Journal of Applied Biology & Biotechnology Vol. 1 (01), pp.006-010, June, 2013
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2013.1102

Molecular characteristics of prickly-pear cactus (*Opuntia*) based on internal transcribed spacer sequences (ITS) of Queretaro State – Mexico

M. C. C. P. de Lyra[1], D. C. Santos[2], C. Mondragon-Jacobo[3], M.L.R.B. da Silva[4] , A. C. E.S Mergulhão[5] and E. Martínez-Romero[6]

[1]Laboratorio de Genômica - Instituto Agronômico de Pernambuco-IPA. Av. Gal San Martin 1371 Bonji – Recife – Pernambuco – Brazil.
[2] Estação Experimental de Arcoverde - Instituto Agronômico de Pernambuco-IPA. Av. Gal San Martin 1371 Bonji – Recife – Pernambuco – Brazil 52761000. [3]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarías-INIFAP. Av. Luis Pasteur 414, 1er. piso, Valle Alameda, Queretaro-Queretaro-México. [4]Laboratorio de Genômica - Instituto Agronômico de Pernambuco-IPA. Av. Gal San Martin 1371 Bonji – Recife – Pernambuco – Brazil
[5]Dra. Laboratorio de Genômica - Instituto Agronômico de Pernambuco-IPA. Av. Gal San Martin 1371 Bonji – Recife – Pernambuco – Brazil 52761000
[6]Ecological Genomics Program, Research Group Environmental and Symbiotic Microbiology Universidad Nacional Autonóma de México -Centro de Ciencias Genómicas Av. Universidad s/n Col. Chamilpa 62210, Cuernavaca, Morelos, México

ARTICLE INFO

Article history:
Received on:30/04/2013
Revised on: 14/05/2013
Accepted on: 22/05/2013
Available online: 28/06/2013

Key words:
Opuntioideae**;** Taxonomy, Hybrid, molecular markers, prickly-pear cactus

ABSTRACT

The prickly-pear cactus has great importance in the human feeding in Mexico and also for export of fruits. Around 8 000 years ago in Mexico, *Opuntia* was domesticated for human consumption took place. The high capacity of prickly-pear cactus for vegetative propagation has contributed to its wide-spread distribution, even to the extent of becoming a weed. In secondary diversification areas of introduction, the genotypic and phenotypic characteristics of *Opuntia* were modified. Studies using biosystematics and morphology demonstrated a relationship with other Opuntoideae. The internal transcribed spacer region nuclear ribosomal DNA was sequenced in seventeen species for phylogeny. These findings indicate that the ITS region in Opuntoideae should be further exploted as a promising source of nuclear phylogeny markers. *Opuntia ficus-indica* (L. Mill),, *Opuntia robusta*, *Opuntia albicarpa*, *Opuntia streptacantha* they are not well characterized in this study, them of they contain in way its would arbitrate. The need of larger studies with molecular and morphologic markers in these species is very important.. The results of phylogeny analysis of *Opuntia* species suggests a new revision in the taxonomy of these plants sees that, the presence of hybrid in this study demonstrates the difficulty of characterizing in a safe way the species of the family Opuntiodeae.

INTRODUCTION

Opuntia ficus-indica (L. Mill), a domesticated prickly-pear cactus [1] is highly efficient to convert water into biomass [2]. *Opuntia* domestication for human consumption took place around 8 000 years ago in Mexico [3] perhaps at the same time that maize and bean were domesticated as well. *Opuntia megacantha*, presumably the wild type from which domesticated cultivars derived is found in Mexico [4]. Only in Mexico there are more than 10.500 ha for the production of young cladodes consumed as vegetables [5; 6]. The genetic improvement of the species led to different clones and cultivars [7 -15], that were even given the status of species. Molecular studies to analyze the gene patterns and sequences have faced the difficulty of obtaining high-quality DNA from these plants [16]. They vary most frequently in fruit size and color, cladode morphology and in phenology, i.e., variations due to environmental conditions such as temperature, light, humidity, and maturation time [17]. Assumptions about natural hybrids were suggested by [18-17] who observed natural crosses between different *Opuntia*e species in the F1 hybrid progeny. The closeness of the different species in natural environments has created a favorable environment for the gene flow between cultivars. Morphological markers are the most widely used for germplasm characterization due to the simplicity, ease of characterization and low costs. However, the advent of molecular biology has created new tools that help enhance the characterization of these banks in the world. The use of morphological characters alone to evaluate a germplasm bank makes evaluations difficult and the genetic markers using DNA have facilitated the classification

* *Corresponding Author*
Maria do Carmo Catanho Pereira de Lyra
Adress: Av. Gal San Martin 1371 Bonji – Recife – Pernambuco – Brazil.

within the genus *Opuntia* [19]. A widely used method is based on the internal transcribed spacer of nuclear ribosomal genes (nrITS) for phylogenetic analysis indicated that *Opuntia ficus indica* should be considered polyphyletic (a group that does not include the common ancestor of all individuals) when derived from multiple lines. Molecular phylogenetic analysis can be useful in evolution studies of the cactus, but a classification based on the limited molecular analysis only would result in an inappropriate classification of this group. Molecular systematics methods to reconstruct plant systematic and resolve systematic problems that are difficult to resolve by way of classical taxonomy [20]. Ribosomal internal transcribed spacer (ITS) sequences (including ITS1 and ITS2) and 5.8S rRNA sequences have conserved lengths and a high degree of variability and are well suited for classification studies. The need to evaluate the accesses for molecular studies of the bank of germoplasm of the plants originating from of the breeding program of opuntias through these markers was what took us to accomplish these studies.

2. Material and methods

The seventeen mexican plants were provided by the Instituto Nacional de Investigaciones Forestales y Agrícolas y Pecuarias (INIFAP, Banco de germoplasma del Programa de Nopal y Frutales), from the experimental field in North Guanajuato (Table 1). The samples were taken from the modified leaves and placed in silica in tubes to lyophilize the whole tissue for latter 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 of double-stranded DNA was performed in ITS1 and ITS2 regions were amplified as described by [21]. 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 de $MgCl_2$, 0.25 mM de 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 30 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. Because of the interference was necessary to make a mucilaginous NESTED-PCR. For this, the bands were cut from the gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). 5 µl of product was purified reamplified using the same reagents and cycling conditions for PCR.The fragments of DNA was 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 viewing the agarose gel were purified using 0.8% for every 100 ul of the amplified product was added to 8 ul ammonium acetate 7.5 M, 208 ul 100% ethanol and centrifuged at 10 000 rpm for 45 min. to 20°C. Then added to cold 70% ethanol, centrifuged for 10 min at 4000 rpm and then the supernatant was discarded and the microtube was reversed leaving dry overnight. The pellet was resuspended puificado DNA in 30 ul of sterile ultrapure water and stored at-20C 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, a pathfinder in genome research (Korea). 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 [22]. The optimal tree with the sum of branch length = 0.08171050 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 [23]. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages [24] 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 [25] 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 pairwise sequence comparisons (Pairwise deletion option). There were a total of 622 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4.0 (http://www.megasoftware.net/) [26].

3. Results and Discussion

The gene fragments were amplified by PCR. The amplified ITS sequences of all seventeen speces of Opuntias were determined with reference to the nine accessions in GenBank (Table 1) The phylogenetic tree (Fig.1) obtained from the analysis of ITS 1 and 2 was used Clustal X Software to align the lengths to the ITS sequences by BioEdit Program ranged from 594 – 656 bp and the ITS sequence phylogenetic tree were construted using MEGA program formed 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; 15,7; 32.8; 32.8. The ITS had high G+C content consistent with earlier observations in the other plant taxa [21;27] As outgroup it used AY181587 *Pachycereus marginatus* sa1372. In the first group is major clusters (bootstrap value (BV=58%) with all species retired from GenBank and ten species studied in this work. The accessions EU428845 (*Opuntia ficus-indica* cv. Milpa alta) and EU428844 (*Opuntia albicarpa* cv. Tuna Mansa) showed identical with similarity of 100% and this *Opuntia robust* acv. El lindero and the hybrid 2-23-15 they formed an independent clade. The accession DQ672551 (*Opuntia ficus-indica* cv. Pico Chulo behaved with monophyletic branch. The species AM946652 (*O. robusta* cv. Reyna), AM946652 (*O. ficus-indica* cv. Lisa) and AM946655 (*O. ficus-indica* cv. Roja Lisa) formed the other clade with 100% of similarity. Still in this same group, this species AM946661 (*O. robusta* cv. Reyna-3), AM946662 (hybrid 2-11-108), AM946660 (*O. ficus-indica* cv.AV), AM946651 (*O. robusta*

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

Species/bases pair (bp)	Author	GenBank Acession no.	Voucher	Specimen/n°
Opuntia sp/638 bp	This study	AM946663	INIFAP Germplasm Bank	2-26-23=hybrid
Opuntia robusta/614 bp	This study	AM946662	INIFAP Germplasm Bank	2-12-108=hybrid
Opuntia robusta/639 bp	This study	AM946661	INIFAP Germplasm Bank	Reyna-3
Opuntia ficus-indica/606 bp	This study	AM946660	INIFAP Germplasm Bank	AV
Opuntia robusta/656 bp	This study	AM946659	INIFAP Germplasm Bank	El Lindero
Opuntia robusta/640 bp	This study	AM946658	INIFAP Germplasm Bank	Reyna-2
Opuntia robusta/652 bp	This study	AM946657	INIFAP Germplasm Bank	2-14-55=hybrid
Opuntia robusta/639 bp	This study	AM946656	INIFAP Germplasm Bank	2-20-15=hybrid
Opuntia ficus-indica/601 bp	This study	AM946655	INIFAP Germplasm Bank	Roja Lisa
Opuntia robusta/617 bp	This study	AM946654	INIFAP Germplasm Bank	Reyna
Opuntia robusta/596 bp	This study	AM946653	INIFAP Germplasm Bank	2-13-90=hybrid
Opuntia ficus-indica/615 bp	This study	AM946652	INIFAP Germplasm Bank	Lisa
Opuntia robusta/594 bp	This study	AM946651	INIFAP Germplasm Bank	2-14-72=hybrid
Opuntia robusta/618 bp	This study	AM946650	INIFAP Germplasm Bank	2-23-15=hybrid
Opuntia robusta/615 bp	This study	AM946649	INIFAP Germplasm Bank	2-20-09
Opuntia robusta/600 bp	This study	AM946648	INIFAP Germplasm Bank	2-20-15
Opuntia robusta/599 bp	This study	AM946647	INIFAP Germplasm Bank	Reyna-1

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

Species/bases pair (bp)	Author	GenBank Acession no.	Voucher	Specimen/n°
Opuntia ficus-indica/617 bp	Luna-Paez et al, 2008 Unpublished	EU428848	Opuntia Germplasm bank CRUCEN-UACH	Pelon Blanco
Opuntia albicarpa/654 bp	Luna-Paez et al, 2008 Unpublished	EU428847	Opuntia Germplasm bank CRUCEN-UACH	Copa de Oro
Opuntia megacantha/544 bp	Luna-Paez et al, 2008 Unpublished	EU428846	Opuntia Germplasm bank CRUCEN-UACH	Lirio Rojo
Opuntia ficus-indica/576pb	Luna-Paez et al, 2008 Unpublished	EU428845	Opuntia Germplasm bank CRUCEN-UACH	Milpa Alta
*Opuntia albicarpa/*615 pb	Luna-Paez et al, 2008 Unpublished	EU428844	Opuntia Germplasm bank CRUCEN-UACH	Tuna Mansa
*Opuntia albicarpa/*665 pb	Luna-Paez et al, 2008 Unpublished	DQ672549	Opuntia Germplasm bank CRUCEN-UACH	Fafayuca
*Opuntia streptacantha/*664 pb	Luna-Paez et al, 2008 Unpublished	DQ672548	Opuntia Germplasm bank CRUCEN-UACH	Cardon de Castilla
Opuntia.albicarpa/664 pb	Luna-Paez et al, 2008 Unpublished	DQ672550	Opuntia Germplasm bank CRUCEN-UACH	Amarilla Oro
Opuntia ficus-indica/664 pb	Luna-Paez et al, 2008 Unpublished	DQ672551	Opuntia Germplasm bank CRUCEN-UACH	Pico chulo
Pachycereus marginatus/752 bp	Arias et al, 2003	AY181587	Jardin Botanico, UNAM	sa1372

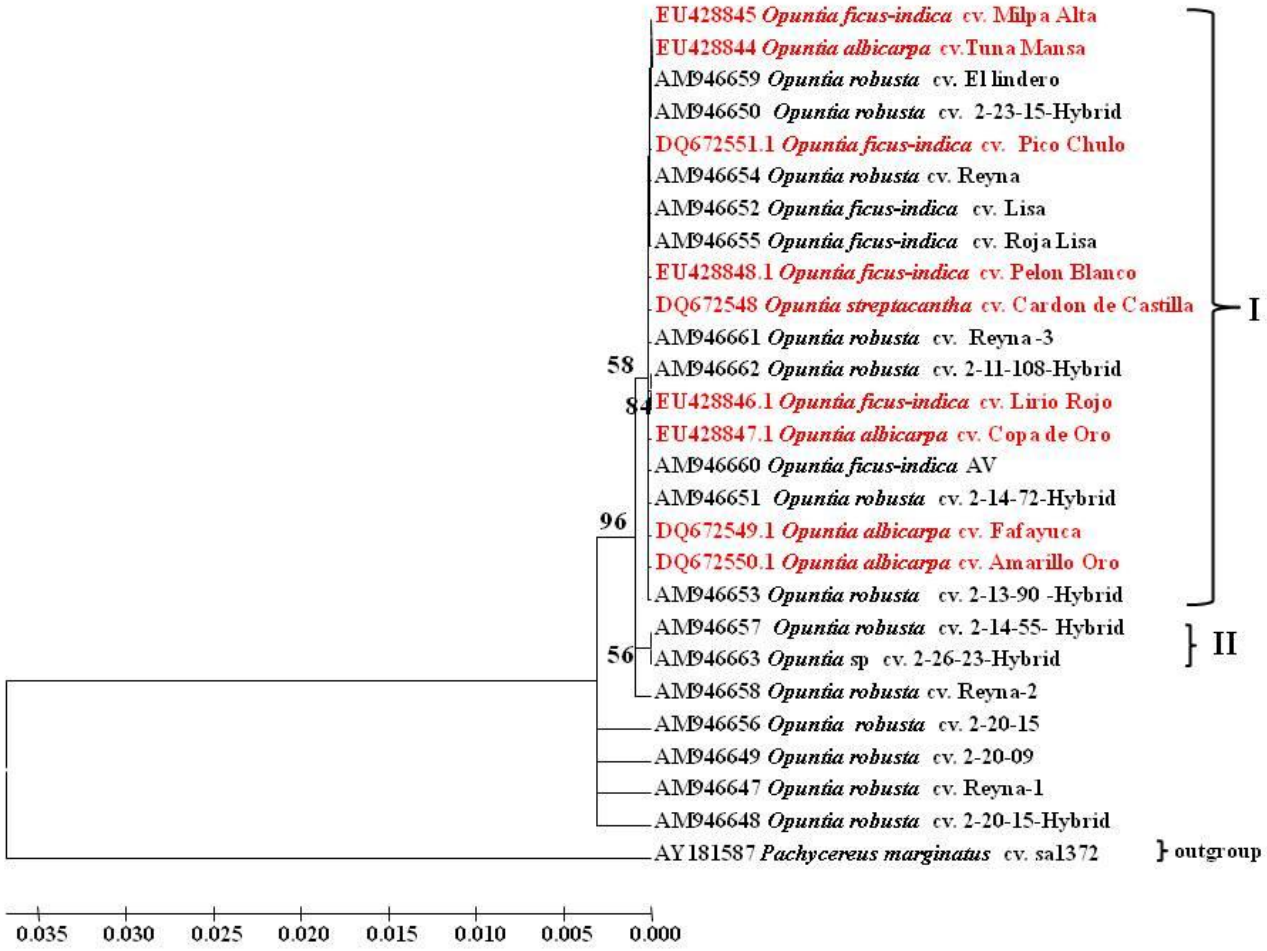

Fig. 1. *Opuntia* group relationship inferred from Tamura-Nei tree based on ITS sequences using Neighbor-Joining (NJ) with 1000 bootstrap tests.

cv. 2-14-72 hybrid), AM946653 (hybrid 2-13-90) formed an independent monohyletic branches. Plant systematic and phylogenetics studies are based mainly on morphological and molecular characters. In this stydy, we sequenced and analysed ITS sequences of 17 species of Opuntia and related plants that have not been reported before. The results provide an initial understanding of the evolutionary relationship. In agreement with [20], the use of morphological characters alone to evaluate a germplasm bank makes evaluations difficult and the genetic markers using DNA have facilitated the classification within the genus *Opuntia*. Kumar [28] and Lin and Rao [29], showed that, in plants, the applied DNA markers for genotypes, lines, varieties and cultivars in determining the purity os seed lots, resolving uncertainties in parentage as well as for legal proteccion of improved varieties throuth definitive identification. The final four species all have a monophyletic branchs with the 95% similarity and bootstrap 96%.

4. CONCLUSION

This study has introduced new information about phylogenetic relationship among Opuntoideae plants.

Molecular methods have demonstrated several advantages but is necessary to unite the morphologic data for a larger understanding of the species of Opuntia. However, in this work we demonstrated that the marker ITS is capable to characterize the studied plants.

5. Acknowledgements

The authors thank Lopez, E, Becerra, S, Yañez, J., Gaytan, P from the Instituto de Biotecnologia, UNAM for preparing the oligonucleotides. They are grateful to the Secretary of the Laboratório de Ecologia Genómica Lucila Lulo Ochoa for her enormous help with the paperwork and to Jorge Muñoz Garcia and Martin Garcia Solis whose endeavour made the every day work of this research a lot easier.

REFERENCES

1. Russell CE, Felker P. The prickly pears (*Opuntia* spp., Cactaceae): a source of human and animal food in semiarid regions. Economic Botany. 1987; 41: 433-445.
2. Kluge M, Ting IP. Crassulacean Acid Metabolism: Analysis of an Ecological Adaptation. Berlin, Springer-Verlag. 1978.
3. Ostolaza C. Cactus y etnobotánica. Quepo. 1994; 8:79-86.

4. Luna-Paez A, Valadez-Moctezuma 1E, Barrientos-Priego AF, Gallegos-Vázquez C. Caracterización de *Opuntia* spp. mediante Semilla con marcadores RAPD e ISSR y su posible uso para diferenciación. Journal of the Professional Association for Cactus Development. 2007; 9:43-59.
5. Flores C. Producción, Industrialización, y comercialización de Nopalitos. Centro de invstigaciones económicas sociales y tecnológicas de la agroindustria y la agricultura mundial. Universidad Autónoma de Chapingo, Mexico, 2001, 27p.
6. Reys-Agüero JA, Aguirre-Rivera JR. Hernández HM. Systematic note and detailed description of Opuntia ficus-indica (L.) Mill (Cactaceae) Agrociencia. 2005; 39(4): 395-908.
7. Benson LH. The cacti of the United States and Canada. Stanford University Press, Stanford, California, USA. 1982.
8. Mandujano, M. C., C. Montaña, I. Méndez, and J. Golubov. The relative contributions of sexual reproduction and propagation in *Opuntia rastrera* from two habitats in the Chihuahuan Desert. Journal of Ecology.1998; 86:911–921.
9. Casas A, Caballero J, Mapes C, Zárate S. Manejo de la vegetación, domesticación de plantas y origen de la agricultura en Mesoamérica. Boletín de la Sociedad Botánica de México 1997; 61: 31-47.
10. Nobel PS. Cacti: biology and uses. University of California, Berkeley, California, USA 2002
11. Nobel PS, De La Barrera E, Beilman DW, Doherty JH, Zutta BR. Temperature limitations for cultivation of edible cacti in California. Madroño. 2002; 49: 228-236.
12. Casas A, Barbera G. Mesoamerican domestication and diffusion. In: P. S. Nobel [ed.], Cacti: biology and uses, University of California, Berkeley, California, USA 2002. p.143–162.
13. Griffith MP. A new Chihuahuan Desert prickly pear, *Opuntia* x *rooneyi*. Cactus and Succulent Journal (U.S.) 2001; 73: 307-310
14. Griffith MP. Experimental hybridization in northern Chihuahuan desert region Opuntia, Rancho Santa Ana Botanic Garden 2001; 20: 37-42.
15. Griffith MP. Using molecular data to elucidate reticulate evolution in *Opuntia*. Madroño. 2003; 50: 162-169
16. Mondragon-Jacobo C. Cactus Pear Domestication and Breeding. In: J. Janick, Plant Breeding Reviews. 2000; 20: 135-165.
17. Pimienta-Barrios E, Muñoz-Urias A. Domestication of Opuntias and cultivated varieties. *In*: Barbera, G., P. Inglese and B.E. Pimienta (Eds.), Agroecology, cultivation and uses of cactus pear, 1995. p 58-63.
18. Grant V, Grant AK, Hurd DP. Pollination of *Opuntia lindheimeri* and related species. Plant Systematics and Evolution. 1979;132: 313-320.
19. Andersen JR, Lübberstedt T. Functional markers in plants. Trends in Plant Sciences. 2003; 8: 554-560.
20. Cai Y-f, Li S-w, Liu Y, QuanS, Chen S, Xie Y-f, Jiang H-z, Wei E-z, Yin N-w, Wang, L, Zhang R, Huang C-l, He X-H, Jiang M-f. Molecular phylogeny of Ranunculaceae based on internal transcribed spacer sequences. African Journal of Biotechnology. 2009; 8: 5215-5224.
21. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for Phylogenetics. *In*. PCR protocols, a guide to methods, and, applications, eds. M.A Innis, D.H. Gelfand, J.J. Sninsky, and, T.J. White. San Diego: Academic Press. 1990. p.315-322.
22. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 1987; 4:406-425.
23. Takezaki N, Rzhetsky A. Nei M. Phylogenetic test of the molecular clock and linearized trees. Molecular Biology and Evolution. 2004;12:823-833.
24. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 1985; 39:783-791
25. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution. 1993; 10:512-526.
26. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution. 2007; 24:1596-1599.
27. Baldwin BG. Phylogenetic utility of the internal transcribed spacers os nuclear ribosomal DNA in plants: An Exemple frm the composite. Molecular Phylogenetics and Evolution. 1992;1:3-16.
28. Kumar LS. DNA markers in plant improvement: a overview. Biotecnology advances. 1999;17: 143:182.
29. Lim CC, Rao V. DNA fingerprinting of oil palm-choice of tissues. J. of oil Palm Research. 2005; 17:136-144.

How to cite this article:

M. C. C. P. de Lyra, D. C. Santos, C. Mondragon-Jacobo, M.L.R.B. da Silva, A. C. E.S Mergulhão and E. Martínez-Romero. Isolation and molecular characterization of endophytic bacteria associated with the culture of forage cactus (*Opuntia* spp.). J App Biol Biotech, 2013; 1 (01): 006-010