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rbcL AND *ITS* BARCODING AND PHYLOGENETICS OF *CYCAS PSCHANNAE* Srivast & Singh

Praveen K. Agrawal[1*], Mirza Zeeshan Beg[2], Masood Akhtar[3] and R.C. Srivastava[4]

[1,2,3] Department of Botany, Shibli National (P.G.) College, Azamgarh, U.P., India
[4] Botanical Survey of India, HQ, Kolkata, West Bengal, India
*E-mail: agrawalpk2015@gmail.com

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ABSTRACT

Barcoding of *Cycas pschannae* Srivast. & Singh from Andaman & Nicobar Islands was done with the help of *rbcL* and *ITS*(Standard Universal Barcode Sequence- CBoL) and submitted to GenBank. Phylogenetic relationship of this species was analyzed and Cladistic study of nine *Cycas species* from India and adjacent country including *Cycas revoluta* thunb had been conducted with help of BLAST tool by using *rbcL* barcode sequence, which reveals that *Cycas pschannae* has close relation with *Cycas circinalis* as compared to other Indian species.

Keywords: *Cycas pschannae*, Barcoding, Phylogeny, *rbcL, ITS.*

INTRODUCTION

DNA Barcoding is a novel technique to provide rapid, accurate species identifications by using short, standardized gene sequences as specific species tags (Hebert *et al.*, 2003; Taberl et *et al.*, 2007) with the help of certain molecular markers. The DNA barcode is an effective tool for the identification and delimitation of plant species in comparison to the complexity of morphological-based identification of plant species. This technique gaining popularity due to the higher precision and simplicity of DNA barcoding, DNA barcode can be used even when the identifying morphological characters are absent and it works for all stages of life. These barcodes can be used as a forensic tool in identification for endangered plant species, e.g. Cycadales (Sass *et al.*, 2007) and an added tool in the discovery of new species. The effectiveness of DNA barcoding depends on the detection and description of new cryptic species and sibling species.

The plant world of the Consortium for the Barcode of Life (CBoL) recommended that the chloroplast DNA sequence for land plants along with nuclear DNA sequence should be used for barcoding. Molecular markers, which are useful in barcoding e.g. plastid genome (*rbcL*) and nuclear genome (*ITS*). It is recommended that *rbcL* and *ITS* gene sequence should be used for plants as standard Universal barcode sequence in the 4th International Barcode of Life Conference, 2011 (Adelaide).

MATERIALS AND METHODS

Cycas pschannae leaflet Sampling was made during a field survey in May 2019 from Cuthbert Bay, Rangat, and Andaman & Nicobar Island. The Sample was collected with the objective to establish its identity and molecular study. Macro-morphological characters were scrutinized to confirm the identity of the taxon (Figure: 1A &B) and compare it with the holotype of *Cycas pschannae* (Srivastava & Singh, 2015).

DNA isolation from *Cycas pschannae* leaflets was worked out by using NucleoSpin® Plant II Kit (Macherey-Nagel).The consistency of the DNA isolated was examined using agarose gel electrophoresis. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Bio-Rad). PCR amplification reactions were carried out in a 20 µl reaction volume which contained PCR buffer, dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, DNA polymerase enzyme, BSA and DMSO, Betaine, forward and reverse primers of *rbcL* and *ITS*2 separately.A PCR thermal cycler was used to conduct the PCR amplification (GeneAmp PCR System 9700, Applied Biosystems).The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) according to the manufacturer's instructions. In an ABI 3500 DNA Analyzer, the cleaned and air-dried product was sequenced (Applied Biosystems). Sequence Scanner Software v1 was used to verify the sequence consistency (Applied Biosystems)

Cladistic study of *Cycas* had been conducted with help of BLAST tool, which was available at www.ncbi.nlm.nih.gov/blast. In BLAST, for nucleotide search, *Blastn* was used to get maximum similarity with query sequence. The most similar sequence obtained from BLAST results were saved in the form of FASTA format for further analysis. Phylogenetic Tree was constructed from these FASTA files by using MEGA X with ClustalW software (Kumar *et al*., 1993).BioEdit software is used to combine the forward and reverse sequences obtained from *rbcL* and *ITS* gene sequencing to create a continuous sequence for

Figure 1: *Cycas pschannae* Srivast. & Singh (A) Apical Cluster of Megasporophylls, (B) A part view of Leaflets. (Photo was taken during a field trip from Cuthbert Bay, Rangat, and Andaman & Nicobar Islands)

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  1 TGGCAGCGTT CCGAGTAATT CCTCAACCTG GAGTGCCGCC TGAGGAAGCG GGAGCTGCAG
 61 TAGCCGCTGA ATCTTCCACT GGTACATGGA CCACTGTTTG GACCGATGGA CTTACCAGTC
121 TCGATCGTTA CAAGGGGCGA TGCTATGACA TCGAGCCCGT TCCTGGGGAG GAAAATCAAT
181 TTATTGCCTA TGTAGCTTAC CCCTTAGACC TCTTTGAAGA AGGTTCTGTT ACTAACATGT
241 TCACTTCCAT TGTAGGTAAT GTATTTGGAT TCAAAGCCCT ACGAGCTCTA CGCCTAGAAG
301 ATTTGCGAGT TCCTCCTGCT TATTCCAAAA CTTTCCAAGG TCCACCTCAT GGTATCCAAG
361 TTGAAAGAGA TAAATTAAAC AAATATGGCC GTCCTCTATT GGGATGTACT ATTAAACCCA
421 AATTGGGTTT ATCTGCCAAA AACTATGGTA GAGCAGTTTA TGAATGTCTT CGTGGTGGAC
481 TTGATTTTAC CAAAGATGAT GAGAACGTAA ATTCCCAACC ATTTATGCGC TGGAGAGATC
541 GTTTCTGCTT CTGTGCAGAA GCAATTTATA AAGCTCAGGC TGAGACGG
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Figure 2: *Cycas pschannae* - FASTA file of *rbcL* Barcode [Accession no.: MT635447 (Genbank)].

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  1 TGGCGCTGTG TTCGTCCCCG CAACGGTCTT CGCATGAGTT TCCCCCCCGC CGTGACTCCG
 61 AGTCGATGTG CGGGCCGCGT CCTCTCGGGG AGGGGGCAGG GTTTTCCGGT TGCGCGGCGG
121 GCGCCGTTAG AGCCAGGGTG GGCCAACAAA CCAAAAGCAC AGGGTGCCCG CGCACCAAG
181 GACTCGAGTT GCTGCACGGA CCCTCGATCC AGTGTTCTGC GGGGGCCCGT GCGGCATATT
241 TGCAGAAATG CACGACTCTC GGCAACGGAT ATCTCGGCCC TGGCCACGAT GAAGAACGTA
301 GCGAAATGCG ATACTTAGTG TGAATTGCAG AATCCCGTGA GTCATCCAGT CTTTGAATGC
361 AAGTTGCGCC CGAGGCTTCG AATGAGGGCA CGTCTGCCTG GGTGTCGCAC ACAATCGAAT
421 CGCCCCATTG CCTGCACGTC GCCTGTGGCG GTGGAGAAGG GCATGCTGGC ATGTCCGTGC
481 TCCCTTGTGA CACCGTCGGC CTTCACCGGA TTGGGGTAGC GCCTTGTCGA GAAGCGATCG
541 AGCGAGCTTG TTGCGTTCAA GGTCGTCTTG GATGATCGAT CACGTCGGCA CCGTAAAAGT
601 TCGCTCGGCA TGCGGGGAAG GCCCCCTCGA CTTGATCACA CTCGGGAGGC CTAGGCTTCG
661 GCCGGAGTTC CTCCCGTCCA CGTCACGCGA CCCCAGGTCA GGCGAGAGCA CCCGCTGAGT
721 TTAAGCATAT CACTAAGCCG AGAAAAGGAA TTCACC
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Figure 3: *Cycas pschannae* - FASTA file of *ITS* Barcode [Accession no.: MT520650 (Genbank)].

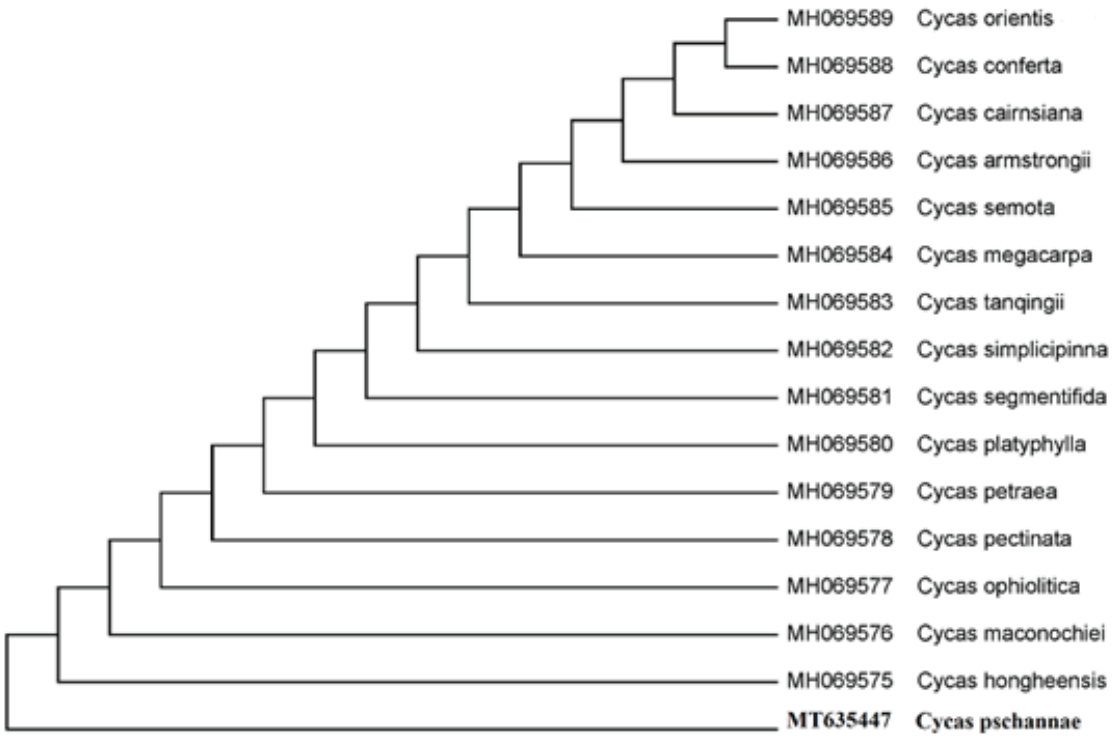

Figure 4: Phylogenetic tree obtained from *rbcL* Barcode (Software MEGA X)

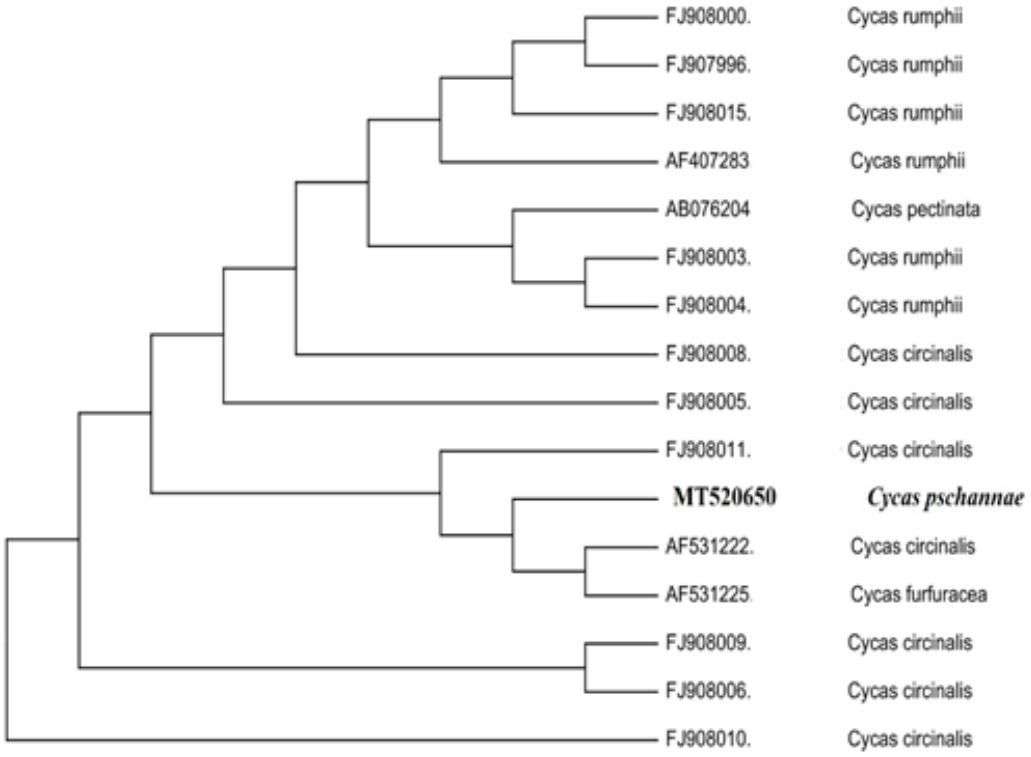

Figure 5: Phylogenetic tree obtained from *ITS* Barcode (Software MEGA X)

submission to GeneBank.

RESULT AND DISCUSSION

The *rbcL* and *ITS* gene barcoding sequences of *Cycas pschannae* Srivast. & Singh has been submitted to GenBank and it gets accession numbers MT635447 and MT520650 respectively (Figure: 2 & 3).

rbcL barcode of *Cycas pschannae* is 588 bp long, a part of a linear mDNA sequence, which contains code for the production of protein 'ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit'. *ITS* barcode of *Cycas pschannae* is 756 bp long linear RNA sequence, which contains internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and large subunit ribosomal RNA.

The phylogenetic tree created by BLAST analysis

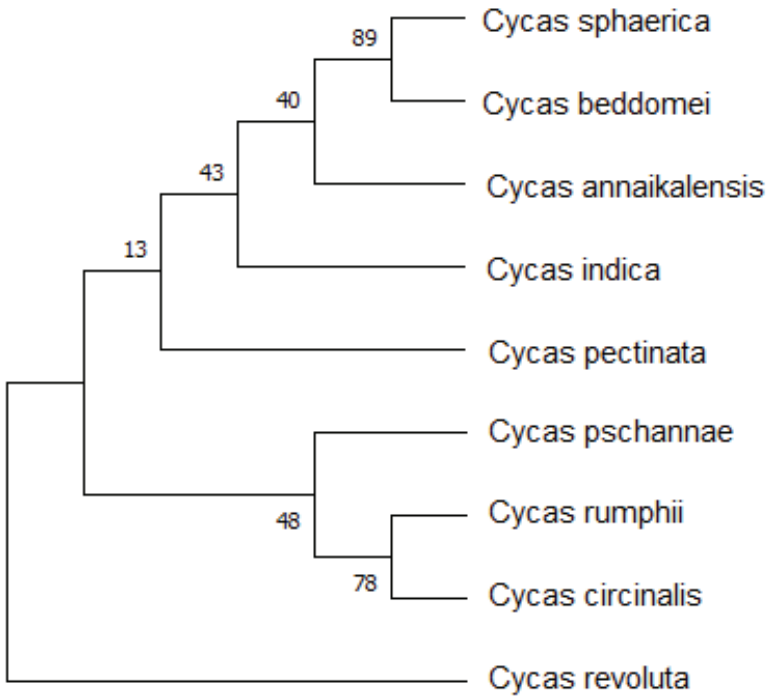

Figure 6: A Maximum likelihood *rbcL* Cladistic of Indian *Cycas* Sp. including *Cycas rumphii* and *Cycas revoluta*. (Software: MEGA X)

Table 1: *rbcL* barcode Accession no. of *Cycas* Species with Authors detail (Source: GenBank Database, NCBI)

S.No.	*Cycas* Species	Accession No.	Authors
1	*C. circinalis* L.	MG009464	Tiwari, N., Kundu, P. and Bast, F.
2	*C. pectinata* Buch. -Ham.	MG009480	Tiwari, N., Kundu, P. and Bast, F.
3	*C. rumphii* Miq.	MG009477	Tiwari, N., Kundu, P. and Bast, F.
4	*C. revoluta* Thunb.	MG009470	Tiwari, N., Kundu, P. and Bast, F.
5	*C. beddomei* Dyer.	KY077558	Rout,G.R., Swain,D. & Jadhao,K.R.
6	*C. sphaerica* Roxb.	KF432022	Saritha, K.V., Khedkar, G.D., Hanumanth Kumar, G., Ughade, B.R., Tiknaik, A.D. & Mohan Reddy,Y.
7	*C. annakalensis* Singh & Khuraijam	MH069560	Forest,F., Moat,J., Baloch, E., Brummitt,N.A., Bachman,S.P., Ickert-Bond,S., Hollingsworth, P.M., Liston,A., Little,D.P., Mathews,S., Rai,H., Rydin,C., Stevenson,D.W., Thomas,P. &Buerki,S.
8	*C. indica* Lindstrom & Hill	MH069561	Forest,F. *et al.*
9	*C. pschannae* Srivast. & Singh	MT635447	Agrawal, P.K. & Akhtar, M.

of the *rbcL* barcode marker confirms that the plant material belongs to the Genus *Cycas*, and the *ITS* barcode marker confirms the same, at the same time also indicating a close relationship to *Cycas circinalis* and a distal relationship to *Cycas rumphii* and *Cycas pectinata*, both of which are found in India and the surrounding areas (Figure: 4&5).

Cycas species (India) including *C. rumphii* and *C. revoluta* (Japan) were sequenced by using *rbcL* gene marker from chloroplast/plastidto examine diversification and evolution at the molecular level. The *rbcL* sequence of *C. circinalis* (MG009464.1); *C. pectinata* (MG009480); *C. beddomei* (KY077558); *C. sphaerica* (KF432022); *C. annakalensis* (MH069560); *C. indica* (MH069561); *C. rumphii* (MG009477) and *C. revoluta* (MG009470) were taken together with *C. pschannae* (MT635447) from Genbank database (Available online at https://www.ncbi.nlm.nih.gov/genbank) (Table: 1).

The Neighbor-Joining approach was used to infer the evolutionary history (Saitou &Nei, 1987). The evolutionary history of the taxa examined is represented by a bootstrap consensus tree inferred from 100 replicates. Branches that correspond to partitions that have been replicated in less than 50% of bootstrap replicates have been collapsed.Next to the branches is the percentage of replicate trees in which the related taxa clustered together in the bootstrap test (100 replicates) [Felsenstein, 1985]. The evolutionary distances were calculated using the Maximum Composite Likelihood method [Tamura, Nei, and Kumar, 2004] and are in the units of the number of base substitutions per site.

The nucleotide frequencies are A = 26.75%, T/U

= 28.19%, C = 21.32%, and G = 23.74% in dataset. This analysis involved 9 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1340 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar, Stecher, Li, Knyaz& Tamura, 2018).

Cladistic analysis (Figure: 6) illustrates the relationship within *Cycas* species. A separate position of *Cycas revoluta* (A Japan origin species) indicates its distance relation with Indian and adjacent country *Cycas* species. *Cycas* species from South India i.e. *Cycas sphaerica*, *Cycas beddomei*, *Cycas annaikalensis*, *Cycas indica* has shown difference with *Cycas* species north east India i.e. *Cycas pecitnata*. *Cycas circinalis* and *Cycas rumphii* show most similarity supported, its evidence of presence and reported from south India and adjacent country. *Cycas pschannae* has shown close relation with *Cycas circinalis* compared to other Indian species.

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Conflict of interest statement: Authors declare that they have no conflict of interest.

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