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Isolation and molecular characterization of endophytic bacteria associated with the culture of forage cactus (*Opuntia* spp.)

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ABSTRACT

In Brazil, the forage cactus, has great economic interest because such plants are the nutritional base for livestock from semiarid regions. However little is known of endophytic diazotrophs in this culture. This work is aimed at characterizing molecularly in different semi-solid media using 22 isolates from seven cultivars of *Opuntia:*IPA 90-92; Marmillon Fodder (1317); F8 (438); F3 Rojo Vigor; Mexico vegetable (1294); Chile Fruit (1371); COPENA - V1 – isolated in different semi-solid media specific. The plant was inoculated with 100 µL of plant crude extract in a semi-solid medium free of nitrogen. The isolates were grown in DYGS medium with malic acid (NFB, JNFb, LGI and LG), and sucrose in DYGS medium for the isolates of LGI-P medium. The Wizard Genomic DNA Purification Kit (Promega) was employed for the extraction of DNA. The use of four ISSR markers (UBC 808, UBC 809, UBC 810 and UBC 812), and the ARDRA technique consisting of five restriction enzymes (*Dde*I, *Ava*I, *Hae*III, *Hinf*I and *Msp*I), followed by amplification of the 16S rDNA region (primers fD1 and rD1). This research points to great diversity of bacteria living in association with cactus and it will be necessary to undertake more detailed studies with molecular markers to obtain a conclusive characterization of the genetic diversity of plants with these endophytes, which could be achieved working with ribosomal genes and subsequent sequencing of these microorganisms.

INTRODUCTION

Within the Genera cacti, forage cactus (*Opuntia* spp) may be the most well known genus in the five continents due to its specific morphology and anatomy [11], besides being considered the most important economic sub-species with regard to its utilisation, principally forage for animals and fruits and vegetables for human consumption. James [9] reports that inside this plant there is a very suitable environment for biological nitrogen fixation (BNF). Such environments present low levels of oxygen and a high source of carbon – an ideal condition in which bacteria could fix nitrogen releasing it directly inside the plants and thereby helping partially their nitrogen requirements [4, 2, 3].

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Diazotrophic microorganisms may play an important role in rehabilitation and sustainability of ecosystems as they incorporate nitrogen through BNF in amounts that regulate the hormones which assist the plant's growth e.g. auxins, gibberellins and cytokinins; as well as improving the mineral condition and the use of water by plants [1, 10]. The methodology based on DNA digestion, or specific sequencing by endonucleases results in a pattern of bands, is called ARDRA (Amplified Ribosomal DNA Restriction Analysis) and it offers the opportunity to review polymorphism in the analysed DNA [12]. The employment of new markers requires extensive understanding of genetic organization. According to Gupta [8] and Sánchez [13], microsatellites are very efficient as they are interspersed in the genome, constituting unobtrusive markers to obtain the DNA fingerprinting, which in turn is an efficient method for signalling several loci and positive discriminants of variants related to genotypes [6].

ISSR markers are semi-arbitrary – a simple and direct primer consisting of 16-18 nucleotides of longitude, including two repetitive units with 2-4 anchors at 3'-5'. The most interesting aspects of such markers are their unnecessary presence to inform genomic sequences and their high polymorphism [16, 14]. An important element for the basis of these studies is endophytic diazotrophic in plants and the lack of knowledge regarding such microorganisms in this medium of culture. Nothing is known about the identity, diversity and population levels of microorganisms in the enigmatic cladode –and the relation of these microorganisms with their hosting plants. As a result, this work aims to use these biological techniques for identifying and assessing the diversity of new isolates associated with the forage cactus (*Opuntia* spp.).

MATERIAL AND METHODS

Biological Material

Twenty-two unidentified isolates from the interior of cladodes of forage palms from different cultivars were used (Table 1). The palm cladodes were subjected to a superficial disinfection process – washed with soap and water and after with sterile distilled water, 70% alcohol was applied on their surfaces, macerated 1 cm^2 in a sterile crucible in saline [5] and inoculated with 100 µL of plant crude extract in the semi-solid mediums devoid of nitrogen in 13 and 5 ml glass flasks with cotton stoppers (Medium NFb, JNFb, LGI, LGI-P and LG) [5]. All inocula were incubated at 32^{o}C over a period of 5-8 days and subjected to colony purification after seeing or detecting a pH change in the media. This purification encompassed several processes from semi-solid to solid media with yeast extract. The isolates were conserved in potato media; those grown in media NFb, JNFb, LGI and LG were conserved in potato-P; others grown in LGI-P were conserved in sterile mineral oil.

DNA Extraction of Isolates

The isolates were grown in DYGS medium with malic acid (NFBb, JNFb, LGI and LG), and with sucrose in DYGS media (glucose – 2.0 g; malic acid – 2.0 g; bacteriological peptone -1.5 g; yeast extract - 2.0 g; potassium phosphate dibasic – 0.5g; magnesium sulphate hydrate – 0.5g; glutamic acid – 1.5 g) was used for isolates from LGI-P media with subsequent agitation at 200 rpm for 24 hours at 30 ºC. 1.5 mL of cultures were then centrifuged at 13.000 rpm for 5 min. and subjected to DNA extraction using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacture's guidelines. The samples were treated with RNAse and kept at - 20 ºC.

Amplification of Region 16S rDNA

The PCR (polymerase chain reaction) were carried out in final volume of 25 µL containing DNA template (150 ng), 1µm of each specific *primer* (fD1 5'-AGA GTTTGATCCTGGCTCAG-3') and (rD1 5'-AAGGAGGTGATCCAGCC-3') [15], and 12.5 µL of GoTaq Colorless (Promega). The temperature cycles were provided by a MJ Research Inc. PTC-^{100} Thermocycler, with initial denaturation at 94 ºC for 4 min.; each cycle lasted 30 s at a temperature of 94 ºC, 30 s of annealing at 50 ºC and 1 min. of extension at 72 ºc, followed by a final extension of 8 min. at 72 ºc per 30 cycles. To determine whether there was amplification, an aliquot was taken from the reaction (5 -10 µL) to detect the presence of DNA in agarose gels. The amplified products were run in 1% electrophoresis agarose gel using 0.5 X of TBE buffer and 100 V. After electrophoresis, the products were reviewed using Sybrgold (Invitrogen) in LPIX-HE Photo-documentation system (Loccus do Brasil).

Amplified Ribosomal DNA Restriction Analysis

The amplified products from gene 16S rDNA of the isolates were purified as follows: for every 25 µL of amplified product, 2 µL ammonium acetate and 52 µL of 100% absolute ethanol was added. To precipitate the DNA, it was centrifuged at 13.000 rpm for 20 min. The supernatants were discarded and the pellet washed with 70% ethanol. To eliminate the ethanol remains, the samples were evaporeted at room temperature for 1 hour and the precipitate was re-suspended in 20 µl of sterile water. A 10 µL of the amplified product was used for the cut reaction added 2.5U of each enzyme, 10% of 10X reaction buffer until final volume of 30 µL.

Table 1. Total number of isolates of diazotrophic endophytes in the culture of forage cactus using the following semi-selective media: NFb; JNFb; LG; LGI-P.

Cultivares	**Número de aislados**					**Total de aislados**
	NFb para *Azospirillum* spp.	**JNFb para *Herbaspirillum* spp**	**LG para *Azotobacter* spp. Y *Azomonas* spp.**	**LGI-P para *Gluconoacetobater diazotroficus***	**LGI para *Azospirillum amazonense***	
IPA 90-92	IPA-IS19	-	IPA-IS15	IPA-IS16	IPA-IS20	04
Marmillon	IPA-IS1	IPA-IS8	-	IPA-IS7	IPA-IS2	04
Fodder (1317)						
F8 (438)	IPA-IS21	-	-	IPA-IS22	-	02
F3 Rojo Vigor	-	IPA-IS6	IPA-IS13	IPA-IS14	-	03
Mexico vegetable (1294)	IPA-IS4, IPA-IS5, IPA-IS3	-	-	-	-	03
Chile Fruit (1371)	IPA-IS11	-	IPA-IS9	IPA-IS12	-	03
COPENA-V1	IPA-IS10	-	IPA-IS17	IPA-IS18	-	03
TOTAL						**22**

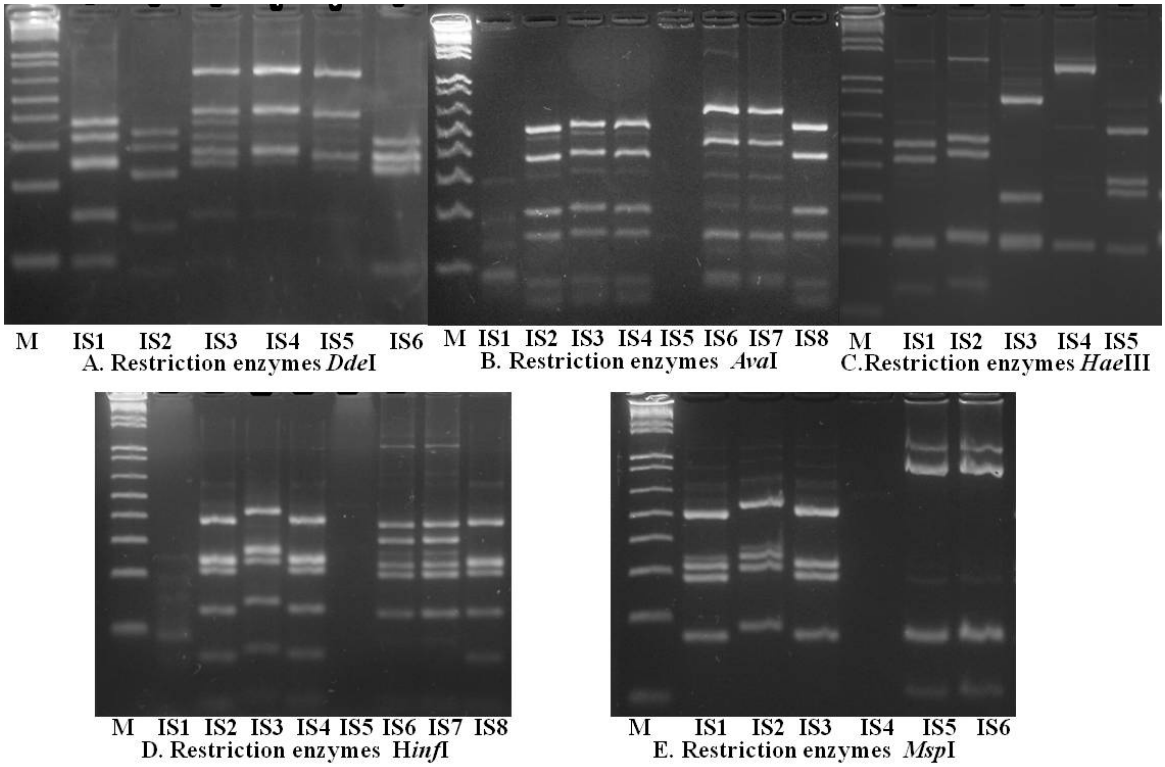

Fig 1. ARDRA analysis with the following restriction enzymes: *Dde*I, *Ava*I, *Hae*III, *Hinf*I and *Msp*I in 2.5% agarose gel. Legend: M-1Kb Plus (Invitrogen), Isolates: IS1: IPA-IS1, IS2: IPA-IS2, IS3: IPA-IS3, IS4: IPA-IS4, IS5: IPA-IS5, IS6: IPA-IS6, IS7: IPA-IS7, IS8: IPA-IS8.

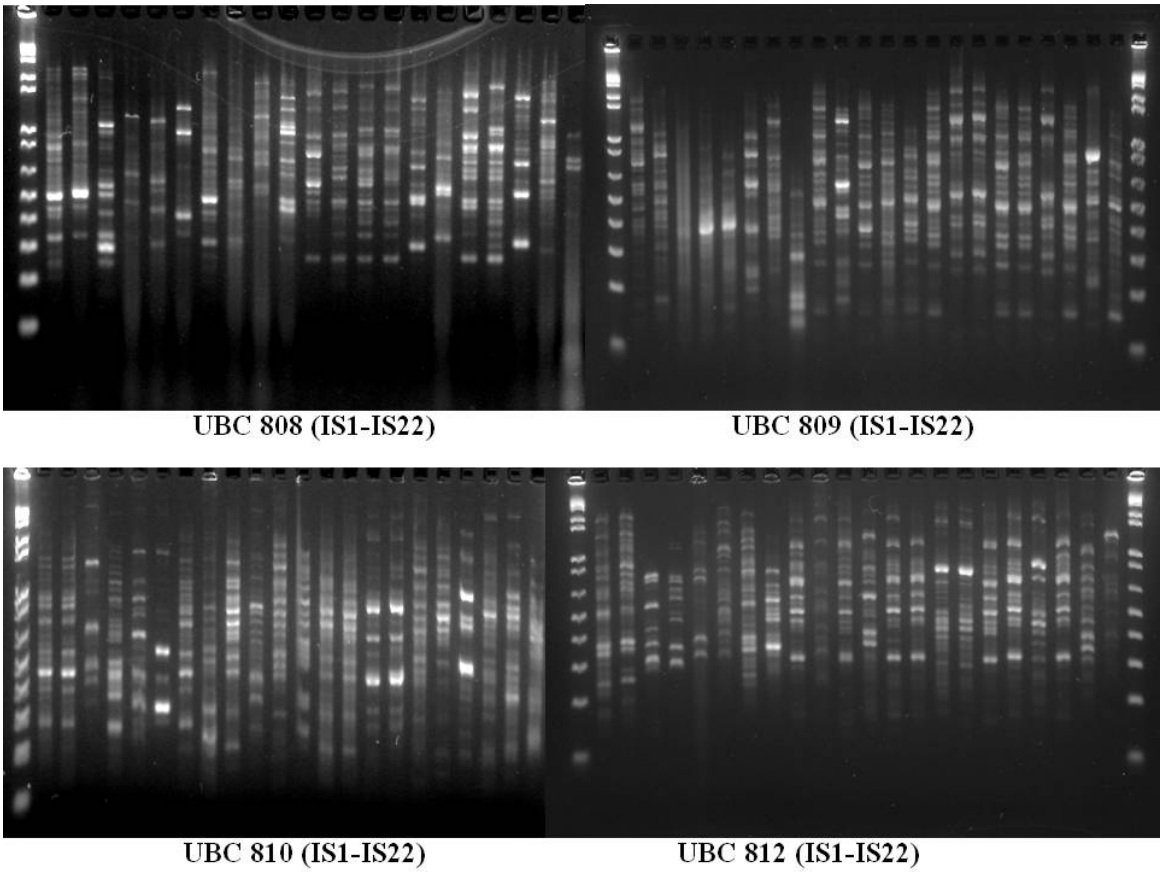

Fig 2. Microsatellite analysis (ISSRs): UBC808, UBC809, UBC810 and UBC812 in 1.5% agarose gel with 1 Kb Plus molecular weight markers (Invitrogen).

They were incubated at 37°C in dry baths for 6 hours with the following endonucleases: *Hinf*I, *Hae*III, *Msp*I, *Dde*I, *AvaI* (Invitrogen). The amplified products were run in electrophoresis at 2.5% agarose gel using a 0.5X TBE buffer at 80V for 4 hours. Afterwards, the products were reviewed using SybrGold (Invitrogen) in an LPIX-HE photo-documentation system (*Loccus do Brasil)* (Figure 1).

ISSR Marker Assays

The ISSR published by University of British Columbia – UBC 808 $(AG)_8C$, UBC 809 $(AG)_8G$, UBC 810 $(AG)_8T$, UBC 812 $(AG)_8A$ – were based on the method described by Zietkiewicz [16] using anchored primers. The DNA amplifications were carried out in a MJ Research Inc. PTC-^{100} Thermocycler with a final volume of 25 µL for each reaction: 2.5 µl of buffer 10X, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 1.0 µM of primers, 0.3 U of Taq DNA polymerase (Invitrogen) and 100 ng of DNA. For PCRs, the samples were subjected initially to 95 ºC for 5 min, and 30 amplification cycles: 94ºC for 30 s, 50°C for 45 s, 72ºC for 2 min and a final extension of 72ºC for 7 min. The reaction products were separated in 1.5% agarose gel in 0.5X TBE buffer and stained with SybrGold (Invitrogen) and visualized using the LPIX-HE Photo-Documentation System (*Loccus do Brasil)* (Figure 2).

Cluster Analysis

The ARDRA and ISSR patterns was analysed and a data matrix generated based on the well-defined band with each primer. This data was recorded as binary discrete variables using 1 to indicate the presence of the band and 0 the absence. A matrix of genetic distance was generated by comparing each pair of genotypes using the NTSYS-pc Windows software program, employing UPGMA cluster analysis byqualitative data of similarity (SIMQUAL), Simple Matching Coefficient (SM), Assembly method (SAHN - Sequential agglomerative hierarchal nested cluster analysis) and phylogenetic tree using Tree Plot software.

RESULTS AND DISCUSSION

In this research, we carried out the isolation of microorganisms in nitrogen-free environments as a first step towards identifying and then analysing, the bacteria, using molecular markers applying restriction techniques through endonucleases from gene 16S ribosomal rDNA and ISSR microsatellites (Non Anchored Inter Simple Sequence Repeats). Of the 22 isolates, eight were grown in NFb medium in the following cultivars of palm: Marmillon Fodder (1317), F8 (438) and México vegetable (1371). Two isolates (IPA-IS6 and IPA-IS8) were grown in JNFb medium in the cultivars Marmillon Fodder and F3 Rojo Vigor. The three isolates IPA-IS2, IPA-IS13 and IPA-IS15 were grown in JNFb medium in Marmillon Fodder, F3 Rojo Vigor and IPA 90-92. Six isolates were grown in LGI-P medium (*Gluconacetobacter diazotrophicus*) in the forage cactus Marmillon Fodder, F3 Rojo Vigor, F8 (438), Chile Fruit (1371) and IPA 90-92. And three isolates in the LGI medium – IPA-IS20 (IPA 90-92), IPA-IS17 (COPENA-V1) and IPA-IS9 (Chile Fruit (1371). Interestingly, in the same forage cactus – cultivar Mexico Vegetable – we found three isolates growing with distinct morphological characteristics. According to Dobereiner [5], in the NFb medium only bacteria from the genus *Azospirillum* as the species – *Azospirillum lipoferum*, *A. brasilense*, *A. irakense* and *A. halopraeferans* grows. – This isolate could originate from one of the four species mentioned, or it could be a new species of this genus since this medium is semi-selective, or even a new genus, growing within this forage cactus, which has not yet been identified.

The ARDRA technique provided a huge number of 1000-100 bp bands cut by restriction enzymes (Figure 1). The molecular weight has varied significantly with regard to the ISSR markers – between 2000 and 200 bp. All four markers amplified in all isolates; however the isolate IPA-IS8 was not cut by any restriction enzyme. The isolates IPA-IS14 and IPA-IS18 (Figure 2) were not cut either. The results obtained from the phylogenetic tree generated by the ARDRA analysis based on the compiled data (Figure 3) demonstrated a huge diversity among the microorganisms isolated. Figure 1 indicates four clusters. The first was formed by isolates IPA-IS1 and IPA-IS2 with 91% similarity. Both were from the palm cultivar Marmillon Fodder (1317) but under different media. IPA-IS1 was grown in NFb medium for growth of *Azospirillum* spp. and IPA-IS2 was grown in LG specific for the genera *Azotobacter* spp. and *Azomonas* spp. The isolate IPA-IS7 is found in a monophyletic branch with 82% similarity (*G. diazotrophicus*); IPA-IS6 represented *Herbaspirillum* spp, but with lower similarity as it was grown in JNFb medium. The second cluster was the largest formed by the studied isolates; however, it has demonstrated a particular characteristic: most of them formed monophyletic branches. The closest isolates were IPA-IS8 (JNFb), IPA-IS14 (LGI-P) and IPA-IS18 (LGI-P) with 98% similarity; these last two could belong to specie *G. diazotrophicus*. In the third cluster, two of the isolates presented a genetic distance of 100%; in other words, identical and coming from the same palm cultivar – IPA 90-92 – but grown in different media. The last cluster was formed by two isolates: IPA-IS3 and IPA-IS4; both were grown in NFb medium and isolated from the same cultivar of forage palm – Vegetable (1294).

The isolate IPA-IS5 (NFb) behaved as an external group. In Figure 4, we observed that the phylogenetic tree compiled with ISSR markers presented a variance between 65% and 89% for the genetic distance between the isolates. The most interesting telling of this tree is that the genetic distance among the isolates has not reached 100%. This indicates tremendous diversity among the studied isolates as with regard to the studied micro-satellites. Five clusters were formed; the first comprised the same isolates that were grouped in cluster 1 of Figure 3 formed by the ARDRA analysis, however with a genetic distance higher than 76%. The isolates IS15 and IS16 behaved identically, but their similarity was lower than 89%. In the cluster IV, the isolates IPA-IS13 and IPA-IS14 presented 83% similarity, but both come from the same cultivar of forage palm – F3 Rojo Vigor – and from different

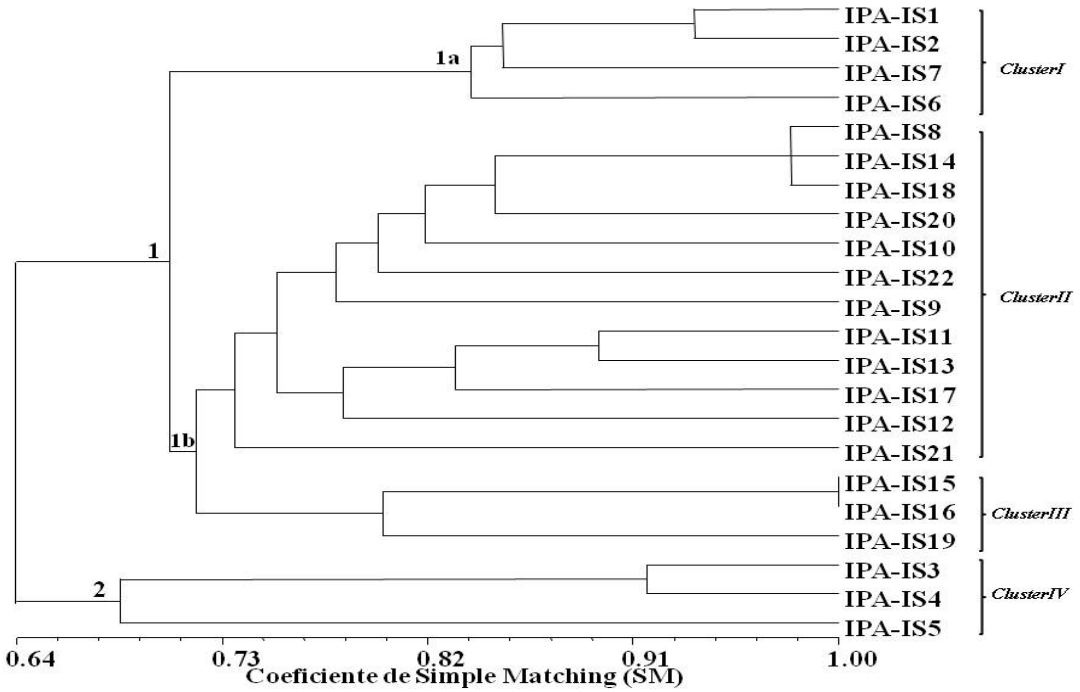

Fig 3. Dendrogram of similarity based on the ARDRA (Amplified Ribosomal DNA Restriction) analysis of the gene 16S rDNA with initiators fD1 and fD2. The isolates were provided by the following cultivars of forage cactus: IPA 90-92; Marmillon Fodder (1317), F3 Rojo Vigor, Mexico vegetable (1294), Chile Fruit (1371) and COPENA-V1, whose characteristics are shown in Table 1.

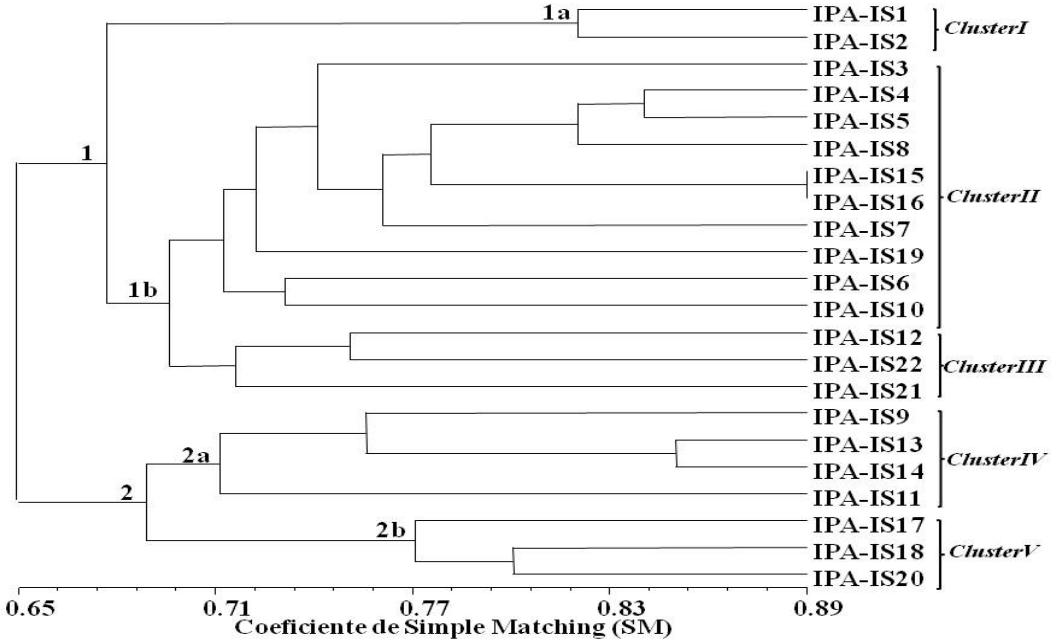

Fig 4. Dendrogram of similarity based on ISSR analysis with the following initiators: UBC808 $(AG)_8C$; UBC809 $(AG)_8G$; UBC810 $(AG)_8T$; UBC812$(AG)_8A$. The isolates were provided from the following cultivars of forage cactus: IPA 90-92; Marmillon Fodder (1317); F3 Rojo Vigor; Mexico vegetable (1294); Chile Fruit (1371); COPENA-V1, whose characteristics are found in Table

media (LG and LGI-P). The greatest divergence was found in the isolate IPA-IS5 which behaved as an external cluster in ARDRA analysis; however, with the ISSR micro-satellites, it was assembled in cluster II – considerably close to the isolate IPA-IS4 (NFb - Mexico Vegetable (1294). Thus, a complete and reliable characterisation of bacterial isolates must be followed by a molecular characterisation such as the ribosomal DNA sequence of the gene 16S rDNA [7]. We concluded that the diversity found in the culture of forage palm regarding the diazotrophic endophytes demonstrates the need for greater research to fully understand the life of such microorganisms in *Opuntia spp*.

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