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GENETIC RELATIONSHIP AMONG SOME GENERA OF THE FAMILY CACTACEAE

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SUMMARY

The family Cactaceae comprises many genera, has taxonomic controversies at the species level, and yet has not been studied at the genetic level in Iraq. The presented study focused on species genotyping based on the RAPD-PCR method. The classification of six species, i.e., *Hylocereus undatus*, *Aloe vera*, *Opuntia ficus-indica*, *Espostoa guentheri*, *Echinocactus grusonii*, and *Mammillaria elongata*, ensued based on phenotypic characters to determine their precise taxonomic names and evaluate kinship by constructing the phylogeny tree RAPD-PCR. The short oligo primers showed the highest polymorphic bands (100%), with no monomorphic and basal bands correlated among these species. This highly polymorphic relationship indicated that each species has a superior identity and unique evolutionary trend. The oligo primers were considered productive by showing highly distinct and sharp bands, while others showed faint bands. This research confirmed the efficacy of RAPD primers in measuring polymorphism, comparing genotypes, and identifying Cacti species using specialized RAPD markers.

Keywords: Cactaceae, genotyping, RAPD-PCR, *Opuntia, Mammillaria*

Key findings: Using RAPD molecular markers, the study revealed a highly polymorphic relationship among the species of the family Cactaceae and has a superior identity and unique evolutionary trend.

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INTRODUCTION

The Cactaceae family is part of the order Caryophyllales and comprises around 300 genera and 2,000 species of spiky succulents with photosynthetic stems (Nyffeler and Eggli, 2010). Several species of the family Cactaceae are ecologically specialized to tolerate salty and desert-like environments and wildly distributed and dominant in alkaline and arid areas of Canada, Chile, Argentina, Africa, India, Australia, Asia, and, especially, Iraq (Perumal *et al*., 2021).

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In the family Cactaceae, the diverse plants revealed fleshy with tufts of thorns, stems flattened, jointed, succulent, epigynous, tepals indefinite with a gradual transition from sepals to petals, stamens indefinite, inferior ovary unilocular, parietal placentae, and fruit berry (Brockington *et al*., 2009; Flowers *et al*., 2010; Qiu *et al*., 2010; Crawley and Hilu, 2012). In Iraq, the family Cactaceae includes many common genera like *Mammillaria*, *Echinocactus*, *Opuntia*, *Hylocereus*, *Espostoa*, and others.

Many popular plants of the family Cactaceae are useful as dyes, stover, medicines, and edibles. For decades, Cacti species have attracted botanists and plant lovers, and many of them are now grown as pot plants because of their peculiar behaviors and colorful, enormous blooms (Stintzing *et al*., 2003). Cactus is not generally consumed due to its acidic flavor but also due to the significant number of seeds within the fruit. Yet, current research has revealed that natural cacti offer some health and medicinal advantages (Soel *et al*., 2007).

The Cactus plants served as essential herbals due to their multiple uses in treating many human complications like diabetes, burns, ulcers, and lung cancer (Qadir, 2009). Prickly pear cactus pads became a food source for the indigenous O'odham people. Moreover, the O'odham divided and cooked cactus pads before applying them to bodily joints to treat arthritis and rheumatism (Lakhssassi *et al*., 2017).

Unfortunately, in Iraq, the *Cactaceae* genera are yet for morphological and genetic level classification, and various taxonomic controversies have appeared in several species in this family. More than a decade of molecular phylogenetic research has yielded a plethora of suggestions about relative association within the family Cactaceae. Most recent discoveries were unexpected and greeted with skepticism, yet, in many other instances, confirmed the prior knowledge.

Hence, Nyffeler and Eggli (2010) captured these ideas in the form of a phylogenetic summary tree of the estimated connections among the major species of the Cactaceae and transformed this hypothesis into a hierarchical suprageneric category device highlighting four subfamilies, i.e., Cactoideae, Opuntioideae, Pereskioideae, and Maihuenioideae, eight tribes, i.e., Cylindropuntieae, Opuntieae, Blossfeldieae, Cacteae, Phyllocacteae, Notocacteae, Rhipsalideae, and Cereeae, and six subtribes, i.e., Corryocactinae, Hylocereinae, Echinocereinae, Rebutiinae, Cereinae, and Trichocereinae.

Molecular tools may also provide essential information on the genetic distances among the various species. The random amplified polymorphic DNA (RAPD) markers have become more popular for analyzing the genetic diversity of different species. Nowadays, molecular markers have shown effectiveness in determining the genetic diversity among and within species (Charcosset and Moreau, 2004; Al-Nema and Abdullah, 2023; Mohsin *et al*., 2023). These molecular markers might be employed as supplementary approaches to morphological descriptors to show their promise (Demey *et al*., 2003). RAPD marker helps compare DNA in biological systems that have received little attention from the scientific community or a limited number of DNA sequences available for comparison (Anderson *et al*., 2018).

In recent years, several studies on the genetic diversity of the cactus pear have undergone by various researchers. Using RAPD and ISSR markers, Valadez-Moctezuma *et al.* (2014) investigated the prevailing genetic diversity within and among several Mexican *Opuntia* species. Labra *et al*. (2003) investigated the genetic diversity of *Opuntia* species using cpSSR and AFLP markers. Many studies emphasized the efficacy of RAPD in diagnosis like Zoghlami *et al*. (2007), who assessed the genetic diversity of *Opuntia ficus-indica* in Tunisia; El-Kharrassi *et al.* (2017) investigated to evaluate the genetic diversity within and among *Opuntia spp*. from different regions of Morocco, using morphological descriptors, as well as, ISSR and RAPD markers, and Rabeh *et al*. (2020) study pointed out that RAPD markers are an effective tool to authenticate eight *Opuntia* species in Egypt. Based on these points, the presented research aimed to offer a comprehensive

overview of the phylogenetic connections among various species of the family Cactaceae as presumed by molecular phylogenetic investigations, such as RAPD-PCR methodologies.

MATERIALS AND METHODS

Plant samples collection and DNA extraction

Procuring six plant species of the family Cactaceae ensued from various arboretums in Babylon province, Iraq (Table 1, Figure 1). Prof. Dr. Huda Altameme, a plant taxonomist, preliminarily classified these species at the Department of Biology, College of Science for Women, University of Babylon, based on phenotypic characterization and by adopting available scientific references, such as, Matthews's description (1972) in Flora of Turkey and East Aegean Islands and Chaudhary (2001) in Flora of the Kingdom of Saudi Arabia. The study period was from September to December 2022 at the Laboratory of Science College for Women, University of Babylon, Iraq. The plants were

Table 1. Plant species with their families used in the study.

No.	Plant Species	Family/Subfamily	Tribe
1	*Hylocereus undatus*	Cactaceae /Cactoideae	Phyllocacteae
2	*Aloe vera*	Asphodelaceae /Asphodeloideae	-
3	*Opuntia ficus –indica*	Cactaceae /Opuntioideae	Opuntieae
4	*Espostoa guentheri*	Cactaceae /Cactoideae	Phyllocacteae
5	*Echinocactus grusonii*	Cactaceae /Cactoideae	Cacteae
6	*Mammillaria elongata*	Cactaceae /Cactoideae	Cacteae

Figure 1. The phenotypic forms: 1) *Hylocereus undatus,* 2) *Aloe vera*, 3) *Opuntia ficus – indica,* 4) *Espostoa guentheri,* 5) *Echinocactus grusonii,* and 6) *Mammillaria elongata*.

Table 2. RAPD primers used in genotyping the plants under study.

No.	Primer	Sequence 5' to 3'	References
1	OP-V19	GGGTGTGCAG	Ismail *et al*. (2020)
2	OP-R06	GTCTACGGCA	Ismail *et al*. (2020)
3	OP-V14	AGATCCCGCC	Ismail *et al*. (2020)
4	OP-V09	TGTACCCGTC	Habeeb *et al*. (2022)
5	OP-L05	ACGCAGGCAC	Habeeb *et al*. (2022)
6	OP-M05	GGGAACGTGT	Habeeb *et al*. (2022)
7	OP-P04	GTGTCTCAGG	Habeeb *et al*. (2022)

cut into small pieces, then placed in a ceramic vase, got crushed by adding liquid nitrogen continuously until they became a fine powder. From the leaves sample (0.05 g), isolating the genomic DNA was according to the protocol of the ZR Plant/Seed DNA MiniPrep kit (Cat No. D6020 /Zymo/USA).

After calibration with elution buffer, a Nanodrop spectrophotometer deposited 1 µl DNA extract on its sensitive lens. Plant extract DNA concentrations were measured at 260 and 280 wavelengths.

RPD-PCR assay

Seven primers were used for RAPD-PCR amplification (Table 2). The 25 µL reaction mixture used for the RAPD-PCR amplification process contained 5 µL of Taq PCR PreMix, 1 µL of each primer (10 pmol), and 2 µL of genomic DNA (20 ng/µL). Adding sterilized distilled water adjusted the final quantities up to 25 µL. The Multi Gene OptiMax Gradient Thermal Cycler (Germany) carried out the RAPD-PCR-based amplification as follows: Initial denaturation was at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 1 min at the annealing temperature of 45 °C, with an extension at 72 °C for 1 min. The final extension came at 72 °C for 5 min.

Gel electrophoresis and data analysis

Separating the findings of the RAPD through gel electrophoresis involved running the PCR product through 2% agarose gel in TBE buffer. Using a 100 bp DNA ladder and the staining process for Red Safe Nucleic Acid Staining Solution (20,000x), the estimation of the size of the DNA bands on the gel continued. Viewing the agarose gel used a UV illuminator (CBS, Scientific Electrophoreses USA), with each primer's number of strips and frequency of polymorphisms determined separately (Al-Tameme, 2018).

According to the existence and absence of the bands (allele), the amplification bands received scores of '1' and '0.' The RAPD fragment frequencies calculation progressed for each species. The PhotoCapt Molecular Weight technique helped estimate the size of amplification bands.

Calculating the percentage of the polymorphism of the primer was according to the following equation (Grundmann *et al*., 1995):

$$\text{Percentage (\%) Polymorphism per primer} = \frac{\text{Number of variant bandles in the primer}}{\text{total number of primer bands}} \times 100$$

Computing the percentage of discriminatory power for each primer was according to the following equation:

$$\text{Discrimination power per primer (\%)} = \frac{\text{Number of differential bands of the primer}}{\text{number of differential bands of all primers}} \times 100$$

As for the percentage of the efficiency of each primer, its calculation comprised the following equation:

$$\text{The efficiency of each primer (\%)} = \frac{\text{Total number of primer bands}}{\text{total number of all primer bands}} \times 100$$

The genetic dimension coefficient between samples' computation was according to Nies' (Nie and Lie, 1979) equation:

G.D. = 1-([2*Nxy]/[Ny+Nx])

Where:
Nxy = number of bands shared by both models X and y are samples.
The sample's total bands are Nx.
The sample's total bands are Ny.

For creating a dendrogram or genetic dimension, the cluster analysis scheme used the UPGMA technique (Sneath and Sokal, 1973) and the ready-made application Numerical Taxonomy System (NTSYS-pc).

RESULTS AND DISCUSSION

The results of the extracted DNA from six species of plants by using the ZR Plant/Seed DNA MiniPrep kit appeared in Figure 2 and Table 3. The results presented *Mammillaria elongata* was responsible for the highest DNA concentration (8.8 ng/µl), whereas the lowest was the species, *Aloe vera* (5.9 ng/µl). The purity of DNA was high in most species under interest, ranging from 1.8 to 2. The DNA concentration's fluctuation may be correlated with the chemical compound that constitutes each species. However, carbohydrates may sometimes cause a reduction of available DNA during extraction methods, and handling can induce fluctuations in DNA concentration, as well as, the age and environmental conditions of the plant material impact the effectiveness of high-quality DNA isolation (Ramos *et al*., 2014).

Isolating DNA from cactus species is a challenging task since these plants have a significant concentration of polysaccharides and secondary metabolites, which may form insoluble complexes with nucleic acids during the process of DNA extraction (Guillemaut and Maréchal-Drouard, 1992). However, these secondary metabolites and polysaccharides have been observed to inhibit enzyme activity in cactus species plants (Porebski *et al.*, 1997).

The presented results also showed that seven primers provided successful fingerprints of the six species of plants, showing the polymorphic bands that depended on genetic variations among these species under genotyping (Table 2). The number of bands per

Table 3. An illustration of measuring DNA concentration and purity.

No.	Species	Nucleic acid Conc. (ng/µl)	260/280 purity
1	*Hylocereus undatus*	6.8	1.8
2	*Aloe vera*	5.1	1.81
3	*Opuntia ficus-indica*	5.9	1.8
4	*Espostoa guentheri*	7.2	1.96
5	*Echinocactus grusonii*	8.5	1.83
6	*Mammillaria elongata*	8.8	2.0

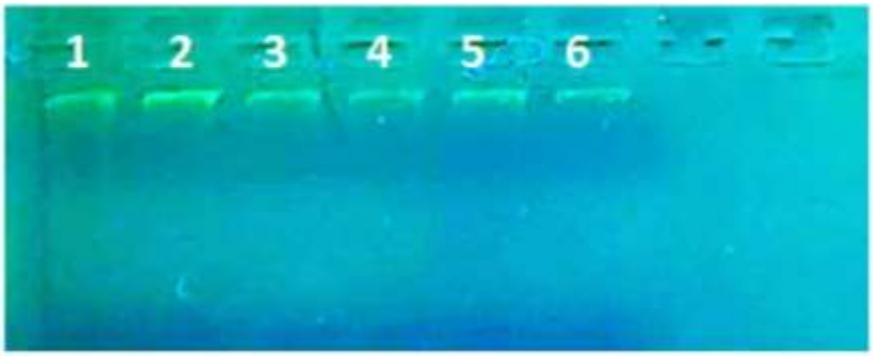

Figure 2. Genomic DNA from six plant species' electrophoresis on a 1% agarose gel at 70 volts for 30 min.

Table 4. Plant species genetic polymorphism based on a technique of RAPD-PCR by seven primers, total bands productivity, heterogenetic, primer efficiency %, and discrimination.

Primer name	Total number of bands	Heteromorphic band number	Polymorphism per primer (%)	Primer efficiency (%)	Discrimination of primer (%)
OP-V19	15	15	100	13.3	13.3
OP-R06	19	19	100	16.8	16.8
OP-V14	21	21	100	18.6	18.6
OP-V09	13	13	100	11.5	11.5
OP-L05	16	16	100	14.2	14.2
OP-M05	18	18	100	15.9	15.9
OP-P04	11	11	100	9.73	9.73
	113	113		100	100

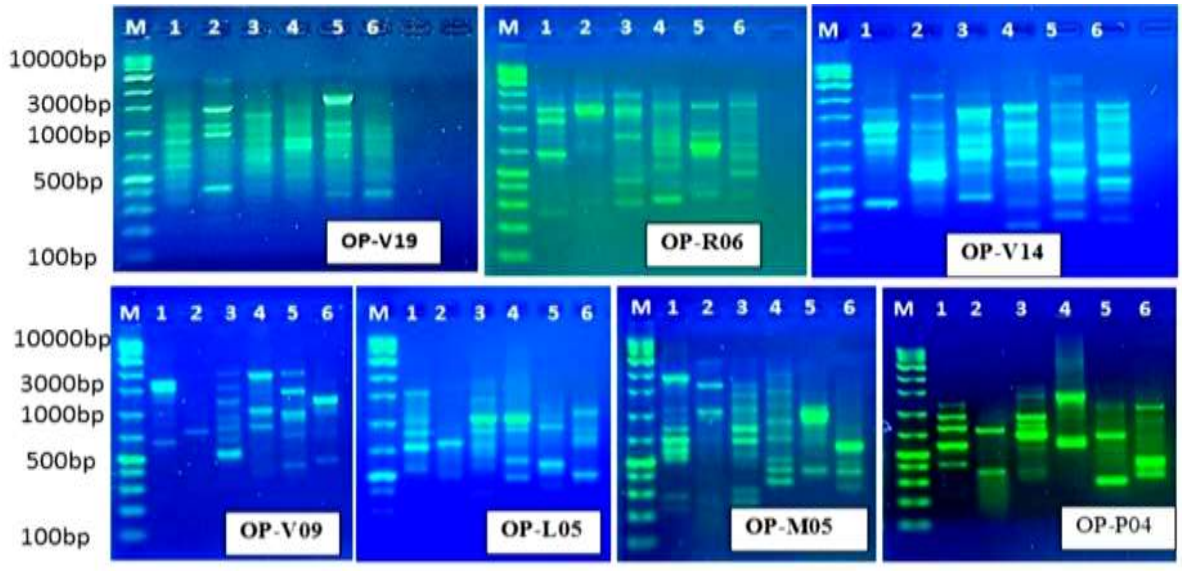

Figure 3. RAPD-PCR product of primers: OP-V19, OP-R06, OP-V14, OP-V09, OP-L05, OP-M05, and P0-PO4, respectively. The product's electrophoresis on 2% agarose at 70 volts. 1x TBE buffer for 1:30 h. M: ladder upper band 10kbp and first band 0.1kbp (100 bp).

plate indicated higher genetic variations in Figure 3. Using seven primers, the genotyping results displayed various band patterns for each studied species. The total polymorphic bands were 113. The novelty of the present research was that all the band sizes and sites exhibited the highest polymorphism. The highest percentage of the polymorphic bands was 100% (Table 4); however, at the same time, the genotyping by seven oligo primers showed the absence of a monomorphic band in two species, indicating that all species were genetically far away from each other.

The productivity of six primers under pursuit showed variability, and some primers showed potentially better for genotyping all the plant species, producing highly distinctive and sharp bands by four primers, i.e., OP-RO6, OP-VO9, OP-MO5, and OP-PO4 (Figure 3). However, other primers showed faint bands and were confused with each other.

The OP-V14 was one of the best primers by showing the most number of bands, as well as, the efficiency of the primer and the discriminating ability (Table 4). It also exhibited distinction by the presence of 21

heteromorphic bands, as reflected in the efficiency of the primer and its discriminating ability, amounting to 18.6%. The primer OP-R06 follows, showing 19 heteromorphic bands with an efficiency and discriminatory capacity of 16.8%, followed by OP-M05 and OP-L05. Meanwhile, the primer OP-P04 displayed the least number of bands amounting to 11, with an efficiency and discriminatory capacity of 9.73% (Table 4).

The phylogeny tree of six plant species was based on the coefficient distance among the species. Based on RAPD-PCR, the tree construction for various plant species showed that three species were clustered in clade-1 and two in clade-2, while *Aloe vera* diverged from these five species. The clades clustering indicated that five species belonged to the family Cactaceae, and, at the same time, the clustering raised a doubt that *Aloe vera* might belong to the family Asphodelaceae and not to Cactaceae. The presented results also got support from the past findings of *Aloe vera* var. Chinensis (Wang *et al*., 2004). Several variables, like the primer design, the template amount, and the small number of linkage sites in the genome, also contributed to this variability (Figure 4).

The five species belonging to the family Cactaceae underwent a comparison in genetic affinity with one of the succulent plants, *Aloe vera*, to find the relationship of affinity among them. It was proven by the evolutionary tree among the six species using the RAPD-PCR method based on the coefficient distance between species. The existing three different clades showed that the first clade combines the two species, i.e., *Echinocactus grusonii* and *Mammillaria elongata,* with a ratio of approximately 27.9 (Figure 4 and Table 5). This convergence was due to the joining of both species to the subfamily Cactoidae and the tribe Cacteae.

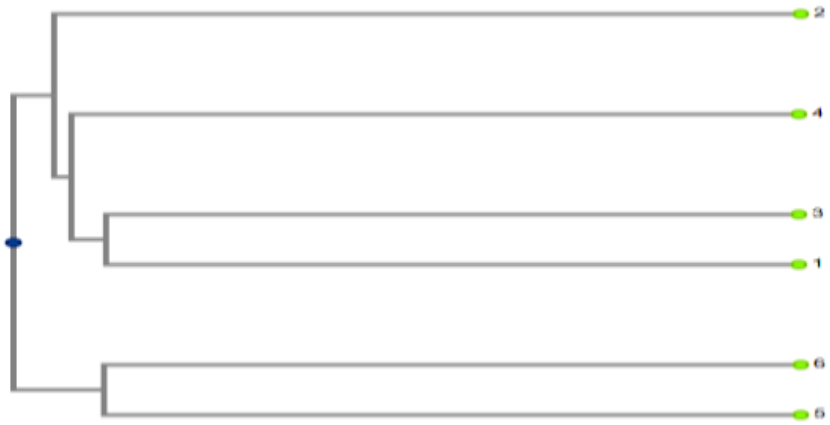

Figure 4. Phylogenetic tree dendrogram (homologous coefficient %) among various plant species based on the RAPD–PCR primers.

Table 5. Similarity Matrix computed with Jaccard's coefficient among various species.

Species	1	2	3	4	5	6
1	100	22.1	28.2	27.1	19	16.7
2		100	23.9	22	16.7	15.5
3			100	21.9	17.5	18.3
4				100	25.8	17.7
5					100	27.9
6						100

1-*Hylocereus undatus* 2 -*Aloe vera* 3 -*Opuntia ficus –indica* 4 -*Espostoa guentheri* 5 -*Echinocactus grusonii* ,6 -*Mammillaria elongata.*

The various morphological and molecular traits' assessment revealed the subfamily Cactoideae as a well-known monophyletic group. Cortical vascular bundles, which create a three-dimensional network across the cortex, served as diagnostic tools for all the taxa except *Blossfeldia*, making up the majority of the family's 1,530 species of cacti (Mauseth and Sajeva, 1992; Anderson, 2005). The globular was depressed to short columnar cacti that make up the Cacteae tribe, ranging in size from miniature to colossal. *Echinocactus* stalks can have ribs, as those of *Coryphantha* (Engelmann) Lemaire, which can have tubercles (Charles *et al*., 2002).

The second clade includes the two species, i.e., *Hylocereus undatus* and *Opuntia ficus-indica,* at a ratio of approximately 28.2, which were associated with the species *Espostoa guentheri*. This association illustrates the similarity in some phenotypical characteristics of the two tribes, Phyllocacteae and Opuntieae, belonging to the subfamily Cactoideae and Opuntioideae, respectively. Several molecular investigations further indicate the monophyly of the taxon and the extremely well-circumscribed subfamily Opuntioideae (Wallace and Dickie, 2002; Griffith and Porter, 2009). Generally, all the species have distinct segments in their stems, and many species exhibited the classic "prickly pear" development shape. In addition to regular, retrorsely barbed spines, the areoles frequently generate large amounts of unpleasant glochids. Although glochids and spines develop simultaneously; however, glochids are not sclerotic at the base, making them more brittle (Robinson, 1974).

Concerning the third clade, it confirms the isolation of the *Aloe vera* species from the cactus group, as it belongs to the family Asphodelaceae and the subfamily Asphodeloideae. The genotyping-based RAPD-PCR revealed that *Aloe vera* does not belong to the family Cactaceae, removing the relationship ambiguity of *A. vera* to the family Cactaceae and confirming it belongs to the family Sphodelaceae. All cactus plants are succulent, but not all succulents are cacti. Distinguishing the cactus plant from the group of succulent plants can be through the presence of thorns arranged in a halo shape, in the middle of which is a focal thorn, surrounded by a smooth area devoid of hairs and spines. These results were also consistent with the past findings, as mentioned in dividing the cactus family into subfamilies and tribes (Wallace, 1995; Wallace and Dickie, 2002; Griffith and Porter, 2009; Nyffeler and Eggli, 2010).

The observed high degree of variation may refer to the fact that the study of DNA variability was based on RAPD markers (Singh *et al*., 2006). The lowest similarity among the studied species arose due to genotyping of different species. Wholly, RAPD-PCR by arbiter primer differentiated among the closed related biotypes or some strains in the same species. Although, these RAPD-PCR genotyping results differ from other genotyping patterns in various past studies (Abdul *et al*., 2014; Al-Tamimi, 2019; Anis and Al-Dulaimi, 2020). The genotyping arrangement also revealed that each plant species in focus was a dependent taxonomic unit with a unique evolutionary line (Greenberg and Donoghue, 2011). These conclusions also accord with Wang *et al*. (2004) that diverse variables, including primer structure, template quantity, and the limited number of binding sites in the genome, produced this variability.

CONCLUSIONS

The RAPD technique proved an authenticated way to know how genetically close and far apart plants are from different places, species, and genera of the family Cactaceae. Comparing the RAPD data with the botanical data can provide accurate conclusions. The dendrogram made by the computer processing of the RAPD data showed how some populations of cacti were related phylogenetically and how genetically different from this family as a whole.

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