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Molecular studies of forage prickly-pear cactus from the semiarid of Pernambuco State-Brazil

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ABSTRACT

The cultivation of forage cactus is of great economic interest in Brazil, due to its then importance for nutrition animal. This crop had been studied for many years but the advent of molecular biology has greatly contributed to shed light on its phylogeny. Twenty-eight prickly pear cactus plants (*Opuntia* and *Nopalea*) - from Brazil were analyzed using the ribosomal marker ITS (internal transcribed space regions) of rRNA ribosomal gene. Three prickly pear cactus varieties are used the forage in Northeastern Brazil: var. palma gigante (*Opuntia ficus-indica*), var. palma redonda (*Opuntia* sp.), var. miúda (*Nopalea cochenillifera* or *Opuntia cochenillifera*). The DNA was extracted from leaves and the ITS1 and ITS2 regions of all plants amplified and sequenced. Results showed que the ITS marker is very efficient to investigate the species studied and examine the level of the genus cactus plants. Interestingly, the species *Nopalea cochenillifera* when submitted to the NCBI its designation was changed to *Opuntia cochenillifera*, however, the organisms continues the *Nopalea cochenillifera*. These plants may show high degree of apomixis, vegetative propagation, ploidy and the high inter and intra-specific hybridization capacity. Plant systematic and phylogenetic studies are based on morphological and molecular mainly characteristics. The ribosomal ITS rRNA markers 1-2 have great ability to characterize species of prickly pear cactus in this work. Additional studies with other phylogenetic markers will deepen the understanding of the phylogeny of these species.

1. INTRODUCTION

Some species of the genera *Opuntia* and *Nopalea* of the Cactaceae family [1] are cultivated and are essential in the diet of human beings and animal. *Opuntia* was introduced in Brazil towards the end of the nineteenth century and is the most important forage crop animals in the Northeast region of Brazil. Prickly pear cactus is cultivated in an area of approximately 500 thousand hectare in northeastern Brazil, Pernambuco and mainly in Alagoas State. In the semiarid region of Pernambuco the lack of water is partial or complete and high quality fodder production is unsustainable. The only suitable crop to feed animals during the dry season under these conditions is prickly pear cactus.

Three cactus varieties are used the forage in Northeastern Brazil: var. palmagigante (*Opuntiaficus indica*), var. palmaredonda (*Opuntia* sp.) and var. miúda (*Nopalea cochenillifera*). The high capacity of prickly pear cactus is vegetative propagation has contributed to its widespread distribution, even to the extent of becoming a weed [2-5]. In secondary diversification areas of introduction, the genotypic and phenotypic characteristics of *Opuntia* were modified [6]. It seems that changes in some genes may lead to phenotypic differences such as presence and absence of thorns [7], phenotypic variation may occur in relation to polyploidy [8] and plant hybridization. The taxonomical and evolutionary studies based on morphology is prickly pear cactus has been hampered by these difficulties [9-10]. The advent of molecular biology has created new tools that help to enhance the characterization of these banks in the world.

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The use of morphological characters alone to evaluate the germplasm bank makes evaluations difficult and the genetic markers using DNA have facilitated the classification within the genus *Opuntia* [11]. A widely used method is based on the internal transcribed spacer of nuclear ribosomal genes (nrITS) for phylogenetic analysis indicated that *Opuntiaficus indica* should not be considered polyphyletic (the group that does not include the common ancestor of all individuals) when derived from multiple lines. Report of Baldwin [12] que the sequencing of the ITS region has one potential source of the the nuclear DNA characterization for phylogenetic reconstruction in plants.

The aim of this study was to characterize some of prickly pear cactus accessions that exist in the germplasm bank of two regions of the state of Pernambuco in semiarid which are based on internal transcribed spacer sequences.

2. MATERIAL AND METHODS

Twenty eight plants of the species Opuntioideae were provided by the germplasm bank of the Agronomic Institute of Pernambuco (IPA); the plants were collected from the experimental fields in Arcoverde and Sertânia municipality (Table 1a and 1b). The samples were taken from the modified leaves and placed tubes with silica to lyophilize the whole tissue for further analyses. Total genomic DNA was isolated from 20 mg of plant tissue lyophilized and macerated in liquid nitrogen using a procedure of the GenomicPrep™ kit from GE Healthcare and following the user's protocol. The ITS region of nuclear ribosomal DNA was amplified using primer specific for ITS1 and ITS2 regions were amplified as described by [13]. The primers used were ITS1 (5´-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5´-TCCTCCGCTTATTGATATGC-3'). The PCRs were carried out in 50 µl solution containing (1.5 mM)$MgCl_2$, (0.25 mM)dNTPs mix, DMSO (10%), (0.4 µM) ITS1, (0.4 µM) ITS4, Taq buffer 10X (10%), Taq polymerase Invitrogen (1U). The genomic DNA concentrations varied from 10 ng to 40 ng. The 30X PCR cycles were: 96°C for 30 s; annealing at 58°C for 30 s, extension step at 72°C of 45 s and a final extension step at 72°C of 10 min. The vegetable prickly pear cactus material has a lot of mucilage, a material that has loads of secondary metabolites such as phenols which greatly complicates the extraction of genomic DNA with a high degree of purity.

In view of this, it was necessary to use the NESTED-PCR technique for obtaining amplicons of the ITS region. For this, the bands were cut from the gel using QIA quick Gel Extraction Kit (Qiagen, Hilden, Germany). A volume of 5µl of the purified product was reamplified using the same reagents and cycling conditions for PCR. The DNA fragments were visualized under UV in 0.8% agarose gel using SybrGold (Invitrogen) using 1 Kb Plus Invitrogen as marker. The nested-PCR was performed three times to a volume of 100 ul and after checking the DNA products in agarose gels, a volume of 100 ul of amplified product was mixed with using of 8 ul ammonium acetate 7.5 M, 208 ul 100% ethanol and centrifuged at 10000 rpm for 45 min. to 20°C. Then, cold 70% ethanol was added, centrifuged for 10 min. at 4000 rpm and then the supernatant was discarded and the microtube was reversed leaving dry overnight. The pellet was re-suspended DNA purified in 30 ul of sterile ultrapure water and stored at-20°C until sequenced. These products of the of region ITS1 and ITS2 purified and sent for sequencing by the Sanger method in an automatic Applied Biosystem sequencer using Macrogen. BioEdit 7.0.9 (http://www.mbio.ncsu.edu/BioEdit) was used for alignments, excluding the end of 18S ribosomal gene and the beginning of 26S rDNA and then cut to 619 pb. Online blast at the NCBI website was used for analysis. The evolutionary history was inferred using the Neighbor-Joining method (NJ)[14]. The optimal tree with the sum of branch length = 0.24113564 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches Felsenstein[15].

The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages [16]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method [17] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons (Pairwise deletion option). There were a total of 597 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.0 (http://www.megasoftware.net/) [18].

3. RESULTS AND DISCUSSION

The phylogenetic tree (Fig.1) obtained from the analysis of ITS 1 and 2 from 28 Brazilian accessions and compared with six species of Opuntias in GenBank. Clustal X Software was used to align the lengths to the ITS sequences by BioEdit Program ranged from 583 – 653 bp and the ITS sequence phylogenetic tree construted with MEGA program using inferred NJ tree and Tamura-Nei short distances based on all pairwase comparison of ITS1, 5.8S and ITS2. The sequence of the 5.8S was well conserved with the length of 162 bp. The average A, T, C, G ratio was 20.5; 15,5; 33.2; 30.9. The ITS had high G+C content consistent with earlier observations in the other plant taxa [12; 19]. As out group it used AY181575.1 *Pachycereus lepidanthus* isolate tcsn. The alignable portions between the in group and out group did not provide any information to resolve in group phylogeny. Interestingly, the species *Nopalea cochenillifera* when submitted to the NCBI its designation was changed to *Opuntia cochenillifera* however as organisms continues as *Nopalea cochenillifera*. All *Nopalea* here studied before were characterized by their morphological characteristics in Brazil. The Fig. 1 shows five major clusters. The first cluster include nine accessions: AM946670.1, AM946671.1 and AM946668.1 with 100% similarity. With a smaller similarity, AM946666.1and AM946667.1 were also found in this cluster.

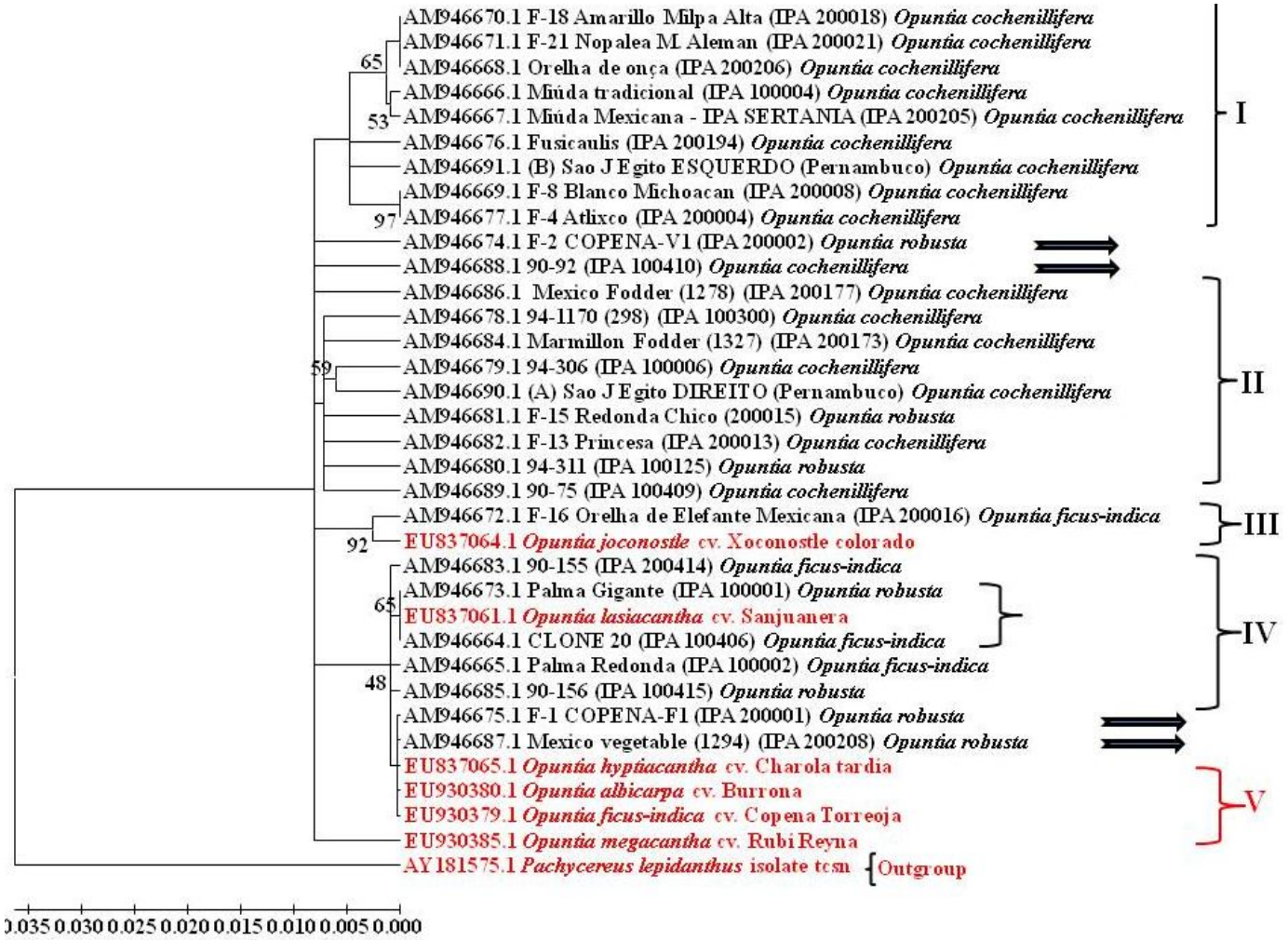

Fig. 1: Phylogenetic tree based on ITS sequences from *Opuntiaficus indica* and *O.cochenillifera* using Neighbor-Joining (NJ) with Tamura-Nei and 1000 bootstrap tests.

Table 1a: Species of the Opuntias tested, species, author, GenBank accession numbers, voucher and specimen for ITS sequences.

Species/bases pair (bp)	Author	GenBankAcession no.	Voucher	Specimen/no
Opuntiaficus indica/623bp	This study	AM946664	ArcoverdeGermplasm Bank	clone-20 IPA100406
Opuntia ficus-indica/643bp	This study	AM946665	ArcoverdeGermplasm Bank	Palma Redonda IPA100002
Opuntia cochenillifera/615 bp	This study	AM946666	ArcoverdeGermplasm Bank	Miúda Redonda IPA100004
Opuntia cochenillifera/653 bp	This study	AM946667	SertâniaGermplasm Bank	Miuda mexicana IPA Sertânia IPA200205
Opuntia cochenillifera/647 bp	This study	AM946668	ArcoverdeGermplasm Bank	Orelha de Onça IPA 200206
Opuntia cochenillifera/607 bp	This study	AM946669	ArcoverdeGermplasm Bank	F8-Blanco Michoacan IPA200008
Opuntia cochenillifera/642 bp	This study	AM946670	ArcoverdeGermplasm Bank	F18-Amarillo Milpa Alta IPA200018
Opuntia cochenillifera/641 pb	This study	AM946671	ArcoverdeGermplasm Bank	F21-Nopalea M. Aleman IPA200021
Opuntia ficus-indica/616 bp	This study	AM946672	ArcoverdeGermplasm Bank	Orelha de elefante Mexicana IPA200016
Opuntia robusta/618 bp	This study	AM946673	ArcoverdeGermplasm Bank	Palma Gigante IPA100001
Opuntia robusta/613 bp	This study	AM946674	ArcoverdeGermplasm Bank	COPENA-V1 IPA200002
Opuntia robusta/653 bp	This study	AM946675	ArcoverdeGermplasm Bank	COPENA-F1 IPA200001
Opuntia cochenillifera/626 bp	This study	AM946676	SertaniaGermplasm Bank	FUSICAULIS IPA200194
Opuntia cochenillifera/635 bp	This study	AM946677	SertaniaGermplasm Bank	F4-Atlixco IPA200004
Opuntia cochenillifera/594 bp	This study	AM946678	SertaniaGermplasm Bank	94-1170 (298) IPA100300
Opuntia cochenillifera/646 bp	This study	AM946679	SertaniaGermplasm Bank	94-306 IPA100006
Opuntia robusta/629 bp	This study	AM946680	SertaniaGermplasm Bank	94-311 IPA100125
Opuntia robusta/583 bp	This study	AM946681	SertaniaGermplasm Bank	F15-Redonda Chico IPA200015
Opuntia cochenillifera/602 bp	This study	AM946682	SertaniaGermplasm Bank	F13-Princesa IPA200013
Opuntia ficus-indica/652 bp	This study	AM946683	ArcoverdeGermplasm Bank	90-155 IPA200414
Opuntia cochenillifera/643 bp	This study	AM946684	ArcoverdeGermplasm Bank	(1327) MarmillonFodder IPA200173

Table 1b: Continued Species of the Opuntias tested, species, author, GenBank accession numbers, voucher and specimen for ITS sequences.

Species/bases pair (bp)	Author	GenBank Acession no.	Voucher	Specimen/n°
Opuntia robusta/629 bp	This study	AM946685	ArcoverdeGermplasm Bank	90-156 IPA100415
Opuntia cochenillifera/658 bp	This study	AM946686	ArcoverdeGermplasm Bank	(1278) MexicoFodder IPA200177
Opuntia robusta/645 bp	This study	AM946687	ArcoverdeGermplasm Bank	(1294) Mexico vegetable IPA200208
Opuntia robusta/654 bp	This study	AM946688	ArcoverdeGermplasm Bank	90-92 IPA100410
Opuntia cochenillifera/642 bp	This study	AM946689	ArcoverdeGermplasm Bank	90-75 IPA100409
Opuntia cochenillifera/652 bp	This study	AM946690	São josé do Egito	DIREITO
Opuntia cochenillifera/646 bp	This study	AM946691	São josé do Egito	ESQUERDO
Opuntia joconostle /665 bp	Luna-Paez et al, 2008 Unpublished	EU837064	Germplasmbank CRUCEN-UACH	Xoconostle colorado
Opuntia lasiacantha/665 bp	Luna-Paez et al, 2008 Unpublished	EU837061	Germplasmbank CRUCEN-UACH	Sanjuanera
Opuntia hyptiacantha/667 bp	Luna-Paez et al, 2008 Unpublished	EU837065	Germplasmbank CRUCEN-UACH	Charola tardia
Opuntia albicarpa/678 bp	Valadez-Moctezuma et al, 2008 Unpublished	EU930380	Germplasmbank CRUCEN-UACH	Burrona
Opuntia ficus-indica/686 bp	Valadez-Moctezuma et al, 2008 Unpublished	EU930379	Germplasmbank CRUCEN-UACH	CopenaTorreoja
Opuntia megacantha/541 bp	Valadez-Moctezuma et al, 2008 Unpublished	EU930385	Germplasmbank CRUCEN-UACH	Rubi Reyna
Pachycereuslepidanthus/751 bp	Arias et al 2003	AY181575	JardinBotanico, UNAM	isolatetcsn

The species AM946669.1 and AM946677.1 showed 100% of similarity and bootstrap values above 97% are considered to be significantly supported. Two monophyletic branches formed by the accessions AM946676.1 and AM946691.1 were detected. In cluster II, the majority of species behaved as monophyletic branches and the species AM946681.1 and AM946680.1 appeared as *Opuntia robusta*. The clusters III, the species AM946672.1 grouped EU837064.1 *Opuntia joconostle* cv. *Xoconostle colorado*. This species *Opuntia ficus-indica* grouped in cluster IV (AM946683.1, AM946664.1, AM946665.1). All the NCBI species were grouped in cluster V. Plant systematic and phylogenetic studies are based mainly on morphological and molecular characters.

As has been previously observed in plants [20], we found that the phenotype is not a straight function of the genotype, but undoubtedly the genotypic characterization should be investigated. There are major incongruences in taxonomic studies of *Opuntia* due to phenotypic variations caused mainly by their high inter and intra specific hybridization capacity, polyploidy, vegetative and sexual reproduction and to the high apomixis rate, which allows the occurrence of hybrids between species [21]. The subfamily Opuntioideae has been challenging the generic and specific classification with few natural exomorphic (external appearance) traits and the existence of asexually propagated hybrids, hampering the recognition of this taxon considerably [6;22]. The phenotypic variability between lines of the genus Opuntioid may result in convergence due to difficulties encountered by taxonomists to examine the morphology of cactaceas especially the genera *Opuntia* and *Nopalea*, resulting in controversial generic circumscription. We also observed that while Opuntia and Nopalea can be separated into two different clades there are exceptions.

4. CONCLUSION

The rDNA ribosomal ITS 1 and ITS 2 markers have great ability to characterize species of forage cactus in this work and proved adequate to separate the accesions studied according to our classified botanically as *Nopalea cochenillifera* or *Opuntia cochenillifera*, *Opuntia robusta* and *Opuntia ficus-indica* which were grouped similarly by morphological traits and by the molecular analysis..

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