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ASSESSMENT OF GENETIC VARIATION OF GENUS *PARACARYUM* (BORAGINACEAE) BY RAPD MARKERS

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Keywords: Gene flow; Endemism; Random Amplified Polymorphic DNA (RAPD).

Abstract

The present study reveals the genetic diversity of Iranian *Paracaryum* based on morphological and molecular characters of 12 species from 11 provinces of Iran. A total of 118 reproducible bands were generated by 10 of 30 random amplified polymorphic DNA (RAPD) primers, with an average of 11.8 bands per primer and 49% polymorphism. The largest number of effective alleles (Ne), Shannon Index (I) and genetic diversity (H) higher level of Shannon Index (I) and genetic diversity (H) were shown by *Paracaryum persicum*. Our data depicted the highest similarity between *Paracaryum cyclhymenium* and *P. persicum* and the lowest between *P. sintenisii* and *P. bungei*. *P. bungei* showed a relatively low level of genetic variation. Finally, the Neighbor Joining (NJ) trees based on RAPD markers data divided the populations into two different clusters, indicating their genetic difference, which is discussed in detail.

Introduction

The family Boraginaceae s.str consists of approximately 131 genera and 2,500 species, distributed throughout the temperate and subtropical regions of the world, but mainly distributed in dry, cliffy and sunny habitats of Eurasia, the Mediterranean region and western North America (Retief and Vanwyk, 1997). They are mainly annual, bi-annual or perennial herbs and shrubs, some trees and a few lianes (Retief and Vanwyk, 1997), with a high distribution in Iran. 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 belong to 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 centre of diversity in western Asia and the Mediterranean (Chacón *et al*., 2016). The genus *Paracaryum* (DC.) Boiss. of the tribe Cynoglosseae of this family is herbaceous and includes approximately 67 species, mostly distributed in the Irano-Turanian phytogeographical region (Riedl, 1967).

Paracaryum is a very complex genus from the point of view of taxonomy and nomenclature 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 *rps*16 and *trn*L-*trn*F DNA sequences, the classification of *Paracaryum* is uncertain within the *Cynoglossum* L. s.l. clade and the genus is not monophyletic.

Amedi *et al.* (2020) determined meiotic chromosome numbers and meiotic behaviour of six populations belonging to four species of *Paracaryum* growing in Iran, namely *P. modestum* Boiss. & Hausskn. (2n = 2x = 24), *P. persicum* subsp. *macrocarpum* (2n = 2x = 24), *P. undulatum* (2n = 2x = 24) and *P. rugulosum* (2n = 2x = 24). All chromosome counts are consistent with a

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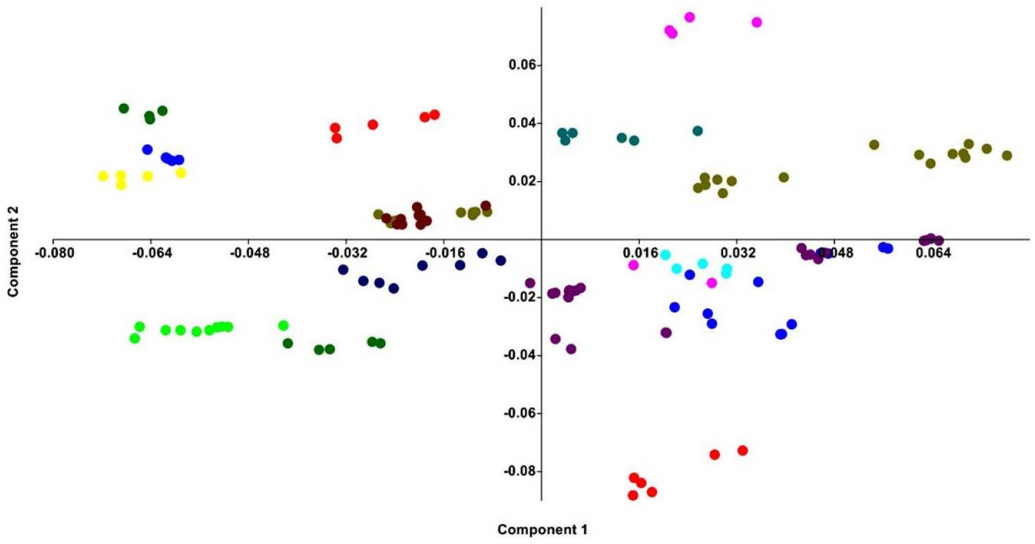
proposed base number of x = 12. The fatty acid compositions of the fruits of ten *Paracaryum* taxa belonging to three different subgenera were investigated for chemotaxonomic allocation using gas chromatography. Among the twenty-two analysed fatty acids, oleic, linoleic and a-linolenic acids were the major fatty acids represented (Amedi *et al*., 2020). For a synthetic approach to the systematics of this family considering both phylogenetic and evolutionary aspects. and in most research fruit morphology has been used as the most important character.

The present study has been carried out to evaluate the genetic diversity and relationships among the Iranian *Paracaryum* species using RAPD markers. This is the first study on the use of RAPD markers in the *Paracaryum* genus and aims at answering the following questions: 1) Is there infra and interspecific genetic diversity among *Paracaryum* species? 2) Is there any genetic distance among these species correlated with their geographical distribution?

Materials and Methods

Plant sampling

A total of 116 individuals were sampled representing 15 distant populations representing 12 *Paracaryum* species in East Azerbaijan, Kermanshah, Esfahan, Tehran, Hamadan, Kurdistan, Khorasan, Kerman, Hormozgan, Semnan and Fars Provinces of Iran during July–August 2017–2019 (Table 1). For morphometric and RAPD analysis, we used 116 plant accessions (up to twelve samples from each population) belonging to 15 different populations with different eco-geographic characteristics and were sampled and stored at -20°C till further use. More information about the geographical distribution of accessions are in Table 1 and Fig. 1.



. 1. PCA plots of morphological characters revealing species delimitation in the *Paracaryum* species*;* sp1= *P. cyclhymenium*; sp2= *Paracaryum persicum*; sp3= *Paracaryum platycalyx*; sp4= *Paracaryum rugulosum*; sp5= *Paracaryum sintenisii*; sp6= *Paracaryum strictum*; sp7= *Paracaryum undulatum*; sp8= *Paracaryum hirsutum*; sp9= *Paracaryum tenerum*; sp10= *Paracaryum bungei*; sp11= *Paracaryum salsum*; sp12= *Paracaryum intermedium*.

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 Semnan, 20 km NW of Shahrud	38 ˚ 52'37"	47 ˚ 23' 92"	1144
Sp2	*Paracaryum persicum* (Boiss.) Boiss. subsp. *persicum*	Kermanshah, Islamabad Tehran, road of Firozkuh	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 Azarbaiejan, 48 km from Tabriz to Marand	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 Hamedan, 20 km S of Nahavand	34 ˚ 52'373	48 ˚ 23' 92"	2200
Sp7	*Paracaryum undulatum* Boiss.	Kordestan, Sanandaj Hamedan, Alvand	38 ˚ 52'373	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; Esfahan, Ghamishleh, protected area, Kooh Dojdoon	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 Hormozgan, Bandar-Abbas;	28◦57′22″	51°28′31″	430

Morphological studies

One to twelve samples from each species were used for morphometric analysis. In total 14 morphological (10 qualitative, 4 quantitative) characters were studied. Data obtained were standardized (Mean= 0, variance = 1) and used to estimate Euclidean distance for clustering and ordination analyses (Podani, 2000). Calyx length, calyx width, corolla length, corolla shape, corolla colour, faucal appendages, nutlet shape, nutlet length, nutlet surface ornamentation, stamens position, style position, nutlet margin and disc, and sepal indumenta.

DNA Extraction and RAPD Assay

Fresh leaves were used randomly from one to twelve plants in each of the studied populations. These were dried with silica gel powder. To obtain genomic DNA, the CTAB-activated charcoal protocol was used Abeshu & Zewdu (2020). The quality of extracted DNA was examined by running on 0.8% agarose gel. A total of 25 decamer RAPD primers of Operon technology (Alameda, Canada) belonging to OPA, OPB, OPC, and OPD sets were used. Among them, ten primers with clear, enlarged, and rich polymorphism bands were chosen (Table 2).

Data analyses

Morphological studies

Morphological characters (Mean = 0, Variance = 1) were first standardized and used to determine the Euclidean distance between taxa pairs (Podani, 2000). The ordination methods of UPGMA (Unweighted paired group using average) were used for grouping the plant specimens (Podani, 2000). To demonstrate morphological variation between populations.

Molecular analyses

The obtained RAPD bands were coded as binary characters (presence = 1, absence = 0) and used for the study of genetic diversity. Using two parameters, polymorphism information content (PIC) and marker index (MI), the discriminatory capacity of the primers used was evaluated to characterise the ability of each primer to detect polymorphic loci among the genotypes.

Results and Discussion

Species identification and interrelationship

Morphometry: ANOVA showed substantial differences ($P < 0.01$) between the studied species in quantitative morphological characteristics. PCA analysis was conducted to determine the most variable characters among the taxa analysed. It showed that over 80 % of the overall variance was composed of the first three variables. Characters such as nutlet shape, nutlet length, nutlet surface ornamentation, stamens position, and style position have shown the highest association (>0.7) in the first PCA axis with 58 per cent of the total variance. Characters affecting PCA axis 2 and 3 respectively were calyx length, calyx width, corolla length, corolla shape, and corolla colour. Different clustering and ordination methods produced similar results, and therefore, PCA plots of morphological characters are presented here (Fig. 2). Plant samples of each species were typically grouped and separate groups were formed. This finding indicates that the studied species belong to different groups based on their quantitative and qualitative morphological features. We did not find intermediate forms in the studied specimens.

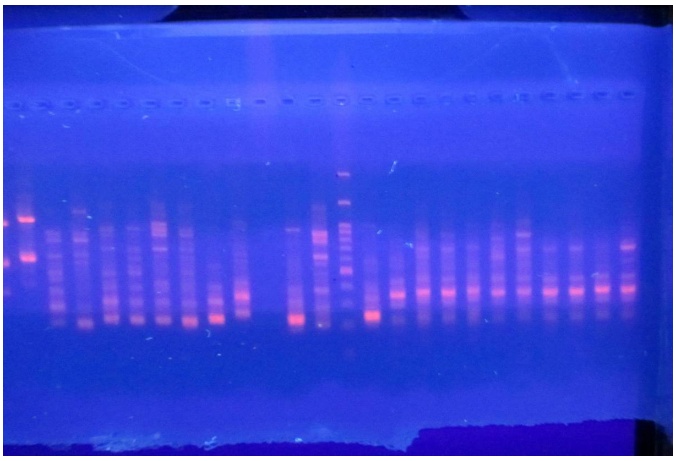


Fig. 2. Electrophoresis gel of studied ecotypes from DNA fragments produced by OPD-02 and OPA-06. sp1= *P. cyclhymenium*; sp2= *P. persicum*; sp3= *P. platycalyx*; sp4= *P. rugulosum*; sp5= *P. sintenisii*; sp6= *P. strictum*; sp7= *P. undulatum*; sp8= *P. hirsutum*; sp9= *P. tenerum*; sp10: *P. bungei*; sp11= *P. salsum*; sp12= *P. intermedium*.

Species Identification and Genetic Diversity

To study genetic relationships among *Paracaryum* species, ten RAPD primers were screened. All the primers generated reproducible polymorphic bands in all 12 *Paracaryum* species. Figure 3 shows an image of the amplification of the RAPD created by the OPD-02 and OPA-06 primer. In total, 114 amplified polymorphic bands were formed across 12 species of *Paracaryum.* The size of the amplified fragments ranged from 100 to 3000 bp. The highest and lowest number of polymorphic bands was 15 for OPC-04, OPD-05 and 7 for OPA-06, with an average of 11.4 polymorphic bands per primer. The PIC of the 10 RAPD primers ranged from 0.34 (OPD-03) to 0.56 (OPA-05) with an average of 0.49 per primer. MI of the primers ranged from 3.33 (OPD-011) to 5.66 (OPC-04) with an average of 4.5 per primer. EMR of the RAPD primers ranged from 8.23 (OPC-04) to 12.55 (OPB-01) with an average of 11.08 per primer (Table 2). The primers with high EMR values were considered to be more informative in distinguishing the genotypes.

Table 2. RAPD primers used for this study and the extent of polymorphism.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPA-05	5′-AGGGGTCTTG-3′	14	14	100.00%	0.56	5.86	10.55	4.77
OPA-06	5′-GGTCCCTGAC-3′	10	7	86.99%	0.43	4.51	9.43	3.85
OPB-01	5′-GTTTCGCTCC-3′	9	9	100.00%	0.54	5.34	12.55	4.44
OPB-02	5′-TGATCCCTGG-3′	12	12	100.00%	0.47	4.18	9.56	3.65
OPC-04	5'-CCGCATCTAC-3'	15	15	100.00%	0.55	5.23	8.23	5.66
OPD-02	5′-GGACCCAACC-3′	14	13	95.74%	0.47	4.66	8.56	4.67
OPD-03	5′-GTCGCCGTCA-3′	15	12	92.31%	0.34	4.21	8.60	3.55
OPD-05	5′ -TGAGCGGACA-3′	13	13	100.00%	0.47	4.32	10.55	3.45
OPD-08	5′-GTGTGCCCCA-3′	10	9	89.89%	0.53	5.56	9.34	4.11
OPD-11	5′-AGCGCCATTG-3′	11	11	100.00%	0.39	4.25	11.19	3.33
Mean		12.8	11.4	96.78%	0.49	5.2	11.8	4.5
Total		128	114					

TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers

The genetic parameters were calculated for all the 12 *Paracaryum* species amplified with RAPD primers (Table 3). Unbiased expected heterozygosity (*H*) ranged from 0.12 (*Paracaryum bungei*) to 0.34 (*Paracaryum persicum),* with a mean of 0.19. A similar trend was observed for Shannon's information index (*I),* with the highest value of 0.35 observed in *P. persicum* and the lowest value of 0.11 observed in *P. bungei* with a mean of 0.29. The observed number of alleles (*N*a) varied between 0.244 in *P. hirsutum* and 0.567 in *P. intermedium.* The effective number of alleles (*N*e) ranged from 1.011 (*P. strictum*) to 1.099 (*P. persicum).*

AMOVA test revealed substantial genetic variation (P = 0.01) among the studied species. It showed that 62% of the total variation was among species and 38% was within species (Table 4). In addition, genetic differentiation of these species was demonstrated by significant Nei's GST (0.66, P = 0.001) and D_est values (0.348, P = 0.01). Compared to within species, these results revealed a greater distribution of genetic diversity among *Paracaryum* species.

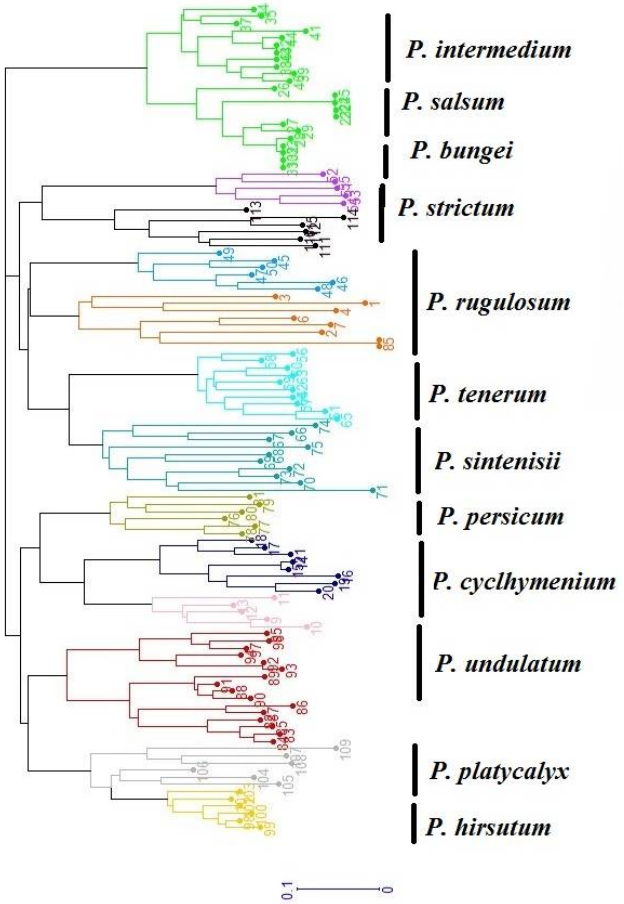


Fig. 3. NJ tree of RAPD data revealing species delimitation in the *Paracaryum*.

Table 3. Genetic diversity parameters in the studied *Paracaryum* species.

SP	N	Na	Ne	I	He	UHe	%P
Paracaryum cyclhymenium	5.000	0.455	1.077	0.277	0.34	0.22	55.05%
P. persicum (Boiss.) Boiss. subsp. *persicum*	8.000	0.499	1.099	0.35	0.43	0.34	69.26%
P.platycalyx	9.000	0.261	1.014	0.242	0.23	0.23	43.15%
P. rugulosum	6.000	0.555	1.021	0.29	0.35	0.31	58.53%
P. sintenisii	4.000	0.344	1.042	0.20	0.23	0.20	27.53%
P. strictum	5.000	0.369	1.011	0.25	0.18	0.22	42.15%
P. undulatum	9.000	0.261	1.014	0.242	0.33	0.23	43.15%
P. hirsutum	6.000	0.244	1.032	0.26	0.23	0.18	55.53%
P. tenerum	4.000	0.314	1.044	0.26	0.18	0.23	43.38%
P. bungei	8.000	0.256	1.066	0.11	0.17	0.12	32.23%
P. salsum	5.000	0.341	1.058	0.27	0.27	0.20	53.75%
P. intermedium	3.000	0.567	1.062	0.29	0.224	0.213	44.73%

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

Two major clusters were formed in the NJ tree (Fig. 3). The first major cluster contained two sub-clusters. Five species namely, *P. cyclhymenium, P. persicum, P. platycalyx, P. undulatum* and *P. hirsutum* were separated from the rest of the species, joined the others with a great distance and comprised the first sub-cluster. The second sub-cluster comprised four species namely, *P. rugulosum, P. sintenisii, P. strictum* and *P. tenerum.* The second major cluster also comprised two sub-clusters: three species including *P. bungei; P. salsum* and *P. intermedium* were placed close to each other, while close genetic affinity between other species. Relationships obtained from RAPD data usually agree well with the relationship of species obtained from morphological data. This is supported by the parameters of AMOVA and the genetic diversity previously presented. The species are genetically well differentiated from each other. The species are well distinguished from each other genetically. These findings show that RAPD molecular markers can be used in the taxonomy of *Paracaryum* species.

Table 4. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	33	1801.364	59.789	13.154	62%	
Within Pops	142	214.443	4.777	3.888	38%	62%
Total	175	1955.777		16.060	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, ($P < 0.001$).

Nei's genetic identity and the genetic distance were determined among the studied species. The results show the highest degree of genetic similarity (0.908) between *P. cyclhymenium* and *P. persicum.* The lowest degree of genetic similarity was shown between *P. sintenisii* and *P. bungei* (0.711). Genetic diversity is a fundamental component of biodiversity and its conservation is essential for the long-term survival of any species in changing environments. Genetic diversity is non randomly distributed among different populations and is influenced by various factors such as geography, breeding systems, dispersal mechanisms, life span etc. Changes in environmental conditions often lead to variation in levels of genetic diversity among different populations, and under adverse circumstances, populations with low variability are generally considered less adapted (Ma, *et al.*, 2021a; 2021b; Peng *et al.*, 2021). Most authors agree that genetic diversity is necessary to preserve the long-term evolutionary potential of a species (Ren *et al.*, 2021). Experimental and field research has shown that habitat fragmentation and population decline have reduced the effective population size in the last decade. Similarly, most geneticists regard population size as a significant factor in preserving genetic variation. In fragmented populations, it is more vulnerable because of the loss of allelic richness and increased population differentiation via genetic drift (decreases heterozygosity and subsequent allele fixation) and inbreeding depression (increases homozygosity within populations). Awareness of genetic variability and diversity between and within different populations is therefore important for their conservation and management (Esfandani-Bozchaloyi *et al*., 2018a, 2018b, 2018c, 2017).

In our study, data on the genetic diversity in the 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* Mill., *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 were recognized in these species. The reticulate type of seed coat and detailed subtypes of reticulate

types were determined based on the 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 (Hou *et al.*, 2021; Huang, *et al*., 2021; Jia, *et al.*, 2020; Karasakal, *et al.*, 2020a; 2020b; Khayatnezhad and Gholamin, 2020a; 2020b).

Morphological studies of the studied *Paracaryum* species showed that both the quantitative (the ANOVA test result) and qualitative characters are well distinguished from each other (The PCA plot result). Furthermore, PCA analysis suggests that morphological characters, such as shape and size of leaves, size and indumenta of the calyx, corolla colour, corolla shape, wing and diameter of nutlets, the shape of nutlet and nutlet surface, may be used in the delimitation of species groups. Quantitative and qualitative characters were accounted for this morphological discrepancy. *Paracaryum* (*Mattiastrum*) *modestum* is cited as an unresolved name in http://www.theplantlist.org. The generic distinction (at least in the Iranian taxa) between *Paracaryum* and *Mattiastrum* (Boiss.) Brand 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 the nutlet or wing is flat or slightly inrolled and the aperture is not evident. *Paracaryum modestum* was transferred from *Paracaryum* to *Mattiastrum*.

Genetic Structure and Gene Flow

A primer's PIC and MI characteristics assist in assessing its usefulness in the study of genetic diversity. Sivaprakash *et al*. (2004) proposed that the ability to overcome genetic diversity by a marker technique could be more explicitly linked to the degree of polymorphism. In general, the PIC value between zero and 0.25 indicates a very low genetic diversity among genotypes, a mid-level of genetic diversity between 0.25 and 0.50, and a value of 0.50 indicates a high level of genetic diversity, between 0.25 to 0.50 shows a mid-level of genetic diversity and value ≥0.50 indicates a high level of genetic diversity (Khayatnezhad, and Gholamin, 2021; Guo *et al.*, 2021; Das *et al*., 2021; Zhao *et al*., 2021). In this study, the RAPD primers' PIC values ranged from 0.34 to 0.56, with a mean value of 0.49, indicating a moderate level ability of RAPD primers in determining genetic diversity among the *Paracaryum* species. Somewhat comparable but low PIC values have been reported with other markers like RAPD and AFLP in African plantain (Karasakal *et al.*, 2020a, 2020b, Khayatnezhad and Gholamin, 2020), ISSR and RAPD in *Salvia* species AFLP in wheat and SCoT markers (Hou *et al.*, 2021, Huang *et al*., 2021; Varamesh *et al*., 2014; Rajaei *et al*., 2020; Fataei *et al*., 2013, 2014; Sadigh *et al*., 2021). In CBDP markers were found to be more effective than SCoT markers about the average PIC which was higher. In our analysis, the RAPD markers were found to be successful in the estimating genetic diversity of *Paracaryum* species in terms of average percentage polymorphism (96.78%), average PIC value of RAPD markers (0.49), average MI (4.5) and average EMR of RAPD markers (11.8). However, various marker methods have been found to have a different resolution of the genome regions and the number of loci that cover the whole genome for genetic diversity estimation (Zheng *et al.*, 2021, Zhu *et al.*, 2021; Yin *et al.*, 2021; Si *et al*., 2020, Wang *et al.*, 2021; Paul *et al*., 2021; Wasana *et al*., 2021).

According to Chacón *et al*. (2016), the phylogenetic analyses based on sequences from three cpDNA regions successfully resolved some major issues about the monophyly of the main tribes of Boraginaceae and provided more detailed insights into the evolution of the Cynoglosseae s.l.

Detailed taxonomic and phylogenetic studies of Subtr. Cynoglossinae are required to resolve this complex group (Chacón *et al*., 2016). However, there is a whole range of segregate genera that have been proposed for *Cynoglossum* and their phylogenetic relationships are not at all resolved. Some of them may be monophyletic, but at present, all of them appear to be nested in *Cynoglossum* based on Chacón *et al*. (2016).

Omphalodes Moench and *Cynoglossum*, were retrieved as either poly or paraphyletic, showing that the morphological characters used in traditional taxonomic classifications are highly homoplasious (Weigend *et al*. 2013). Although the polytomies obtained in Weigend *et al*. (2013) are here largely resolved, most nodes have remained unsupported, and *Lindelofia*, *Mattiastrum*, *Microparacaryum*, *Paracaryum*, *Pardoglossum*, *Rindera*, *Solenanthus* and *Trachelanthus* are retrieved as either para-, or polyphyletic and/or nested in *Cynoglossum* s.str. as they also suggested in Selvi *et al*. (2011).

According to Ahmad *et al*. (2021) SRAP marker's genetic structure revealed that despite the existence of limited gene flow, two distinct ecotypes were produced which may be the consequences of reproductive isolation caused by altitudinal gradient and different niches through parapatric speciation. The heterozygosity (*H*) and Shannon index (*I*) reflect diversity and differentiation among and within the germplasm collections, respectively and the higher the indices, the greater the genetic diversity. The degree of variability among *N*a, *N*e, *H* and *I* indices using studied RAPD markers demonstrated a high level of genetic diversity among and within *Paracaryum* species. In conclusion, the findings of this study showed that the primers derived from RAPD were more effective than the other molecular markers in assessing the genetic diversity of the *Paracaryum*. In addition, the *Paracaryum* species in the dendrogram and PCA were clearly distinguished from each other, suggesting the greater efficiency of the RAPD technique in the identification of the genus.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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