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Species Identification and Population Structure of *Paracaryum* (Boraginaceae) of Iran using SCoT molecular markers

Abstract

Several of the endemic taxa of the distinctive genus Paracaryum, which are found in the Irano-Turanian phytogeographical area, are of unique passion. 139 bands have been generated from the amplification of genomic DNA using 10 primers, 124 of which were polymorphic (91.22%). The high capacity of SCoT primers to identify polymorphic loci among Paracaryum species was demonstrated by the acquired high average PIC and MI values. A range of 0.688 to 0.93 was estimated for the genetic similarity of 12 collections. The examination of SCoT markers revealed that the species Paracaryum bungei and Paracaryum salsum had the most similarity, while P. cyclhymenium and P. intermedium had the least. The aims of the present study are: 1) can SCoT markers identify Paracaryum species; 2) what is the genetic structure of these taxa in Iraq; and 3) to investigate the species inter-relationship. The current investigation has demonstrated that the species may be identified using SCoT markers.

Keywords: *Iraq, Species Identification; Structure, Paracaryum, SCoT (Start Codon Targeted)*

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Introduction

About 131 genera and 2,500 species make up the family Boraginaceae s.str, which is primarily found in arid, cliff-side, and sunny environments in western North America, Eurasia, and the Mediterranean region (Binzet and Akcin 2009). With over 900 species and 50 genera, the largest subfamily is Cynoglossoideae Weigend. Numerous previously known tribes fall within this subfamily, according to recent genomic research (Chacón et al. 2016). A molecular phylogenetic analysis of the generic complex Nonea/Elizaldia/Paraskevia/Pulmonaria (Boraginaceae-Boragineae) was carried out by Selvi et al. (2006) using ITS1 and trnLUAA sequences from non-coding nuclear and plastid DNA, respectively. Based on monophyletic groupings, their molecular results validate a redefinition of Nonea s.l. and are consistent with morphological, karyological, and chorological features. The Cynogolossinae Dumort subtribe of the Cynoglosseae tribe

Materials and Methods

Plant materials

Samples of 135 people from 12 different Paracaryum species were taken in July and August of 2018–2019, representing 15 different geographical populations. 135 plant accessions were sampled for the SCoT study from 15 different populations with varying eco-geographic features (five to twelve samples from each community), and they were stored in -20 until they were requested again.

DNA Extraction and SCoT Assay

In every population under study, one to twelve plants were chosen at random to receive fresh leaves. Powdered silica gel dried them. To extract genomic DNA, the CTAB-activated charcoal procedure was employed. By running the isolated DNA on a 0.8% agarose gel, the quality of the DNA was assessed. Ten primers with clear, enlarged, and diverse polymorphism bands were selected out of the 22 SCoT primers produced by Collard & Mackill (2009) (Table 1).

TABLE 1. SCoT primers used for this study and the extent of polymorphism.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
SCoT-1	CAACAATGGCTACCACCA	11	10	82.89%	0.43	5.56	6.34	3.11
SCoT-3	CAACAATGGCTACCACCG	17	17	100.00%	0.29	5.25	12.34	5.37
SCoT-6	CAACAATGGCTACCACGC	10	10	100.00%	0.37	4.88	10.56	2.85
SCoT-11	AAGCAATGGCTACCACCA	18	18	100.00%	0.35	5.23	6.23	4.47
SCoT-14	ACGACATGGCGACCACGC	12	12	100.00%	0.36	5.86	8.55	3.45
SCoT-15	ACGACATGGCGACCGCGA	10	9	84.99%	0.33	3.91	8.43	3.85
SCoT-16	CCATGGCTACCACCGGCC	19	19	100.00%	0.44	3.34	10.55	2.44
SCoT-17	CATGGCTACCACCGGCCC	10	10	100.00%	0.37	4.88	9.56	4.85
SCoT-18	ACCATGGCTACCACCGCG	15	14	93.74%	0.47	4.66	5.56	3.67
SCoT-19	GCAACAATGGCTACCACC	13	12	92.31%	0.34	5.21	8.60	3.55

Mean		13.9	12.4	91.22%	0.42	5.3	11	4.8
Total		139	124					

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

Data Analyses

Morphological Studies

The Euclidean distance between taxonomic pairings was originally calculated using morphological characters (Mean = 0, Variance = 1), that were initially standardized (Podani 2000). The plant specimens were grouped using the ordination technique of UPGMA (Unweighted paired group using average) (Podani 2000).

Results

Species identification and inter-relationship

Morphometry

ANOVA revealed statistically significant (P <0.01) variations in quantitative morphological traits across the examined species. To find the most variable characteristics among the taxa under investigation, PCA analysis was used. It was discovered that more than 62% of the variation was accounted for by the first three components. Characters that account for 44% of the total variance on the first PCA axis have the greatest correlation (>0.7): corolla color, faucal appendages, nutlet shape, nutlet length, and nutlet surface ornamentation. PCA axes 2 and 3 are influenced by corolla length, leaf length, and leaf width, respectively. Since several clustering and ordination methods produced equivalent results, the PCA plot of the morphological characteristics is displayed here (Figure 1). Plant samples from each species were often grouped to establish distinct groupings. This outcome shows that distinct groups were formed from the tested species according to both quantitative and qualitative morphological traits. Transitional forms were not present in any of the specimens that were being studied. The nutlets of Paracaryum are four, sub-orbicular, and infrequently ovate. The wings are incurved, membranous, and partially cover the disc. In P. strictum, the interior margin of the nutlets is denticulate, but in other species, it is not distinguishably denticulate. Broad, incurved wings with two rows of teeth on their borders are characteristic of Paracaryum sintenisii Hausskn. ex Bornm. The nutlets are 5-8 mm in diameter and can be smooth or have a few spinules on the keel. P. strictum has a disc echinulate, an incurved wing margin, three rows of teeth, and nutlets that are 3.6–3.9 mm in diameter. The nuts of P. rugulosum are 4-6 mm in size, smooth or sparsely echinulate, and the wing margins are incurved, rugulose, whole, or irregularly denticulate. Nutlets are ovate, 3-5 mm broad, with a papillose, broadly winged margin, and a small to large aperture in Paracaryum bungei, Paracaryum salsum, and Paracaryum intermedium, the middle of the dorsal area is smooth or glochidiate.

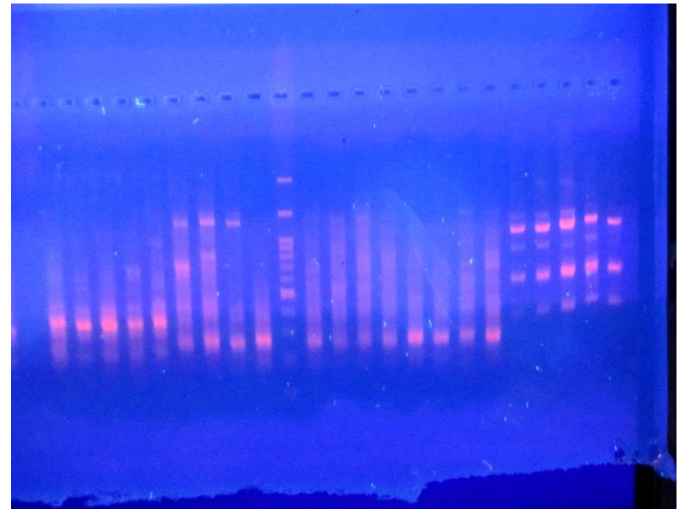

FIGURE 1. Electrophoresis gel of studied ecotypes from DNA fragments produced by SCoT-3, scot-11. sp1= *P. cyclhymenium*; sp2= *P. persicum*; sp3= *P. platycalyx*; sp4= *P. rugulosum*; sp5= *P. sintenisii*; sp 6= *P. strictum*; sp7= *P. undulatum*; sp8= *P. hirsutum*; sp9= *P. tenerum*; sp10: *P. bungei*; sp11: *P. salsum*; sp12= *P. intermedium*.

Species Identification and Genetic Diversity

The genetic connection between the 12 Paracaryum species was investigated using ten SCoT primers, all of which exhibited repeatable polymorphic bands. Figure 1 illustrates the SCoT amplification produced by the SCoT-3 and SCoT-11 primers. A total of twelve amplified polymorphism bands were generated by twelve distinct species of Paracaryum. The size of the amplified fragments ranged from 100 to 3000 bp. For SCoT-16, which has 19 polymorphism bands, and SCoT-15, which has 9 polymorphic bands, there were an average of 12.4 polymorphic bands per primer. The PIC of the 10 SCoT primers ranged from 0.29 (SCoT-3) to 0.47 (SCoT-18), with an average of 0.42 per primer. Each primer had a mean interval (MI) of 4.8, with a range of 2.44 (SCoT-16) to 5.37 (SCoT-3). The EMR of the SCoT primers ranged from 5.56 (SCoT-18) to 12.34 (SCoT-3), with an average of 11 per primer. (Table 1). Primers with the greatest EMR values were expected to be more effective in distinguishing between genotypes

The genetic characteristics of all 12 amplified Paracaryum species were calculated using SCoT primers (Table 2). With a mean of 0.20, the unbiased projected heterozygosity (H) varied from 0.11 (Paracaryum sintenisii) to 0.38 (Paracaryum strictum). Shannon's information index (I) revealed a similar trend, with a mean of 0.21 and the highest value of 0.37 for

Paracaryum strictum and the lowest value of 0.15 for Paracaryum sintenisii. In Paracaryum platycalyx, the number of alleles (Na) was found to be 0.29, while in Paracaryum bungei, it was 1.244. For Paracaryum tenerum, the effective number of alleles (Ne) was 1.457, whereas for Paracaryum persicum, it was 1.026. A significant genetic difference between the studied species was found using the AMOVA test (P = 0.001). According to Table 3, 74% of variance occurred across species, whereas 26% of variation was identified within species. Moreover, these species' genetic divergence was demonstrated by substantial Nei's GST (0.77, P = 0.001) and D_est (0.344, P = 0.001) values. These findings indicated a larger distribution of genetic variability within Paracaryum species compared to genetic diversity within species.

UPGMA clustering is displayed here since the outcomes of the other clustering and ordination methods looked comparable (Figure 2). Plant samples from different species were frequently arranged into different categories. This result suggests that it is possible to distinguish between the two primary clusters or groupings of Paracaryum species using the molecular features that were examined. We were unable to find any transitional forms in any of the specimens under study. The UPGMA tree's first primary cluster (Figure 2) included populations of Paracaryum bungei, Paracaryum salsum, and Paracaryum intermedium. These populations were generally located quite far apart from one another. There were two sub-clusters within the second major cluster. Plants of Paracaryum strictum, Paracaryum rugulosum, and Paracaryum sintenisii comprised the first sub-cluster, whereas P. cyclhymenium plants were present.

Relationships discovered with SCoT data often correspond well with species relationships discovered through morphological analysis. This aligns with previously released AMOVA and genetic diversity metrics. The species differ greatly from one another genetically. These findings suggest that Paracaryum species taxonomy can benefit from the application of SCoT molecular markers. The Popgene software's Nm analysis similarly generated a mean Nm=0.456, which is regarded as a very low value of gene flow among the species under study.

There was isolation by distance (IBD) among the Paracaryum species under study, as indicated by the Mantel test with 5000 permutations, which revealed a strong correlation (r = 0.56, p = 0.0002) between genetic distance and geographic distance.

Nei's genetic distance and the species' genetic distance were ascertained (Table 2). The results indicated that Paracaryum salsum and Paracaryum bungei had the highest level of genetic similarity (0.93). Between P. cyclhymenium and P. intermedium, there was the least genetic similarity (0.68). The low Nm value (0.123) signifies substantial genetic divergence both within and within Paracaryum species and suggests restricted gene flow or ancestrally shared alleles between the species under study.

TABLE 2. Genetic diversity parameters in the studied *Paracaryum* species.

SP	N	Na	Ne	I	He	UHe	%P
Paracaryum cyclhymenium	8.000	0.477	1.187	0.256	0.233	0.148	41.26%
Paracaryum persicum (Boiss.) Boiss. subsp. *persicum*	8.000	0.313	1.026	0.244	0.23	0.26	49.23%
Paracaryum platycalyx	12.000	1.244	1.322	0.29	0.284	0.192	50.91%
Paracaryum rugulosum	5.000	0.358	1.117	0.23	0.25	0.22	44.30%
Paracaryum sintenisii	6.000	0.458	1.039	0.15	0.18	0.11	39.38%
Paracaryum strictum	5.000	0.455	1.077	0.377	0.34	0.38	65.05%
Paracaryum undulatum	8.000	0.499	1.067	0.24	0.23	0.14	49.26%
Paracaryum hirsutum	12.000	0.347	1.199	0.271	0.284	0.192	55.91%
Paracaryum tenerum	5.000	0.358	1.457	0.234	0.30	0.31	56.50%
Paracaryum bungei	6.000	0.299	1.029	0.231	0.28	0.23	44.38%
Paracaryum salsum	3.000	0.567	1.062	0.24	0.224	0.213	44.73%
Paracaryum intermedium	8.000	0.499	1.067	0.20	0.281	0.14	49.26%

Abbreviations:

(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).

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

Source	df	SS	MS	Est. Var.	%	***ΦPT***
Among Pops	33	1601.364	88.789	14.134	74%	74%
Within Pops	130	274.443	2.805	3.858	26%	

Total	163	1855.80		17.060	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, ($P < 0.001$).

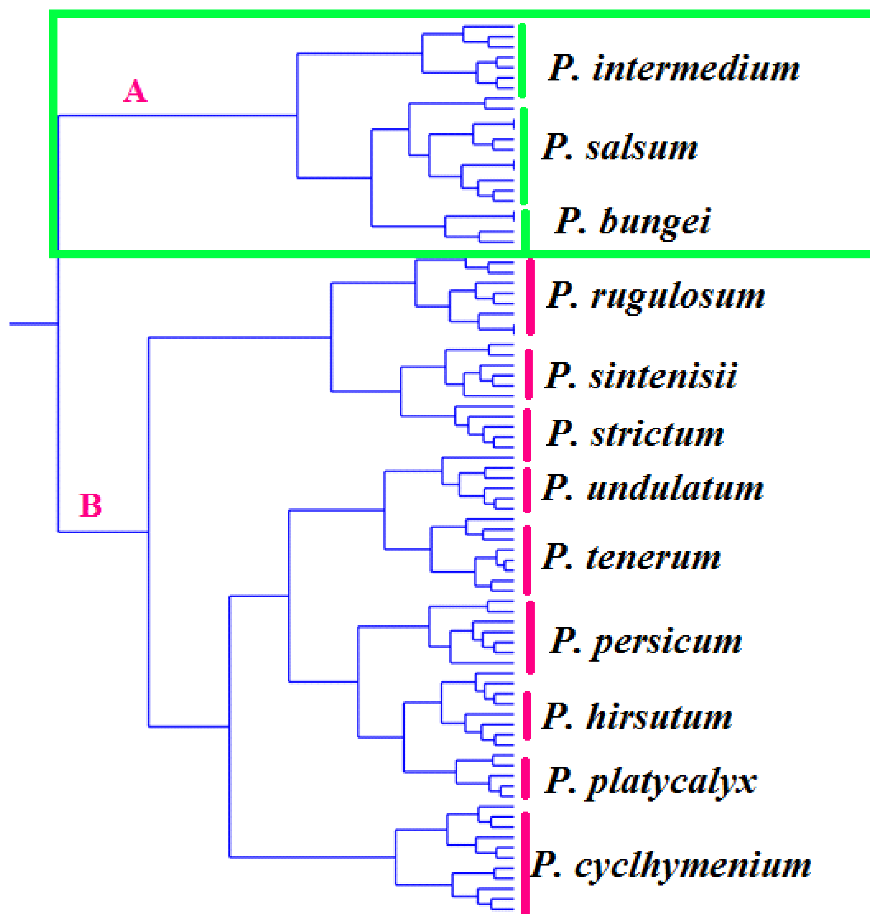

FIGURE 2: UPGMA tree of SCoT data revealing species delimitation in the *Paracaryum*.

Discussion

In the biology of a taxon's or population's long-term evolution, genetic diversity is crucial. the foundation for a taxon's emergence, growth, and evolution. Thus, understanding the taxonomy, genesis, and evolution of taxa requires an understanding of genetic diversity. Additionally, this research will provide a theoretical foundation for the development, utilization, conservation, and breeding of germplasm resources (Lubbers et al., 1991).

For the first time, morphology and genetic diversity in 12 Paracaryum taxa are provided in depth in our work. The current study set out to identify distinguishing characteristics between the several Paracaryum species found in Iraq. As previously mentioned, morphological characteristics are thought to be a helpful aid for species identification (Akcin, 2008). The morphological analyses of the examined Paracaryum species demonstrated that qualitative characteristics and quantitative measures (the outcome of the ANOVA test) could be separated from one another (the PCA plot result). Additionally, morphological traits like leaf size and shape, calyx size and indumentum, corolla color and form, nutlet wing and diameter, nutlet surface, and nutlet shape may be utilized to distinguish between different species groups, according to PCA analysis.

Genetic Structure and Gene Flow

The usefulness of a primer for researching genetic diversity is determined, in part, by its PIC and MI characteristics. Sivaprakash et al. (2004) suggest that the degree of polymorphism might have a direct correlation with the efficacy of the marker approach to overcome variation in genetics. PIC values often display the information below: According to Tams et al. (2005), a value of 0.50 indicates a high degree of genetic diversity; a value between 0.25 and 0.50 represents a mid-level of genetic diversity; and a value ≥0.50 indicates a high level of genetic diversity. Very low diversity in genetics exists between genotypes, as shown by the PIC value between zero and 0.25. The PIC values of the SCoT primers in the present investigation varied from 0.29 to 0.47, with a mean value of 0.42, suggesting that SCoT primers had a mid-level capacity

for determining diversity in genetics among the Paracaryum species. Ten primer pairs exhibited good polymorphism in the Paracaryum taxon. 139 distinct alleles were identified for the species under investigation. The range of polymorphic bands per primer was 9 to 19, with a mean allele number of 12.4 at each locus. The majority of research only uses a few vegetative accessions as the population size (Meusel et al., 1965; Uotila, 1996). Genetic drift may have occurred in this population, as evidenced by the low degree of genetic diversity and high FIS. The Paracaryum populations became fragmented due to demographic isolation and a lack of gene flow. Positive associations were found between genetic diversity metrics and population size, supporting the findings of several studies (Leimu et al. 2006). There are two reasons why genetic variation and population size have a positive association (Leimu et al., 2006). 1. A positive connection may indicate the existence of an extinction vortex, in which a decline in population size reduces genetic variety and causes a fall in inbreeding. Plant fitness distinguishes populations according to differences in habitat quality, which is the second explanation (Vergeer et al., 2003).

Low genetic variety may limit a population's capacity to adapt and select in response to shifting environmental conditions, which could lower plant fitness, based on Booy et al. (2000). 74% of the genetic variance was detected between the populations under evaluation, whereas only 26% of the diversity in genetics was found within populations. One of the main factors influencing the distribution of genetic diversity in plant species is their breeding strategy (Duminil, 2007). One migrant in each generation cannot assure the long-term survival of minor populations, based on Couvet (Booy et al., 2000). Instead, the number of migrants is dictated by life history attributes and population genetics (Vergeer et al., 2003).

The three groups' genetic variations were statistically significant but quite similar. There are two possible explanations for why isolated populations do not differ from one another. According to the first theory, gene flow mechanisms are demonstrated by genetic variety both inside and across populations, which causes larger populations to become fragmented (Dostálek et al., 2010). According to the second theory, populations that are closer together geographically are more effectively linked through gene flow than ones that are farther apart.

In the current study, we developed and used retrotransposon markers based on the SCoT technique in Paracaryum for the first time, taking advantage of the method's prevalence and abundance in plant genomes as well as its roles in genomic diversity.

In conclusion, the study's findings demonstrated that the primers produced from SCoT were more successful than other molecular markers in assessing the genetic diversity of the Paracaryum genus. Additionally, the dendrogram and PCA showed a strong separation of Paracaryum species from one another, demonstrating the greater identification effectiveness of the SCoT approach.

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Conflict of Interests

The authors have not declared any conflict of interest.

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In conclusion, the findings of this study highlight the utility of SCoT markers in species identification, the genetic structure of Paracaryum species in Iraq, and the elucidation of species inter-relationships. These findings contribute to both the fields of molecular ecology and biodiversity conservation, providing valuable information for the management and conservation of Paracaryum species in the Irano-Turanian phytogeographical area.

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