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MOLECULAR IDENTIFICATION OF CACTACEAE PLANT USING rbcL DNA BARCODE

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Abstract: DNA barcoding is a technique of employed in short DNA sequences of the standard genome segment to identify species on a large scale. Compared to morphology-based taxonomy identification, which is challenging and prone to subjective biases, DNA barcoding is becoming more and more popular due to its ease of use and high accuracy rate. The standard chloroplast DNA barcode a terrestrial plant, which was developed by the Consortium for the Barcode of Life (CBOL) plant working group, has to be evaluated for knowing variation of plant species. The amplicons were created by amplifying the isolated genomic DNA using the rbcL gene in a PCR. The recovered amplicons, which are ranged in length from 500 to 800 bp, were subjected to sequencing for the rbcL gene area. Therefore, in the present investigation the rbcL marker's potential for identifying plants in the cacti family is undertaken. To assess the rbcL gene's discriminating capacity, maximum neighbourhood tree analysis was used. The result obtained from DNA barcoding matches up to the genus level including Turbinicarpus and species up to 99 % but these species though it is having 98% rbcL gene similarly with existing species it has 1 % of individual specific genomic component that supports to be identified a new variant species hence these Cactaceae plant family species is considered as new species.

Keywords: DNA Barcoding, rbcL, species identification, DNA extraction, Phylogenetics, Cactaceae.

I. INTRODUCTION

The plant Cactaceae tribe is monophyletic, comprising about 400 species that have globose, globose-depressed, cylindrical, and short columnar development patterns. Distribution of this plant species across the globe. It is found in the USA, Colombia, and Venezuela, but it is most commonly found in Mexico, where the lineage has become more diverse (Vazquez-Sanchez *et al*., 2019). Cacti's metabolic, physiological, and morphological traits are influenced by the harsh environments that frequently make up their habitats, such as scarce water supplies, deficient soil nutrients, and sharp temperature swings (Martinez-Vazquez *et al*., 2021).

The Cactaceae provide a good test of the utility of barcodes for the identification of plants. Cacti are popular with collectors, often illegally traded, difficult to identify and likely to be a group where investment in molecular identification tools will pay dividends in terms of conservation outcomes (Hardesty *et al*., 2008; Hughes *et al*., 2008). Although the family as a whole show a wide range of morphology, closely related species are morphologically similar and are difficult to identify when not in flower (Anderson 2001; Hunt *et al*., 2006).

A small, uniform DNA sequence is used in the species identification process known as a "DNA barcode"(Amandita *et al*., 2019). Recently, barcoding has progressed most in animals while there has been less progress in plants. In particular the order of operations is to identify an appropriate sequence region has been complicated by the desire for a variable sequence providing species specific detail while also being conserved enough to be able to relate the sequences from different organisms (Chase *et al*., 2007; Cowan *et al*., 2006; Newmaster *et al*., 2008). It is hard for categorizing and identifying so many species accurately so it remains a significant challenge, even for highly skilled taxonomists. The advancement of DNA barcoding has helped with biodiversity classification and identification. DNA barcoding is a method for identifying species of organisms by utilizing a brief DNA sequence from a predetermined standard location in the genome. Hence Taxonomists have been interested in DNA barcoding. There are numerous other types of DNA barcoding as well to uphold the right of ownership of intellectual property. Global DNA barcoding was once heralded as a "big science" initiative and even as the return of taxonomy (Li *et al*., 2015). The application of DNA barcoding could facilitate the identification of species more quickly and help to overcome the limitations of morphological traits. Many barcoding approaches have already been developed to explore whether barcoding can effectively be used to verify plant products, including yet not limited to, medicinal plants, kitchen spices, berries, olive oil, and tea (de Vere *et al*., (2015).

rbcL is frequently used sequence in phylogenetic analyses, and Genbank contains over >50000 sequences. This gene has the benefit of being an excellent DNA barcoding region for plants at the family and genus levels, and it is simple to amplify, sequence, and align in the majority of terrestrial plants (Iqra *et al*., 2025). While rbcL alone does not full fill the requirements

for a barcoding locus, it is acknowledged that rbcL can accurately identify plastid or nuclear loci when combined with them (Li *et al*., 2015).

The present investigation is aimed to identify the Cactaceae plant using the rbcL marker through DNA barcoding.

II. MATERIALS AND METHODS

2.1 Plant materials

The plant sample was collected from Green Paradise Nursery, Kalimpong, West Bengal, India. Evolutionary lineage of the selected plant was reported to be unknown.

2.2 Isolation of DNA and rbcL gene amplification

All molecular studies were carried by out sourcing at Barcode Biosciences, Bengaluru, India. Genomic DNA was isolated from the stem of fresh plant sample using QIAGEN DNeasy Blood & Tissue Kit. Its quality was evaluated on 1.0 % agarose gel. Mitochondrial, Chloroplasts, and Nuclei rbcL universal marker gene have been amplified using forward and reverse oligonucleotides.

Synthetic oligonucleotides for polymerase chain reaction (PCR) primers were synthesized by Barcode Biosciences, Bengaluru, India. The following primer sets were utilized to amplify the rbcL gene:

Forward ATGTCACCACAAACAGAGACTAAAGC

Reverse GTAAAATCAAGTCCACCRCG

PCRs were conducted in a total reaction volume of 25 µL containing 6 µL of nuclease free water, 2 µL of DNA template, 2 µL of rbcL forward primer and reverse primer each and 13 µL Amplicon master mix (Kress and Erickson, 2007; Fazekas *et al*., 2008). The PCR for rbcL was performed with an initial denaturation of 30 min at 95^{o}C followed by 35 cycles under the following conditions: 95^{o}C for 30 seconds, 50^{o}C for 30 seconds and 72^{o}C for 45 seconds, terminated by an extension of 72^{o}C for 3 min.

PCR Samples were loaded after mixing with gel loading dye along with 10 µl of DNA Ladder (1500bp DNA Ladder). The PCR amplicon was purified to remove contaminants QIAGEN QIAquick PCR Purification Kit. The PCR product was then preserved at 4^{o}C until sent for sequencing.

2.3 Cycle sequencing, DNA Barcode and Phylogenetic analysis

PCR rbcL products were sequenced according to the method described by Sanger's method 1977; Sanger sequencing of amplicons were carried out using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The nucleotide sequence data of the rbcL Consensus sequence were deposited in the Genbank, NCBI nucleotide sequence databases.

The Cactaceae plant were identified using rbcL amplicon gene sequence in the Bioinformatics sequence analysis tools BOLD (Barcode of life data system) database and a homology search for rbcL genes was performed using Basic Local Alignment Search Tool (BLAST). The identification was correct if the highest identity percentage of searched sequences was derived from expected species or genus. On the other hand, the identification was ambiguous when the highest identity percentage of searched sequences was not derived from expected species or genus, or family.

The genetic distance was calculated using the similarity indices and the phylogenetic trees were constructed using MEGA 11 software (Tamura *et al*., 2011). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993).

III.RESULTS

3.1 Unknown plant sample

The plant sample collected from Green Paradise Nursery, Kalimpong, West Bengal, India were raised in the green house at Department of Biotechnology, Gulbarga University, Kalaburagi for further study and identification of plant is achieved through DNA barcoding using rbcL gene.

Fig1: Cactaceae plant

3.2 rbcL gene amplification and sequencing

Genomic DNA extraction from the stem of fresh plant sample using QIAGEN DNeasy Blood & Tissue Kit was successful, revealing high molecular weight and comparable concentration (Fig. 2A). The efficacy of DNA barcodes relies on the efficiency of PCR amplification and the accuracy of PCR primers. In this study, rbcL markers demonstrated a 100% success rate in amplification. The amplifications of DNA barcode regions were highly specific, yielding the expected product size of 494 bp with a clear background in the agarose gel (Fig. 2B). Subsequently, sequence was successfully obtained by Sanger method with a 100% sequencing success rate. The obtained sequences were aligned, annotated and submitted in GenBank with Accession number: PQ227793 with description Turbinicarpus sp. RL-2024a.

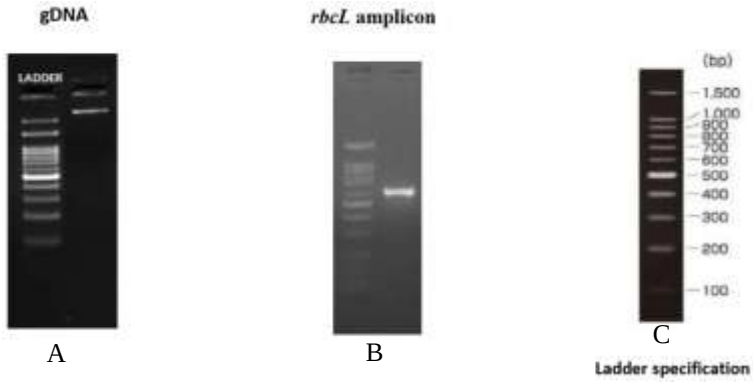


Fig 2: Electrophoretic profile depicting amplified genomic DNA regions through PCR reactions. A- Genomic DNA; B- rbcL gene; C- Ladder specification.

3.3 DNA barcode analysis

The unknown Cactaceae plant is identified by the Consensus sequence data of rbcL gene in the BLAST search, along with their respective accession numbers, are presented in Fig 3. The results highlight the percentages of the greatest similarity in the subject strains, with most samples yielding sequences designated as the best hits of 99.80% with 100% query cover. Furthermore, the findings demonstrate a strong correlation at the genus *Turbinicarpus*, *Strombocatus* and *Mammilaria* for rbcL marker, with a relatively low incidence of misidentification through a best-close match analysis. Based on the effectiveness of the marker in BLAST searches and the analysis of the closest genetic distance and rbcL marker achieved a remarkable 99% accuracy at the genus level, highlighting their reliability in capturing broader taxonomic categories.

BOLD analytical result shown the 100% confidence level of classification of unknown plant is presented in Fig 4. BOLD database combined hits result of our query sequences, shown the highest hit to the genus *Mammillaria* of the Cactaceae family with 99.76% and with a lowest hit of 98.82% to the *Turbinicarpus beguinii* of the Cactaceae family are presented in Fig 5.

The analytical results of the BLAST and BOLD indicates that, the rbcL DNA barcode from unknown Cactaceae plant shown ambiguous outcomes with multiple hits to genus *Turbinicarpus* and *Mammillaria* of 99.80% and 99.76% identity respectively.

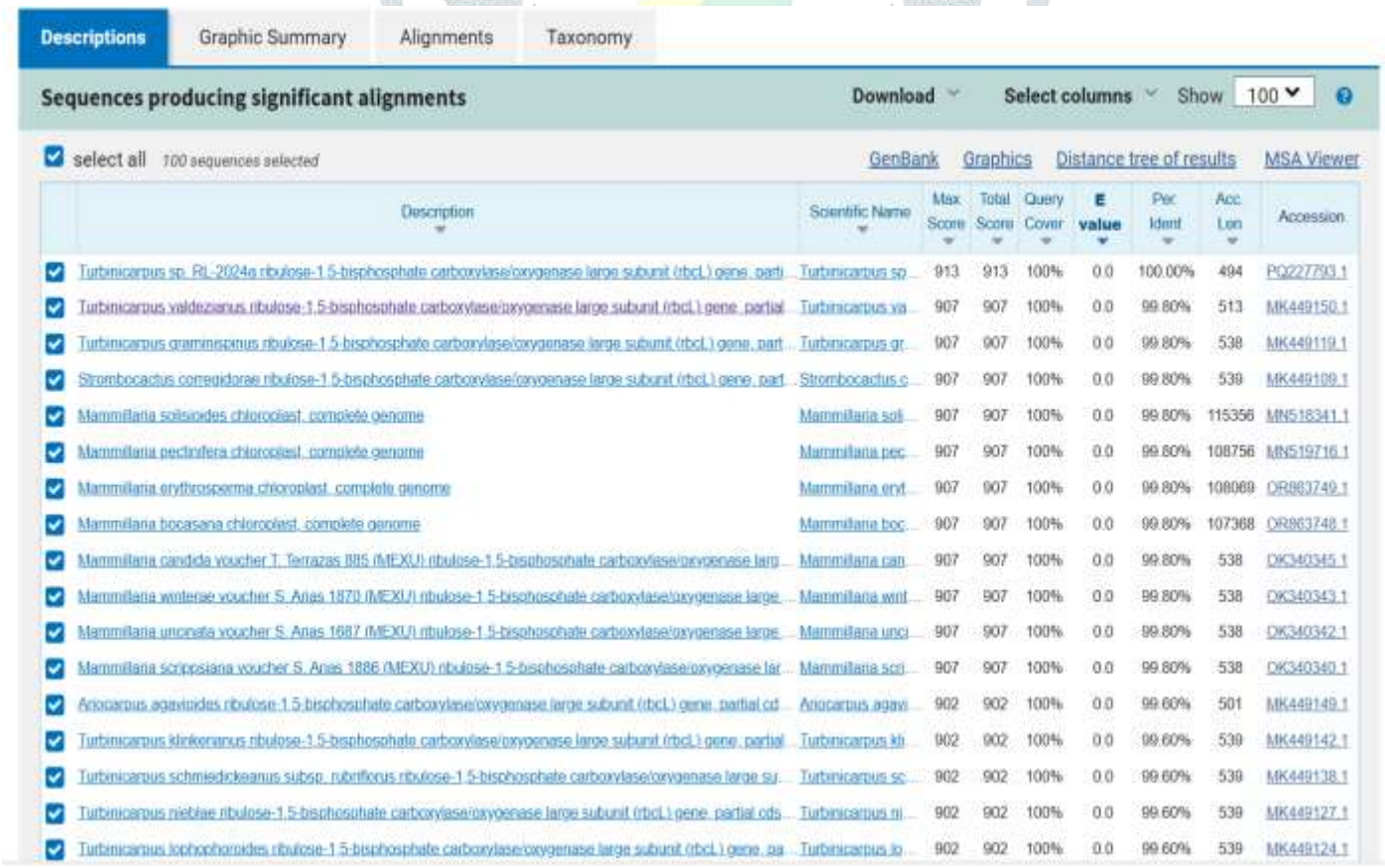
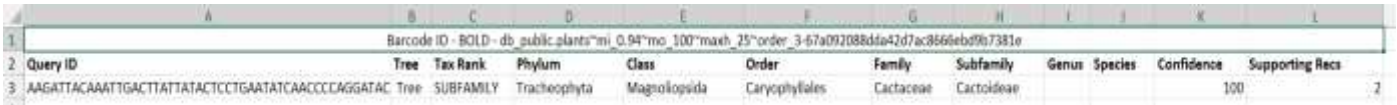


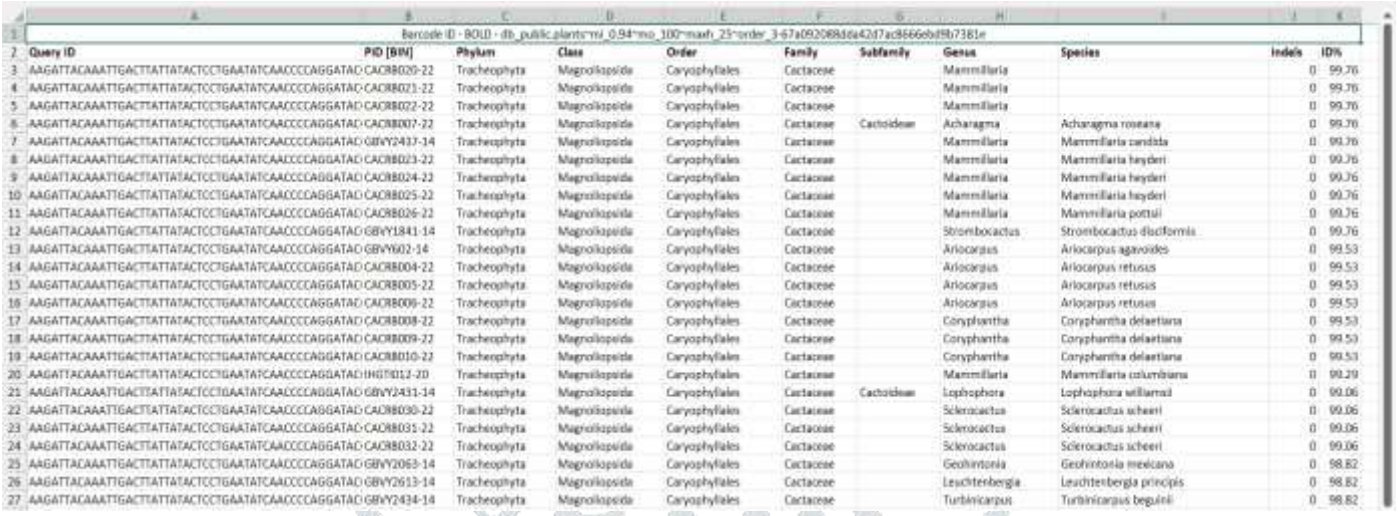
Fig 3: Sequences producing significant alignments for rbcL gene through BLAST



Barcode ID - BOLD - db_public,plants"mi_0.94"mo_100"maxh_25"order_3-67a092088dda42d7ac8666ebd9b7381e

Query ID	Tree	Tax Rank	Phylum	Class	Order	Family	Subfamily	Genus	Species	Confidence	Supporting Recs
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	Tree	SUBFAMILY	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae	Cactoideae			100	2

Fig 4: Classification of Unknown plant by using rbcL marker through BOLD



Barcode ID - BOLD - db_public,plants=ml_0.94=mo_100=maxh_25=order_3-67a092088d6e42d7ac8666e6d9b7381e

Query ID	PID [BIN]	Phylum	Class	Order	Family	Subfamily	Genus	Species	Indels	ID%
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB020-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Mammillaria		0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB021-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Mammillaria		0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB022-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Mammillaria		0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB007-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae	Cactoideae	Acharagma	Acharagma roseana	0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	GBVY2437-14	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Mammillaria	Mammillaria candida	0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB023-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Mammillaria	Mammillaria heyderi	0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB024-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Mammillaria	Mammillaria heyderi	0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB025-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Mammillaria	Mammillaria heyderi	0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB026-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Mammillaria	Mammillaria pottsii	0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	GBVY1841-14	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Strombocactus	Strombocactus disciformis	0	99.76
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	GBVY602-14	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Ariocarpus	Ariocarpus agavoides	0	99.53
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB004-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Ariocarpus	Ariocarpus retusus	0	99.53
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB005-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Ariocarpus	Ariocarpus retusus	0	99.53
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB009-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Ariocarpus	Ariocarpus retusus	0	99.53
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB008-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Coryphantha	Coryphantha delaetiana	0	99.53
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB009-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Coryphantha	Coryphantha delaetiana	0	99.53
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB010-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Coryphantha	Coryphantha delaetiana	0	99.53
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	IHGT9012-20	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Mammillaria	Mammillaria columbiana	0	99.29
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	GBVY2431-14	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae	Cactoideae	Lophophora	Lophophora williamsii	0	99.06
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB030-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Sclerocactus	Sclerocactus scheeri	0	99.06
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB031-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Sclerocactus	Sclerocactus scheeri	0	99.06
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	CACRB032-22	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Sclerocactus	Sclerocactus scheeri	0	99.06
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	GBVY2063-14	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Geohintonia	Geohintonia mexicana	0	98.82
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	GBVY2613-14	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Leuchtenbergia	Leuchtenbergia principis	0	98.82
AAGATTACAAATTGACTTATTATACTCCTGAATATCAACCCCAGGATAC	GBVY2434-14	Tracheophyta	Magnoliopsida	Caryophyllales	Cactaceae		Turbinicarpus	Turbinicarpus beguinii	0	98.82

Fig 5: BOLD database producing significant alignments for rbcL gene

3.4 Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-687.41) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 494 positions in the final dataset (Fig 6).

Phylogenetic evaluation of rbcL nucleotide sequences showed a well-resolved tree with multiple clades, whereas PQ227793.1 and MK449150.1 both belonged to a one clade. It indicates that, the unknown Cactaceae plant species is very similar to *Turbinicarpus valldezianus* but morphologically different.

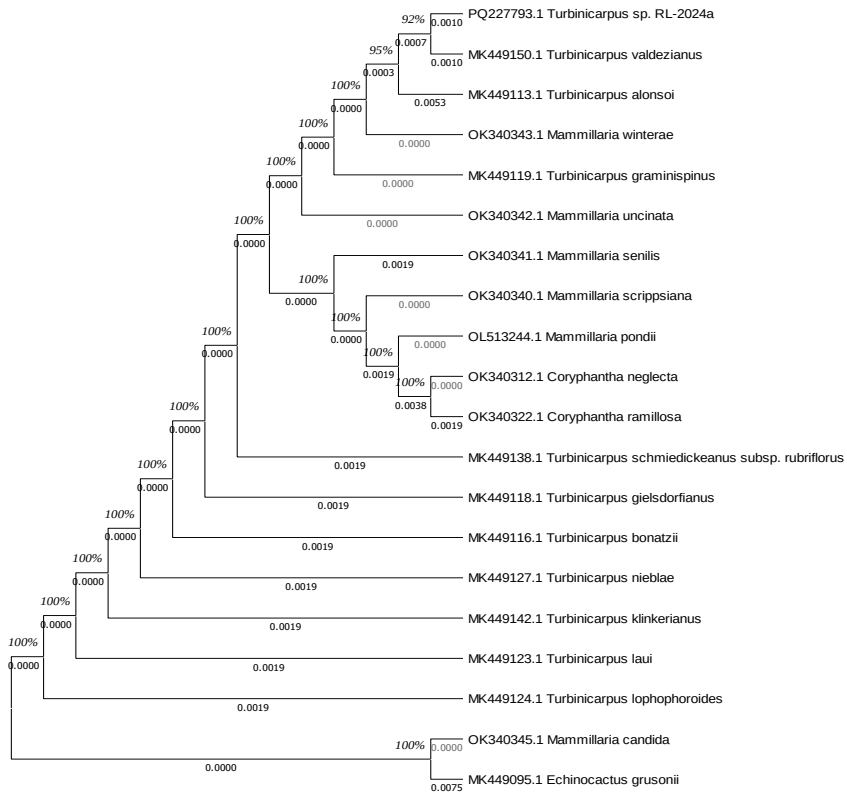


Fig 6: Molecular Phylogenetic analysis by Maximum Likelihood method

IV. DISCUSSION

Understanding the evolutionary history of many significant plants depends on accurately identifying and assessing biologically significant plant species and their families (Shinwari *et al.*, 2018). DNA barcoding soon became a reliable method for identifying species, spotting adulteration, quality control, and ecological assessments. However, it cannot replace the conventional taxonomic identification technique, but it will be easier to understand phylogeny and its origins with the help of this information. The purity of the DNA is crucial for PCR based amplification. Even closely related species may need distinct DNA isolation procedures because metabolites in medicinal plants can occasionally affect DNA integrity during isolation (Khanuja *et al.*, 1999). The main principle of plant species identification is the sequencing variation from the reference sequence and phylogenetic reconstruction (Altschul *et al.*, 1997). In this study, the chloroplast rbcL DNA barcoding region is used to identify the unknown Cactaceae plant variety by amplifying the rbcL gene and sequencing. The 494bp sequence of rbcL gene was subjected to BLAST and BOLD sequence analysis tools for the similarity check. The result showed the high homology similarity with the genus *Turbinicarpus* and *Mammillaria* with similarity of 99.80% and 99.76% respectively. The high universality of the rbcL region in terms of sequence amplification and recoverability was similar to those obtained in other research work (CBOL Plant Working Group *et al.*, 2009 and

Kang *et al*., 2017). Good recoverability of the rbcL region has been mentioned (Roy *et al*., 2010 and Amandita *et al*., 2019). Several authors have demonstrated the high universality of rbcL primers and showed that the sequence generated by this locus was sufficient to function as the central region of DNA barcoding, as it provided sufficient variation for distinguishing species among different plant groups (Kress and Erickson, 2007).

An important factor limiting the success of species identification with DNA barcode technology is the availability of sequences of the corresponding taxa in databases. In this study, the high identification rates of rbcL regions are not only because of the discriminatory power of the barcode itself but also because of the rich sequence resources in the database. Other authors have mentioned that samples belonging to species that have not been registered in the reference databases lead to an increase in the rates of unidentified samples, which is mainly due to incomplete molecular datasets rather than the type of data analysis (Cowan and Fay, 2012).

The neighbour-joining method is based on a grouping algorithm that creates a phylogenetic tree; however, its creation is influenced by the parameters used, mainly by the distance matrix and index, and by the effects of lineage classification between species with very recent divergences (Saitou & Nei, 1987 and Yan et al., 2015). The tree-based method evaluated the discrimination efficiency at the species level, so if the species of a genus formed a monophyletic clade, it was considered a successful identification. In this study, the phylogenetic tree generated with rbcL gene sequence showed the highest discrimination success rate of species in the individual barcode regions analysis.

Our findings showed that, DNA barcoding using rbcL gene can be a promising identification tool primarily at the family and genus level. Thus, further studies using machine learning algorithms that use the whole molecular, morphology, biogeography and barcode Cactaceae data available in open databases could significantly improve the correct Cactaceae species classification.

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