

# Genetic Diversity and Relationships Among *Nopalea* sp. and *Opuntia* spp. Accessions Revealed by RAPD, ISSR and ITS Molecular Markers

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## Research Article

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# Abstract

## Background

*Dactylopius opuntiae* (Cockerell) (carmine cochineal) is an insect pest highly noxious that has spread through cactus pear crops in the Brazilian semiarid region. Knowledge of diversity and genetic relationships of the cactus pear accessions is fundamental to create new varieties resistance to carmine cochineal. Therefore, this investigation was undertaken to assess the genetic diversity and genetic relationships that existed in between cactus pear (*Nopalea* sp. and *Opuntia* spp.) accessions with contrasting resistance to *D. opuntiae*.

## Methods

We conducted a molecular analysis in seven cactus pear accessions from the “reference collection” of the Agronomic Institute of Pernambuco, Brazil using RAPD, ISSR and ITS molecular markers.

## Results

A total of 242 bands were detected from 26 polymorphic primers. The high percentage of polymorphism by RAPD (89.8%), ISSR (81.2%) and ITS (75%) markers suggests that the cactus pear accessions have high genetic diversity. The combined analysis of markers enabled the accessions discrimination of about the genus and ploidy, but were incongruous in relation to resistance level to *D. opuntiae*.

## Conclusions

The resistant accessions (IPA-200016, IPA-200149, IPA-100004, IPA-200205) are genetically divergent, therefore could be potentially incorporated into any further breeding programs directed to create new varieties of cactus pear resistant to *D. opuntiae*.

## 1. Introduction

The cactus pear *Opuntia* and *Nopalea* are widely cultivated for forage purposes in several arid and semi-arid regions of the world [1]. The succulent stems (cladode) of cactus pear are rich in water, sugars, ash, vitamins A and C, but are poor in crude protein and fiber [2].

In the Brazilian Semiarid Region, cactus pear became the basic food for domestic ruminants. However, since 2001 the main cultivated varieties of the *Opuntia* (Giant, Round and IPA-20) have been decimated by carmine cochineal (*Dactylopius opuntiae* Cockerell), a pest insect that has caused great economic losses to local cattle breeders [3, 4].

The Agronomic Institute of Pernambuco (IPA), a Brazilian research and rural extension agency located in the country semi-arid region, has several accessions of cactus pear with contrasting resistance to carmine cochineal in its germplasm bank [5], but its diversity and genetic relationships have not yet been sufficiently clarified. Therefore, it is vital that the germplasm of this collection is properly characterized, as they will be useful to plan future improvement strategies aimed at creating new varieties resistant to carmine cochineal.

The basic characterization of cactus pear germplasm collections is generally performed using morphological descriptors, but they may be insufficient to distinguish varieties or related species. This characterization has been enhanced with the use of polymerase chain reaction (PCR)-based molecular markers, such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR) and internal transcribed spacer (ITS), allowing studies of intraspecific genetic variation in several organisms [6], with applications in several studies in the field of genetics such as diversity, phylogeny, and evolutionary biology.

This investigation was undertaken to assess the genetic diversity and genetic relationships that existed in between seven cactus pear (*Nopalea* sp. and *Opuntia* spp.) accessions with contrasting resistance to *D. opuntiae*.

## 2. Material And Methods

### 2.2. Sampling of accessions

Seven cactus pear accession (Table 1) from the germplasm bank of the Agronomic Institute of Pernambuco (IPA), Arcoverde city, Pernambuco state, Brazil was evaluated. The classification of chromosome number was based to Majure et al. [7] and the level of resistance to carmine cochineal was based on Silva et al. [8] and Lopes et al. [9].

Table 1  
Code, species, chromosome number and resistance level of seven cactus pear accessions

Accession	Specie	Chromosomal number	Resistance level
IPA-100003	<i>Opuntia ficus-indica</i> Mill	2n = 88	Susceptible
IPA-200008	<i>Opuntia atropes</i> Rose	2n = 22	Tolerant
IPA-200016	<i>Opuntia stricta</i> Haw	2n = 66	Resistant
IPA-200149	<i>Opuntia larreyi</i>	2n = 44	Resistant
IPA-100004	<i>Nopalea cochenillifera</i> Salm-Dyck	2n = 22	Resistant
IPA-200021	<i>Nopalea cochenillifera</i> Salm-Dyck	2n = 22	Tolerant
IPA-200205	<i>Nopalea cochenillifera</i> Salm-Dyck	2n = 22	Resistant

## 2.3. Genomic DNA extraction

Genomic DNA was extracted from tissue samples from the young stems (cladodes) epidermis based on the protocol of Doyle and Doyle [10]. The DNA concentration in the samples was estimated by electrophoresis on a 0.8% agarose gel, comparing the fluorescence intensity of the ethidium bromide-stained DNA bands with a known DNA standard (Invitrogen™ 1 Kb Plus DNA Ladder).

## 2.4. DNA amplification via PCR using RAPD, ISSR and ITS primers

PCR-RAPD reactions were performed using 12 primers (Table 2) from Operon Technologies Inc., Alameda, CA, USA (OP06, OPG07, OPG10, OPC11, OPS03, OPG19, OPA02, OPG03, OPG13, OPG15, OPG06 and OPM12) suggested by Mondragon-Jacob et al. [11] for detection of genetic polymorphism in cactus pear. RAPD reactions were prepared with a final volume of 25 µl containing 50.0 ng DNA, 1X PCR buffer [100 mM Tris-HCl (pH 8.5)], 3.0 mM MgCl<sub>2</sub>; 200 µM dNTP's; 1 µM of primer, 1 unit of Taq DNA polymerase) and ultra-pure water to complete the volume. The protocol for DNA amplification, after preliminary tests, consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 minute; annealing the primers at 40°C for 1 min; extension at 72°C for 1 min and final extension at 72°C for 5 min using a thermal cycler model TC-Plus Techne Bibby Scientific Ltd.

Table 2  
List of RAPD, ISSR and ITS primers used

Marker	Primer	Sequence 5'— 3'
RAPD	OPG 07	GAA CCT GCG G
RAPD	OPG 10	AGG GCC GTC T
RAPD	OPC 11	AAA GCT GCG G
RAPD	OPS 03	CAG AGG TCC C
RAPD	OPG 19	GTC AGG GGC A
RAPD	OPA 02	TGC CGA GCT G
RAPD	OPG 03	GAG CCC TCC A
RAPD	OPG 13	CTC TCC GCC A
RAPD	OPG 15	ACT GGG ACT C
RAPD	OPG 06	GAA CGG ACT C
RAPD	OPM 12	GGG ACG TTG G
ISSR	UBC 01	ACA CAC ACA CAC ACA CT
ISSR	UBC 02	GAG AGA GAG AGA GAG AT
ISSR	UBC 857	ACA CAC ACA CAC ACA CYG
ISSR	UBC 808	AGA GAG AGA GAG AGA GC
ISSR	UBC 810	GAG AGA GAG AGA GAG AT
ISSR	UBC 827	AC AC AC AC AC AC AC AC G
ISSR	UBC 830	TG TG TG TG TG TG TG TG G
ISSR	UBC 842	GA GA GA GA GA GA GA GA YG
ISSR	UBC 849	GT GT GT GT GT GT GT GT YA
ISSR	UBC 855	AC AC AC AC AC AC AC AC YT
ISSR	UBC 862	AGC AGC AGC AGC AGC AGC
ISSR	UBC 866	CTC CTC CTC CTC CTC CTC
ITS	ITS 1	TCCGTAGGTGAACCTGCGG
ITS	ITS 4	TCCTCCGCTTATTGATATGC

PCR-ISSR reactions were performed using 12 primers (Table 2) from the Nucleic Acid-Protein Service Unit, University of British Columbia, USA (UBC01, UBC02, UBC857, UBC808, UBC810, UBC827, UBC830,

UBC842, UBC849, UBC855, UBC862 and UBC866) suggested by Mergulhão et al. [12] and Alves et al. [13] for detection of genetic polymorphism in cactus pear. PCR reactions were prepared with a final volume of 25 µl containing 50.0 ng of DNA, 1X PCR buffer [100 mM Tris-HCl (pH 8.5)], 3.0 mM MgCl<sub>2</sub>, 200 µM dNTP's, 1 µM primer, 1.0 unit of Taq DNA polymerase and ultra-pure water to complete the volume.

The protocol for DNA amplification consisted of an initial denaturation of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 seconds; annealing the primers at 52°C for 45 seconds; extension at 72°C for 2 min and final extension at 72°C for 5 min [12] using a thermal cycler model TC-Plus Techne Bibby Scientific Ltd.

The ITS region of nuclear ribosomal DNA was amplified using the ITS1 and ITS4 primers (Table 2) suggested by Lyra et al. [14] for molecular studies in cactus pear. PCR reactions were prepared with a final volume of 25 µl containing 20.0 ng of DNA; 1.0 µl 1X PCR buffer [100 mM Tris-HCl (pH 8.5), 0.25 mM dNTP's, 3.0 mM MgCl<sub>2</sub>; 1.0 µM of primers, 1.25 units of Taq DNA polymerase and ultra-pure water to complete the volume [15]. The protocol for DNA amplification consisted of an initial denaturation of 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 seconds; annealing at 62°C for 1 min; extension at 72°C for 2 min and final extension at 72°C for 5 min using a thermal cycler model TC-Plus Techne Bibby Scientific Ltd.

## 2.5. Amplicons separation by gel electrophoresis

Amplicons separation was performed by horizontal electrophoresis in agarose gel (1.5%) with ethidium bromide dye (0.5 ug mL<sup>-1</sup>). Amplicon migration was performed in 0.5X TBE running buffer (Tris, boric acid, EDTA) at a voltage of 100 V for 120 min. A 100 bp molecular weight standard (Invitrogen™ 1 Kb Plus DNA Ladder) was used. The gel was visualized under ultraviolet light and photodocumented with the aid of the Gel Logic 112Pro imaging system (Carestream, Rochester, NY).

## 2.6. Data analysis

The bands generated by each primer were classified as monomorphic and polymorphic and their presence/absence computed for all sampled. Subsequently, the total number of amplified bands, number of monomorphic bands, number of polymorphic bands and percentage of polymorphism for each primer were calculated.

The presence/absence data were organized into a binary matrix (1 = presence and 0 = absence) which was used to determine the binary Sokal distance using the Genes software [16]. Using the Sokal binary distance values, a dissimilarity dendrogram was constructed based on Ward [17] clustering method. The definition of the number of groups was performed based on the criterion proposed by Mojena [18] using bootstrap analysis to verify and provide statistical support to the internal nodes of the dendrograms. The validation of the clusters was determined by calculating the cophenetic correlation coefficient [19]. Additionally, a principal component analysis was performed using the Past program [20].

## 3. Results

The 26 primers (12 RAPD, 12 ISSR and 2 ITS primers) used for the evaluation of DNA polymorphism in cactus pear accessions produced 242 polymorphic reproducible bands. The amplification patterns of some of the RAPD (OPG15), ISSR (UBC 810) and ITS (ITS 1 and ITS4) primers can be seen in Fig. 1.

RAPD primers amplified from 5 to 12 bands with a mean polymorphism of 90.6% (66.7-100%) and generated 98 bands of which 88 (89.8%) were polymorphic. The ISSR primers amplified from 8 to 19 bands with a mean polymorphism of 78.7% (25-100%) and generated 140 bands, of which 115 (82.1%) were polymorphic. ITS primers amplified 4 bands of which 3 (75%) were polymorphic. These results present that the set of cactus pear accessions has high molecular polymorphism.

The Sokal binary distance between accession pairs ranged from 0.46 to 0.65 with a mean value of 0.57, indicates high genetic dissimilarity between accessions. However, the *Nopalea* sp. accessions were more similar to each other than the *Opuntia* spp. Accessions. The *Nopalea* sp. accessions IPA-100004 and IPA-200021 (0.46) were the most similar and IPA-100004 and IPA-100005 (0.52) were the most genetically dissimilar. The *Opuntia* spp. accessions IPA-200008 and IPA-200149 (0.53) were the most similar and IPA-100004 and IPA-100005 (0.61) were the most genetically dissimilar.

In the cluster analysis, it was found that the cophenetic correlation coefficient obtained from the genetic distance matrix and the cophenetic distance matrix was high and significant ( $r = 0.80$ ;  $P \leq 0.01$ ), indicating that there was high consistency in the grouping patterns [19]. Furthermore, the distortion (10.04%;  $P \leq 0.01$ ) and stress (7.10%;  $P \leq 0.01$ ) values according to the Kruskal scale [21] also indicate that the graphical representation of the dissimilarity in the dendrogram was highly faithful to the matrix of dissimilarity.

Taking the dissimilarity of 1.37 as the cutoff point in the dendrogram, the accessions formed two main clusters (Fig. 2). This grouping pattern was consistent with the genus of accessions, as those to *Opuntia* (IPA-100003, IPA-200008, IPA-200016 and IPA-200149) form a distinct grouping of *Nopalea* (IPA-100004, IPA-200021 and IPA-200205) accessions. However, there was no consistent grouping pattern regarding the level of resistance of accessions to *D. opuntiae*, as we found that resistant and susceptible accessions were allocated to similar subgroups (Figure 2).

The distribution of accessions in the components (CP1 x CP2) of the Principal Component Analysis (Figure 3) corroborated with the Cluster Analysis, as the accessions were also allocated in groups consistent with gender. There was less dispersion among the *Nopalea* cluster accessions in relation to the cluster formed by *Opuntia* spp. in which the IPA-2000149 and IPA-200008 accessions formed a distinct subgroup of IPA-100003 and IPA-200016.

## 4. Discussion

The plant breeding requires a profound knowledge about the diversity and genetic relationships from basic population. Efforts in reaching this goal can be supported by the application of molecular markers.



In this study, we used three molecular marker systems (RAPD, ISSR and ITS) to detect genetic polymorphism among forage cactus accessions with contrasting resistance to *D. opuntiae*.

All RAPD and ISSR primers produced consistent and reproducible amplification profiles and with higher informativity than in other similar studies. For example, the average percentage of polymorphic bands revealed by the RAPD (90.6%) and ISSR (78.7%) primers in our study was higher than that verified in the research by Luna-Paez et al. [22] (RAPD = 58.3%; ISSR = 44.0%), Valadez-Moctezuma et al. [23] (RAPD = 37.0%; ISSR = 28.6%), Bendhifi et al. [24] (RAPD = 73.21%) and Tütüncü et al. [25] (RAPD = 72%). We also demonstrated that being able to detect greater genetic polymorphism of RAPD (90.6%) was higher in relation to ISSR (78.7%) in cactus pear, corroborating the data reported by Valadez-Moctezuma et al. [23]. The high polymorphism of ITS markers (75%) was also similar to that verified by Lyra et al. [14], who were able to discriminate cactus pear accessions in relation to genus, similarly to what was observed in this study.

The high genetic polymorphism detected by RAPD (89.8%), ISSR (82.1%) and ITS (75%) markers and the high dissimilarity between accession pairs (binary Sokal distance) suggest that there is high genetic diversity between accessions being, therefore, a suitable reference set for breeding programs. The high genetic diversity of this set can be explained by its composition that includes different species. The greatest genetic similarity between accessions IPA-100004, IPA-200021 and IPA-200205 is due to the fact that both belong to the species *N. cochenillifera*.

The structuring of accessions in clusters according to genus indicates that the analysis of RAPD, ISSR and ITS markers can also be useful for phylogeny studies in the *Opuntiae* family. The structuring of the accessions was also consistent in relation to the ploidy, because when observing the dendrogram (upper part towards the base) there was an increase in the number of chromosomes in the accessions (22, 44, 66 and 88 chromosomes, respectively). The PCA also supported this, as the IPA-200021, IPA-200149 e IPA-200205 (*N. cochenillifera*) accessions with the lowest ploidy ( $2n = 22$ ) are grouped into a single group in the lower left part of the graph, while the IPA-200016 (*O. stricta*) and IPA-100003 (*O. ficus-indica*) accessions with the highest ploidy ( $2n = 66$  and  $2n = 88$ , respectively) are grouped in a single group in the lower right part of the graph. The other accessions IPA-200008 (*O. artropes*) and IPA-200146 (*O. larreyi*) are forming a group with intermediate ploidy level ( $2n = 22$  and  $2n = 44$ , respectively), in the upper part of the graph. However, this clustering pattern was not consistent with the susceptibility degree to *D. opuntiae*. The development of molecular markers linked to the loci encoding important physiological and morphological traits related to cochineal resistance will allow design of more powerful strategies for early identification of resistant genotypes.

Accession pairs that are resistant to *D. opuntiae* IPA-100004 and IPA-200205 (*Nopalea*) and IPA-200016 and IPA-2000149 (*Opuntia*) are genetically divergent and therefore promising to generate segregating populations for intraspecific and interspecific crossing, respectively. However, the differences in chromosome number between *Opuntia* accessions suggests chromosomal incompatibility (Table 1).

Based on the results, the combined analysis of RAPD, ISSR and ITS molecular markers proved to be a valuable tool to accessing genetic diversity and discriminate cactus pear accessions. We conclude that is an important genetic diversity at the DNA level among the cactus pear accessions from Agronomic Institute of Pernambuco, Brazil, which could be potentially incorporated into any further breeding programs directed to create new varieties of cactus pear resistant to carmine cochineal.

## Declarations

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### Competing Interests

*The authors have no relevant financial or non-financial interests to disclose.*

### Author Contributions

Janieire Dorlamis Cordeiro Bezerra, Albericio Pereira de Andrade, Mailson Monteiro do Rêgo, Riselane de Lucena Alcântara Bruno and Divan Soares da Silva contributed to the study conception and design. Material preparation, data collection and analysis were performed by Janieire Dorlamis Cordeiro Bezerra, José Ribamar Silva do Nascimento Júnior, Elizanilda Ramalho do Rêgo and Angela Maria dos Santos Pessoa. The first draft of the manuscript was written by anieire Dorlamis Cordeiro Bezerra, Fernando dos Santos Araújo, Roberta de Lima Valença and Ariosvaldo Nunes de Medeiros and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

### Ethics approval

Not applicable.

### Consent to participate

Not applicable.

### Consent to publish

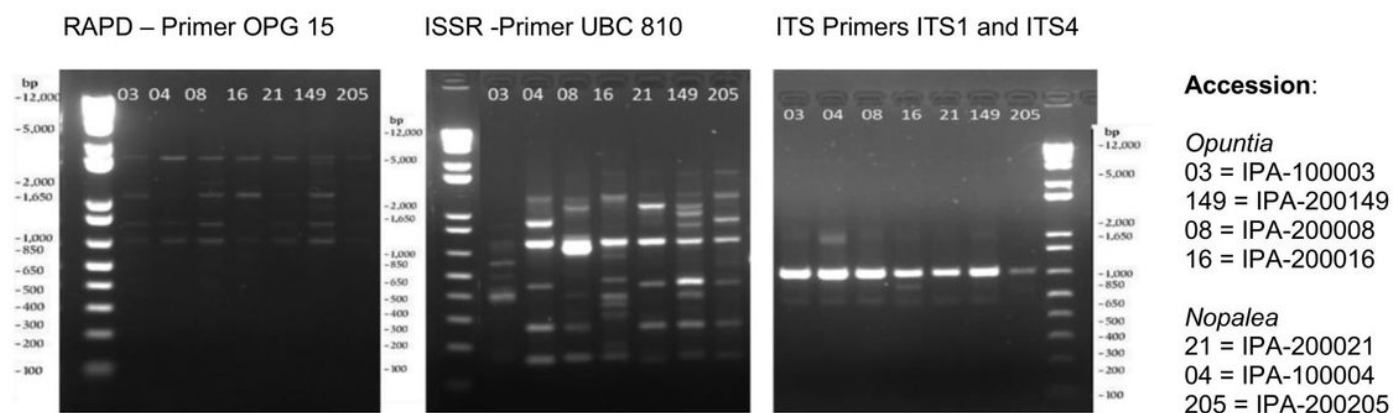
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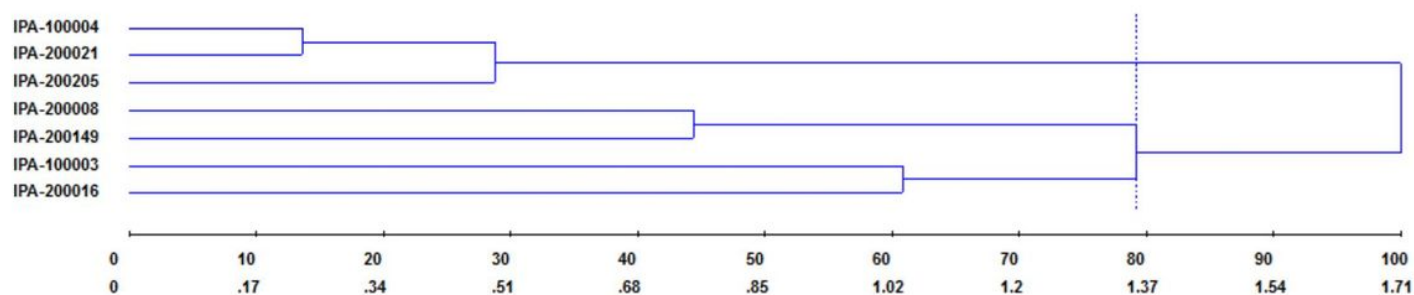
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## Figures



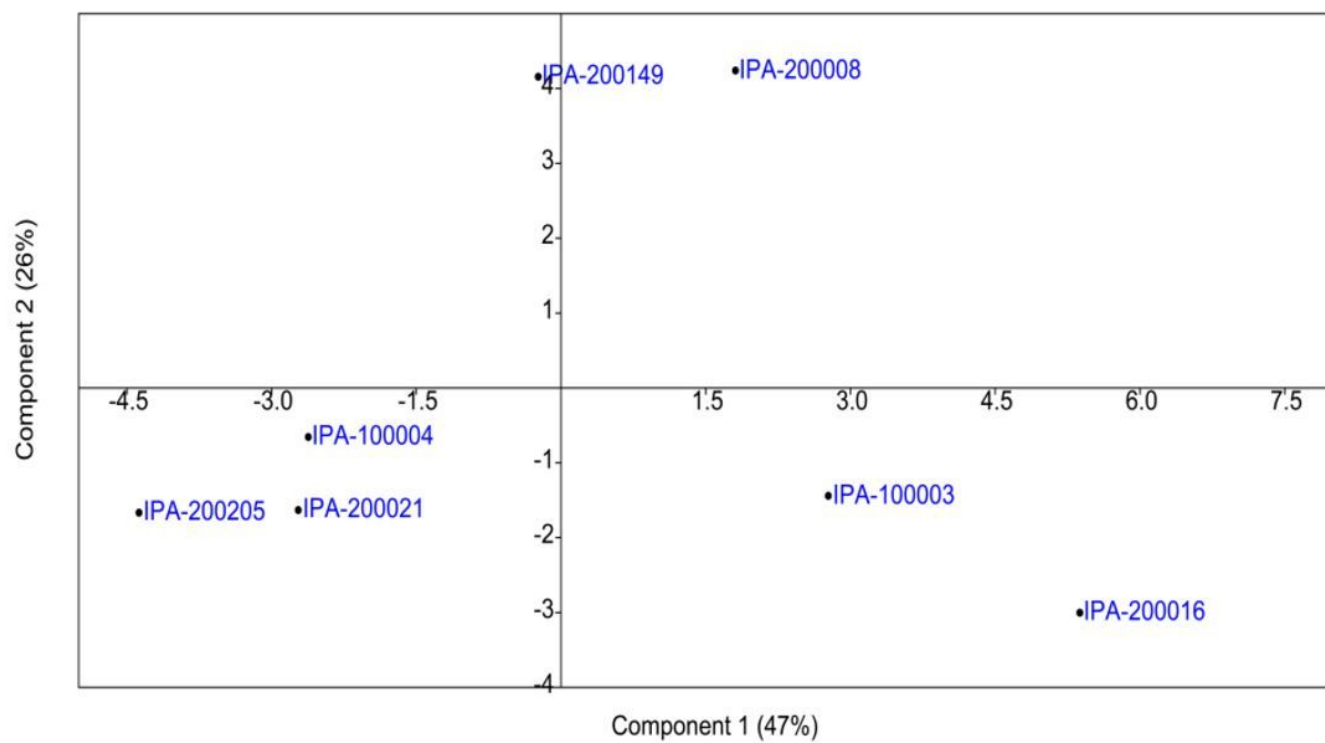
**Figure 1**

Amplification product generated by RAPD, ISSR and ITS markers in seven cactus pear accessions



**Figure 2**

Cluster analysis based on Ward's method derived from 242 bands amplified by RAPAD, ISSR and ITS primers among seven cactus pear accessions. Percentage values are derived from the bootstrap analysis with 100 repetitions



**Figure 3**

Principal component analysis based on the two first principal components derived from 242 bands amplified by RAPAD, ISSR and ITS primers among seven cactus pear accessions