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Survey the Genetic Diversity of Eight *Opuntia* Species from Egypt using Random Amplified Polymorphic DNA (RAPD) Technique

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Abstract

Opuntia is a large genus of succulent shrubs characterized by unique attractive flowers known as prickly pears by virtue of their characteristic edible fruits. They are used as laxative, diuretic, antipyretic, and anti-inflammatory.

This study aims to authenticate eight different *Opuntia* species, *Opuntia brasilliensis* (Willd.) Haw (O_1), *Opuntia dillenii* (ker Gawl.) Haw. (O_2), *Opuntia dejecta* Salm-Dyck (O_3), *Opuntia ficus indica* (L.) Mill. (O_4), *Opuntia tomentosa* Salm-Dyck (O_5), *Opuntia phaecantha* Engelm. (O_6), *Opuntia leucotricha* DC. (O_7) and *Opuntia microdasys* (Lehm.) Pleiff. (O_8), growing in Egypt by using random amplified polymorphic DNA technique in which five primers are used. Accurate identification of the eight *Opuntia* species is an urgent need to maintain herbal formulations potency and peculiarity.

Similarity coefficients of 40.0 - 67.6 % classified *Opuntia* species into two main groups, one of them contains two species and the other one contains six species. Consequently, this technique helps the identification of these *Opuntia* species showing great morphological similarity.

1. Introduction

The genus *Opuntia* belongs to family Cactaceae, comprises about 250 species [1] and is widely spread in Africa, Mediterranean basin as well as American hemispheres and Mexico [2]., it has been used in traditional folk medicine [3], as hypoglycemic [4], stomach ulcers treatment [5], has anti-inflammatory neuroprotective effects through antioxidant actions [6], and also used for treating burns, bronchial asthma, diabetes and indigestion in many countries over the world [7, 8]. These plants are rich in antioxidant compounds (phenols, flavonoids, betaxanthin and betacyanin) and vitamins (ascorbic acid, vitamin E) [1, 2, 3].

Based on species, cultivars or geographical origin, DNA fingerprinting provides an objective evaluation of genetic identity of plants to establish genetic uniformity of raw herbal materials. For medicinal herbs, synthesis and agglomeration of active constituents depend on both genetic makeup as well as environmental conditions.

Random amplified polymorphic DNA (RAPD) analysis is rapid, inexpensive and easy to perform PCR-based method in comparison with other DNA–based markers and extensively used for authentication of closely related plant species that are medicinally important [9], as well as to assure activity and prevent adulteration which is crucial to be achieved at the international trade scale of medicinal plants where the identification of herb by morphological [10] and anatomical [11] approaches require a professional taxonomist's expertise.

The rigorous identification of the eight *Opuntia* species is a compelling need to maintain efficacy as well as quality of herbal formulations. Our chief objective is to establish an accurate identification of the eight *Opuntia* species using DNA-based marker.

2. Materials and methods

2.1. Plant materials

The cladodes of different *Opuntia* species (**Figure 1**), *Opuntia brasilliensis* (Willd.) Haw. {recently known as *Brasiliopuntia brasiliensis* (Willd.) A. Berger} (O_1), *Opuntia dillenii* (ker Gawl.) Haw. {recently known as *Opuntia stricta* var. dillenii (ker Gawl.) L.D. Benson (O_2), *Opuntia dejecta* Salm-Dyck {recently known as *Nopalea dejecta* (Salm-Dyck) Salm-Dyck (O_3), *Opuntia ficus indica* (L.) Mill. (O_4), *Opuntia tomentosa* Salm-Dyck (O_5), *Opuntia phaecantha* Engelm. (O_6), *Opuntia leucotricha* DC. (O_7) and *Opuntia microdasys* (Lehm.) Pleiff. (O_8), were gathered in December 2017 from El-Orman Botanical Garden, Giza, Egypt. The plants were kindly identified and authenticated by Eng. Trease Labib Yousef, National Gene Bank and El-Orman Botanical Garden taxonomy consultant. Voucher specimens were deposited in the Pharmacognosy Department herbarium of Faculty of Pharmacy,

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Figure 1: Photos of the eight *Opuntia* species

Cairo University, Cairo, Egypt (voucher no. 24.12.17.1-8). Prior to DNA isolation, samples of cladodes are freeze – dried, and ground to a fine powder using a small grinder. DNA analysis was conducted at the Food Technology Research Institute, Agriculture Research Center, Ministry of Agriculture and Land Reclamation, Giza, Egypt in 2018.

2.2. Materials for DNA analysis

2.2.1. Buffers

Extraction buffer: using 100 Mm Tris (PH 7.5), 0.01 M EDTA, 0.7 M NaCl, 1% (W/V) N-cetyl- N,N,N-trimethylammonium bromide (CTAB), and 1% (v/v) β-mercaptoethanol (added immediately before use).Washing buffers 1:76% ethanol, 0.2 M Na – acetate; washing buffer 2: 10 mM ammonium acetate buffer, 76% ethanol. TE buffer: 10 mM tris (pH 8.3), isopropanol, d NTP, 500 mM KCl, 0.01% (w/v) gelatine, chloroform / isoamyl alcohol 24:1 (v/v), and Taq DNA polymerase.

2.2.2. Primers

Five primers were used for random amplified polymorphic (RAPD) analysis purchased from Operon Technologies Inc. with the following sequences. OP-A02 [TGCCGAGCTG], OP-B04 [GGACTGGAGT], OP-B07 [GGTGACGCAG], OP-B11 [GTAGACCCGT], OP-C04 [CCGCATCTAC].

2.2.3. Molecular weight marker

100 bp ladder, from Promega Corporation.

2.2.4. Apparatus

An amplification of DNA was done using a DNA thermocycler (Hybaid PCR Express). Separation of RAPD fragments according to size was done using an agarose gel electrophoresis tool (Bioard wide Mini Sub Cell), and visualization of the RAPD fragments by a UV Polarid camera.

2.3. Methods for molecular investigations

2.3.1. DNA extraction

Extracted DNA using the CTAB method [12].Sample of frozen cladodes (50 mg, each) were powdered in liquid nitrogen, separately extracted with 0.8 ml CTAB, precipitated with isopropanol, washed in 70 % ethanol and dissolved in deionized water.

2.3.2. Estimation of DNA concentration

To determine the DNA concentration, DNA was diluted in ratio 1:5 in distilled water. DNA samples was subjected to electrophoresis in 1 % agarose gel against 10 µg of a DNA size marker. This marker covers a range of concentrations between 95 ng and 11 ng. Hence, DNA concentration estimation in a given sample was obtained by comparing the degree of fluorescence of the unknown DNA band with the different bands in the DNA size marker.

2.3.3. Amplification of the RAPD markers

100 ng of genomic DNA template following a thermal cyclic program were used to in performing the PCRs.

2.3.4. Thermo cycling profile

A Perkin- Elmer / Gene Amp PCR system 9700 (PE Applied Biosystems) programmed to fulfil 40 cycles after an initial denaturation cycle for 5 min at 94 ^{0}C was used to perform PCR amplification. Each cycle consisted of a denaturation step at 94^0 C for 1 min., an annealing step at 36 ^{0}C for 1 min. with an elongation step at 72 ^{0} C for 1.5 min. the primer extension segment was extended to 7 min at 72 ^{0}C in the final cycle. Electrophoresis in 1.5 % agarose gel supplemented with ethidium bromide (0.5 µg/ml) in 1 % TBE buffer at 95 volts was used to resolve the amplification products.

2.4. RAPD extraction method

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Extraction of DNA from fresh cladodes was achieved by using the Qiagen DNeasy Kit and a Perkin-Elmer/GeneAmp PCR system 9700 (PE Applied Biosystems) to attain PCR amplification. Resolve the amplification products by electrophoresis, examined visually, and scored for the absence (0) or presence (1) of DNA bands. A similarity matrix was obtained via the cluster analysis of data using unweighted pair group method with arithmetic average (UPGMA). Each of the DNA bands was treated as a unit character for estimating genetic distance among the tested samples, [13].

2.5. Data analysis

RAPD bands were scored as absent (0) or present (1) and treating each of which was as an independent character regardless of its intensity. Only consider prominent and reproducible bands obtained for each RAPD primer. Species-specific bands were identified by comparing the banding patterns of species for a primer. Faint or unclear bands were not treated or considered.

2.6. RAPD analysis

The CTAB method [12] was adopted to extract DNA from fresh cladodes by using the Qiagen DNeasy Kit. Performing PCR amplification by a Perkin-Elmer/GeneAmp PCR system 9700 (PE Applied Biosystems) followed by electrophoresis to resolve amplification products, examined visually, and scored for the absence (0) or presence (1) of DNA bands. Cluster analysis of data using unweighted pair group method with arithmetic average (UPGMA) was used to obtain the similarity matrix. Each of the DNA bands was treated as a unit character for estimating genetic distance among the tested samples [13].

3. Results and Discussion

Eight species of *Opuntia* from Egypt was chosen for testing the reliability of authentication and quality using RAPD techniques. The fresh cladodes of the eight species are more or less similar in morphology to each other and not easily identified (**Figure 1**). Genomic DNA of five decamer primers was used to carry out the RAPD technique. The banding profile produced by the five primers (OP-A02, OP-B04, OP-B07, OP-B11 and OP-C04) is illustrated in (**Table 1**), (**Table 2**) and (**Figure 2**). Considering RAPD bands as present or absent, without considering their percentage. The number of RAPD-PCR fragments designated that the five primers were reproducible. Each DNA band was considered as a unit character. Sixty-five different fragments have been recorded. OP-A02 primer Showing 8 bands ranging from 0.355 kbp to 2.61 kbp divided into 2 monomorphic bands, 2 polymorphic bands, 4 unique bands and the percentage of polymorphism is 75%. OP-B04 primer showing 12 bands ranging from 0.215 kbp to 1.0 kbp divided into 2 monomorphic bands, 9 polymorphic bands, 1 unique band and percentage of polymorphism is 83.33%. OP-B07 primer showing 10 bands ranging from 0.125 to 1.13 divided into 3 monomorphic, 7 polymorphic and percentage of

polymorphism is 70%. 0P-B11 primer showing 19 bands ranging from 0.35 to 1.73 divided into 2 monomorphic band, 8 polymorphic bands, 9 unique bands and percentage of polymorphism is 89.47%. OP-C04 showing 16 bands ranging from 0.33 to 1.9 divided into 3 monomorphic bands, 7 polymorphic bands, 6 unique bands and the percentage of polymorphism is 81.25%. All *Opuntia* species were conceivably discriminated by the presence or absence of unique fragments in the RAPD profile and the total number of unique fragments specific to each species with different primers was summarized in (**Figure 2**), (**Tables 1 and 2**). Dendrogram used in computational biology to illustrate genes clustering and can be used to differentiate medicinal species from their adulterants or closely related species including *Aloe* species (Cactacea) [14], *Echinacea* species (Asteraceae) [15].

A dendrogram using unweighted pair group method with arithmetic average (UPGMA) analysis. The eight *Opuntia* species under investigation were clustered into two main groups with Jaccard's similarity coefficient ranging from 0.40 to 0.542 ((**Figure 2**). and (**Table 2**). *Opuntia leucotricha* DC. and *Opuntia microdasys* (Lehm.) Pleiff were contined in the first main group. While the second main group divided into two sub main groups one of them contains two species, *Opuntia brasilliensis* (Willd.) Haw and *Opuntia dillenii* (ker Gawl.) Haw. The other one divided into two sub groups.

The first group contains *Opuntia dejecta* Salm-Dyck and *Opuntia ficus indica* (L.) Mill. and the second one contains *Opuntia tomentosa* Salm-Dyck and *Opuntia phaecantha* Engelm.

DNA is a constitutional building component of all living cells. The specific arrangement of DNA base-pair sequences leads the production of proteins and enzymes. These in turn decide features such as flower color, leaf shape and direct the synthesis of wide range of phytochemicals in plants [16]. Analysis of unique genetic structure are higher level markers based on DNA marker is considered as fingerprint and usually independent of environment and is consistent throughout different parts and developmental stage of the organism. DNA fingerprints similarity depend on genetic closeness of the tested samples. DNA fingerprinting can distinguish plants from different families, genera, closely related species and cultivars. It can assure genetic uniformity of crude herbal materials. For medicinal herbs, production of chemical constituents depend on both genetic map as well as environmental conditions. DNA profiling can be applied in Individual identification of plant in a very small amount, differentiation between closely related species, detection and production of genetically engineered plants, follow up the effects of environmental conditions on DNA, forensic detection of toxicity caused by certain plant and mutation studies.

In the present study the eight *Opuntia* species were exposed to RAPD assay of their DNA genomes for identification and authentication, this was performed using five RAPD primers produced 65 polymorphic bands. The RAPD-PCR fragments number proves that the five were reproducible. DNA sequence variation led to polymorphism, and greater the polymorphism
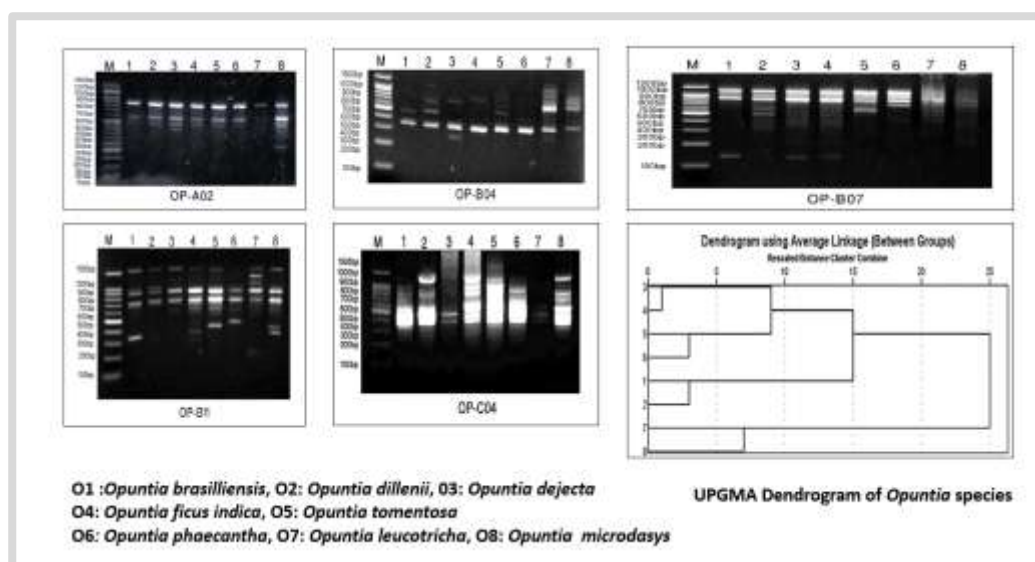
Figure 2: RAPD analysis carried out with primers OP-A02, OP-B04, OP-B07, OP-B11, OP-C04 and unweighted pair group method with arithmetic average (UPGMA) dendrogram showing the clustering of the eight *Opuntia* species based on RAPD data.

Table 1: Polymorphism degree and polymorphic information content for the interspecies genetic relationship in the eight *Opuntia* species.

Primer code	Primer Sequence	Monomorphic	Unique bands	Polymorphic (without unique)	Polymorphic (with unique)	TNB	% P	Mean of band frequency
OP-A02	TGCCGAGCTG	2	4	2	6	8	75	0.516
OP-BO4	GGACTGGAGT	2	1	9	10	12	83.33	0.396
OP-BO7	GGTGACGCAG	3	0	7	7	10	70	0.725
OP-B11	GTAGACCCGT	2	9	8	17	19	89.47	0.395
OP-CO4	CCGCATCTAC	3	6	7	13	16	81.25	0.445

TNB; Total number of bands, % P: % polymorphism.

Table 2: Genetic similarity matrix using RAPD data among eight *Opuntia* species (O1-O8)* estimated following Jaccarrd's method.

				Similarity matrix				
	O1	**O2**	**O3**	**O4**	**O5**	**O6**	**O7**	**O8**
O1	**100**							
O2	59.5	**100**						
O3	40.0	54.1	**100**					
O4	48.8	51.2	67.6	**100**				
O5	59.0	53.7	53.8	61.9	**100**			
O6	52.8	47.4	47.2	52.5	59.5	**100**		
O7	40.0	39.0	50.0	44.2	46.3	47.2	**100**	
O8	44.7	46.8	50.0	54.2	50.0	51.2	53.5	**100**

* (O₁), *Opuntia brasilliensis* (Willd.) Haw, (O₂), *Opuntia dillenii* (ker Gawl.) Haw. (O₃), *Opuntia dejecta* Salm-Dyck, (O₄) *Opuntia ficus indica* (L.) Mill., (O₅), *Opuntia tomentosa* Salm-Dyck, (O₆) *Opuntia phaecantha* Engelm., (O₇) *Opuntia leucotricha* DC., (O₈) *Opuntia microdasys* (Lehm.) Pleiff.

can indicate a greater genetic diversity. All eight species of *Opuntia* were differentiated from each other on the basis of unique band obtained in PCR amplification. O_8 had three unique bands (260,360, 380 bp) with OP-A2 and one unique band (325bp) with OP-CO4, O_3 had unique band (355bp) with OP-AO2 and unique band (215bp) with OP-BO4, O_6 had unique band (455bp) with OP-B11 and unique band (970bp) with OP-CO4, O_1 had unique band (308bp) with OP-B11, O_2 had unique band (320bp) with OP-B11, O_5 had two unique bands (1070, 1900bp) with OP-CO4, O_4 had three unique bands (560, 565, 1035bp) with OP-CO4 and three unique bands (295,365, 425 bp), O_7 had two unique bands (215,1200 bp) with OP-AO2.

Among the different primers used OP-BO4, OP-B11 and OP-CO4 composed maximum number of polymorphic bands and may be used for the identification of these *Opuntia* species. OP-B11 primer was found to be the most effective in generating polymorphic bands with 89.47% on application of RAPD technique followed by OP-B04 with 83.33%, OP-CO4 with 81.25%. From the aforementioned findings, it can be wrap up that this primers can be used to discriminate between eight species of *Opuntia* present in Egypt depending on their low similarity coefficients values and high level of polymorphism.

Cluster analysis showed genetic divergence among these species, the eight species clustered into two main groups, the first main group contains two species, *Opuntia leucotricha* DC. and *Opuntia microdasys* (Lehm.) Pleiff. While the second main group divided into two sub main groups one of them contains two species, *Opuntia brasilliensis* (Willd.) Hawa nd *Opuntia dillenii* (ker Gawl.) Haw. The other one divided into two sub groups. The first group contains *Opuntia dejecta* Salm-Dyck and *Opuntia ficus indica* (L.) Mill. and the second one contains *Opuntia tomentosa* Salm-Dyck and *Opuntia phaecantha* Engelm. Genetic divergence among the eight species in cluster analysis can be correlated to similarities in morphological features in each single group like flowers color, cladodes shapes or fruits color.

4. Conclusion

The plant identification is the first step to assurance efficacy, quality, and safety of a drug or an extract. Our present study clearly point out that RAPD markers as effective tool to authenticate our eight *Opuntia* species in the local herbal markets.

Acknowledgements

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