixture model to illustrate interspecific gene flow or / and ancestrally shared alleles in the species studied.

K-Means clustering showed k = 6 according to pseudo-F and k = 10 according to BIC. K = 10 is in agreement with NJ grouping and AMOVA. K = 6 reveal the presence of 6 genetic group. Similar result was obtained by Evanno test performed on STRUCTURE analysis which produced a major peak at k = 8 (Fig. 3).

The STRUCTURE plot (Fig. 3) produced more detailed information about the genetic structure of the species studied as well as shared ancestral alleles and / or gene flow among *Paracaryum* species. This plot revealed that genetic difference of species 3 and 4 (differently colored), as well as 5 and 6. This is in agreement with Neighbor joining dendrogram presented before. The other species are distinct in their allele composition and differed genetically from each other.

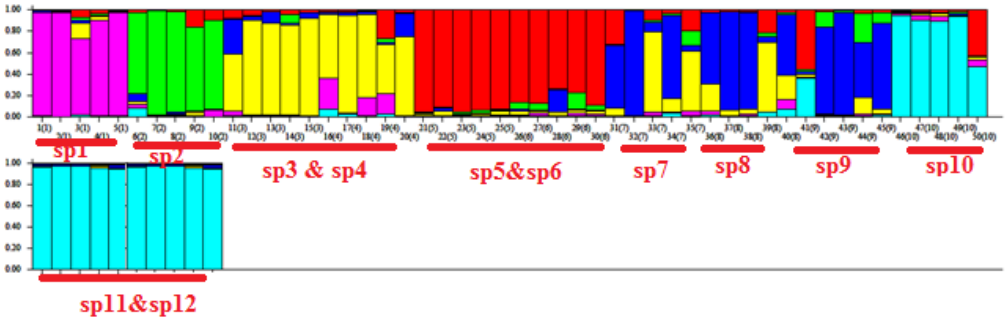

Figure 3. STRUCTURE plot of the *Paracaryum* species based on ISSR data.

The low Nm value (0.56) indicates limited gene flow or ancestrally shared alleles between the species studied and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. Population assignment test also agreed with Nm result and could not identify significant gene flow among members of the studied species.

DISCUSSION

Genetic diversity

Genetic diversity is one aspect of biological diversity that is extremely important for conservation strategies (KALJUND and JAASKA, 2010; GORDON *et al.*, 2012). Population size is considered an important factor for maintaining genetic variation. Small populations are more vulnerable than large ones to extinction because of environmental stochasticity, genetic drift and inbreeding. Genetic drift decreases heterozygosity and eventual fixation of alleles, and inbreeding increases homozygosity within populations (FRANKHAM, 2005; ESFANDANI-BOZCHALOYI *et al.*, 2017 a, 2017b, 2017c, 2017d). In general, a drop in population size may cause the decline of genetic diversity by genetic drift and inbreeding. In the longer term, diminished genetic diversity may cause a loss of fitness and evolutionary capacity to adapt to environmental changes (LANDE, 1993; KALJUND and JAASKA, 2010). Therefore, quantifying patterns of genetic variability and diversity within and among different populations is very important for small population species conservation and management planning.

In our study, genetic diversity in 12 taxa of *Paracaryum* are given in detail for the first time. The aim of the present study was to find diagnostic features to separate species of *Paracaryum* in Iran. Morphological characters are considered as a useful tool for the identification of the species, as indicated previously (AKCIN, 2008). Also, fruits and seeds are known to be useful characters in the identification of *Cynoglossum creticum, C. officinale, C. montanum* and *C. glochidiatum* (AKCIN, 2008). However, due to variation in seed coat and fruit surface, two types of tuberculate and granulate, and two subtypes of granulate-punctuate and granulate-tuberculate was recognized in these species. Reticulate type of seed coat and detailed subtypes of reticulate types were determined based on ornamentation of the seed coats (AKÇIN, 2008). In previous studies, the micro-morphology of seed and fruit was performed in several species and their importance in plant taxonomy was emphasized (KHALIK *et al*., 2008).

Paracaryum (*Mattiastrum*) *modestum* is an unresolved name (see The Plant List: http://www.theplantlist.org). The generic distinction (at least in the Iranian taxa) between *Paracaryum* and *Mattiastrum* is not clear cut in some taxa. In the former, the margin of the nutlets is distinctly inrolled to form an aperture; whereas in *Mattiastrum* the margin of nutlet or wing is flat or slightly inrolled and the aperture is not evident. *Paracaryum modestum* was transferred from *Paracaryum* to *Mattiastrum* by BRAND (1915). Morphological and cytogenetic features of this species confirm its placement in *Paracaryum* because the margins of the nutlets are indistinctly denticulate. All populations of this genus were diploid and polyploidy was not observed in this species. Polyploidy is probably an infrequent phenomenon in perennial *Paracaryum* species based on cytological evidence.

Genetic structure is affected by several factors, such as breeding systems, genetic drift, population size and natural selection (HAMRICK and GODT, 1990). Our analyses of genetic structure showed that the 98 individuals formed a clear separation between all populations; this result indicated differentiation (Fig. 3). This conclusion is also supported by the PCoA (Fig. 1). Analysis of molecular variance analysis of all populations showed that 60% of the genetic variation occurred between populations, whereas 40 % of the genetic variation occurred within these populations (Table 3). To conclude, the present study revealed the use of ISSR molecular markers along with morphological characters in *Paracaryum* species identification. Some degrees of interspecific genetic admixture occur in *Paracaryum*, but the studied species are strongly differentiated during the speciation process and invasion in new habitats.

ACKNOWLEDGMENT

The research is supported by: Natural Science Foundation of Heilongjiang Province (C2018038)

Received, September 21st, 2020

Accepted March 22nd, 2021

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GENETIČKA DIFERENCIJACIJA *Paracaryum VRSTA* (Boraginaceae) DETEKTOVANA SA ISSR

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Izvod

Identifikacija vrsta je od fudamentalnog značaja u biologiji, biografiji, ekologiji i konzervaciji. Rod *Paracaryum*, pripada Cynoglosseae faniliji Boraginaceae, uključuje oko 67 vrsta uglavnom rasprostranjeni u Irano-Turanian fitogeografskom regionu. Uprkos širokoj rasprostranjenosti mnogih vrsta Paracarium koje rastu na različitim staništima, nema dostupnih izveštaja o njihovoj genetskoj raznolikosti, načinu razilaženja i obrascima širenja. Stoga smo izvršili analizu sa ISSR markerima 98 uzoraka od 12 vrsta Paracariuma prikupljenih iz različitih staništa. Korišćen je set od 10 ISSR markera. Genetička rastojanja procenjena su na osnovu Jacardovog koeficijenta sličnosti, a takođe su urađene deskriptivne statistike populacija za procenu genetskih parametara. Dobijeno je ukupno 90 polimorfnih traka. Sadašnja studija otkrila je da podaci ISSR mogu ograničiti vrstu. Analiza AMOVA i STRUKTURE otkrila je da su vrste Paracarium genetski diferencirane, ali imaju određeni stepen zajedničkih zajedničkih alela.

Primljeno 21.IX.2020.
Odobreno 22.III 2021.