Annals of Plant Sciences 8.4 (2019) pp.3541-3546

ANNALS OF PLANT SCIENCES
ISSN: 2287-688X
OPEN ACCESS
www.annalsofplantsciences.com

Research Article

DNA Barcoding of endangered *Ceropegia odorata* Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn. species of Gujarat

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Abstract: In the present study, DNA Barcoding was made for the endangered plants *Ceropegia odorata* Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn. DNA barcoding is used to authenticate their reliable identification, conservation, discrimination, similarities and evolutionary trend among them and with their related taxa for future use. Two species, viz., *Ceropegia odorata* Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn are reported from Gujarat Dediyapada. The identification of both the species depends on the knowledge which held by taxonomists. Non-specialist cannot cover all the knowledge of the species. To handle with these difficulties' DNA Barcoding aims to develop a standardized, rapid, and inexpensive species identification method approachable to non- specialists and will become easy for taxonomist. DNA isolation from leaf samples of present study species was carried out by applying a modified CTAB method and good isolation was just for the species considered. Gradient PCR amplification was performed for the isolated DNA using rbcL gene and the primers rbcLF and rbcLR were used. The amplification success rate was 90-95%. PCR amplification was tested with 1 % agarose gel electrophoresis using ethidium bromide and the products were confirmed. The PCR products were further processed for DNA sequencing and sequences were good for the species with the success rate of 95 %. Pairwise sequence alignments were made with BLAST and multiple sequence alignments are made with ClustalW, and Neighbor joining method to study the phylogenetic aspects of the species studied and with their related taxa this study was to apply to support conservation efforts of *Ceropegia* Species in Gujarat and all over India. We used to recommend rbcL marker for both the sample. We specifically tested whether the markers could be used to solve taxonomic confusion concerning the *Ceropegia* species. This Barcoding system will be utilized specifically to identify and create phylogeny among the selected endangered species.

Key words: DNA Barcoding, rbcL, endangered plants, identification, Phylogenetic tree, conservation

Introduction

Shoolpaneshwar Wildlife Sanctuary situated in the Narmada district in the Southerly region of Gujarat state covering 607.70 Km^2 areas. The Sanctuary falls in Dediyapada and Nandod Talukas of the Narmada District (Vyas, 2011). Originally the Sanctuary was established in 1982, in the name of Dumkhal Sloth Bear Sanctuary. In following notifications made in 1987 and 1989, the area of the sanctuary was distended and now it is known as Shoolpaneshwar Wildlife Sanctuary. The name was derived from the traditionally and myth logically famous Shoolpaneshwar temple, which had gone under submergence of Sardar Sarovar Dam. The forest area of Rajpipla, Kevadiya, Sorapda, Dediyapada and Sagbara ranges surround Shoolpaneshwar Wildlife Sanctuary. One such critically endangered species *Ceropegia odorata* Nimmo ex J. had gone extinct from its type locality soon after the type collection (Graham, 1839). After a lapse of almost a hundred and a half rediscovered *Ceropegia odorata* from the Pavagadh Hills, Panchmahal District in Gujarat but did not comment upon the fragrant aspect of the flowers (Sabnis and Bedi., 1971). Consequently, species was not found in this locality. A decade later, in locating this species, with fragrant flowers, on a new site at Melghat, Amravati District in Maharashtra (Ansari, 1982). This led to the authentication of Nimmo"s observation of "fragrant yellow" flowers of *C. odorata*. Finally, after almost one and a half century of uncertainty confirmed that the flowers in *C. odorata* were fragrant. The species of *Ceropegia* is whole under threat, owing to either their destructive collection or habit degradation. (Rupesh *et al.*, 2018). During our serious botanical explorations in various parts of dediyapada forest in Narmada district, two individuals of *Ceropegia* sp. were recorded. Growing amongst grasses on slopes. Specimen were collected. After critical examination however it was very challenging for us to identify of both the species. After that species were identified by taxonomist but it was very challenging for them. Both the species were put under genetic study with the help of DNA Barcoding method to correct identification. It was described as a new disc to the flora of Gujarat from only one locality (i.e., pavagadh hills in panchmahal district) (Sabnis and Bedi., 1971). The only documented evidence about its occurrence in Gujarat. However, several explorations made from time to time by field botanists from different academic and research institutions (Punjani 1997; Patel 2003, 2013; Pandey 2011; Meena 2012; Parmar 2012; Desai 2013) could not recollect this taxon from pavagadh. It was also reported as a first record of this species as a new distribution in Sabarkantha district and range

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extension record to the flora of Gujarat (Patel *et al.*, 2017). *Ceropegia odorata* Nimmo ex J. Graham, and *Ceropegia hirsuta* Wight & Arn were collected from dediyapada forest but it is still challenging to correct identification because of dissected floral parts, other morphological features and perusal of relevant literature survey. Both sample put under genetic study using DNA Barcoding method. DNA Barcoding is a new tool for the science of taxonomy at the genotypic level which was first initiated and it has attracted international attention for its effect in coming the taxonomy of life forms (Paul Herbert., 2003). Today, DNA barcodes have been nominated for many factors of plant applications include, for example, identifying plant leaves even when flowers or fruit are not available, identifying the diet of an animal based on stomach contents or feces, and identifying products in Herbal supplements or wood.

IUCN threat status:

Based on taxation and field observations from 2007 to 2013, *Ceropegia odorata* ex J. Graham is presently categorized as Critically Endangered [B2b(i,ii,iii,v) c(i,ii,iv)] (Singh *et al.*, 2014). Threat status allocated to the listed species by several authorities, regulatory instruments, and international treaties such as IUCN, CITES, CAMP, RDB14, ENVIS, WPA and state biodiversity notifications under The Biological Diversity Act, 2002. The gathered data were observed for contradictions in the threat status assigned to the species by CAMP, ENVIS and RDB, where the status assigned to a particular species differed among the nations. In such cases, we considered *Ceropegia hirsuta* Wight & Arn the highest threat category assigned to a particular species. (Barik *et al.*, 2018)

Materials and Methods

Sample collection:

Ceropegia odorata Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn were identified. Leaves were cut and collected. Both the sample stored in zip lock bag contain silica gel with sample id. After that both samples were transported to the laboratory and kept in (−20 °C) refrigerator.

Figure 1: *Ceropegia odorata* Nimmo ex J. Graham.

Figure 2: *Ceropegia hirsuta* Wight & Arn

DNA Isolation:

Stored Plant leaf samples were used. Sample were macerated using a sterile mortar and pestle under liquid nitrogen. DNA isolation was carried out by using modified CTAB (Cetyl Trimethyl Ammonium Bromide) method. It takes minor modification. (Bishoyi *et al.*, 2016). The concentration and quality of the extracted DNA were determined using gel electrophoresis. The isolated genomic DNA was stored at -20°C until used (Bafeel *et al.*, 2012).

PCR Amplification:

Gradient PCR was performed using isolated genomic DNA of this study to determine the optimum annealing temperatures of the Primers used, namely rbcL-F (5'-ATGTCACCACAAACAGAAAC-3') rbcL-R (5'-TCGCATGTACCTGCAGTAGC-3'). This primer was obtained from Xcelris Lab Ahmedabad. The PCR reaction mixture was consisted of 10xTaq buffer, dNTP mix 0.3 µl, Taq polymerase 0.3 µl and 5-50 ng of template DNA. Thermal cycling conditions for gradient PCR were as follows: Initial DNA denaturation at 95°C for 5 min, followed by 25/30 cycles of 95°C for 30 sec 45°C for 30 sec, DNA strand extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The PCR products were verified by electrophoresis in 1 % agarose gel stained with ethidium bromide. PCR products were mixed with 1µl of gel loading dye (Bromophenol dye) in a 2 % agarose gel containing ethidium bromide along with 5 µl of DNA ladder. Electrophoretic separation was performed at 100 v for 30 min. Resulting DNA fragments were visualized using an ultraviolet Trans-illuminator. PCR products were purified using a QIA quick PCR Purification Kit (Qiagen) before being sequenced using the dideoxynucleotide chain termination method with a DNA sequencer (ABI 3730XL, Applied Biosystems) and a Big Dye Terminator version 3.1 Cycle Sequencing RR-100 Kit (Applied Biosystems). All sequences were submitted to BOLD System. (Bafeel *et al.*, 2012).

Cycle sequencing:
According by providing protocol, All the DNA regions were sequenced by using the Big Dye Terminator v3. 1 cycle sequencing Kit. Cycle sequencing was done by the 10µl volume. Reaction mixture was developed for each reaction by adding reagents. Two reaction tubes were developed. One is for forward sequencing primers and other one is for reverse sequencing primers. RbcL gene amplification primers (rbcLF and rbcLR) served as sequencing primers in this experiment.

The CTAB method protocol resulted in contamination free, good quantity DNA yield of fresh tissue. The GEL image specified the purity of the DNA. This gel image shows the good DNA product of *Ceropegia odorata* Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn. Both species show good isolation activity and for the isolation of genomic DNA of the species studied. The modified protocol of Cetyl Trimethyl Ammonium Bromide method (CTAB method) was used and good isolation was obtained for the sample. The gradient PCR amplification was performed for the isolated genomic DNA of two species of *Ceropegia* genus mentioned earlier by using rbcL gene and its primers and the amplifications were obtained from the species examined. The amplification of gradient PCR product was solid enough for isolation of bands or direct sequencing and in the present work. The DNA sequences were done at Xcelris lab, Ahmedabad. The methods of ABI-3730 X1 sequencer gave a success rate of 90-95% and read length of 700 bases or more.

Results

Ceropegia odorata Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn were found in dediyapada forest Narmada district. Both the species were identified. Leaves were collected. GEL Electrophoresis image of *Ceropegia odorata* Nimmo ex J.Graham and *Ceropegia hirsuta* Wight & Arn

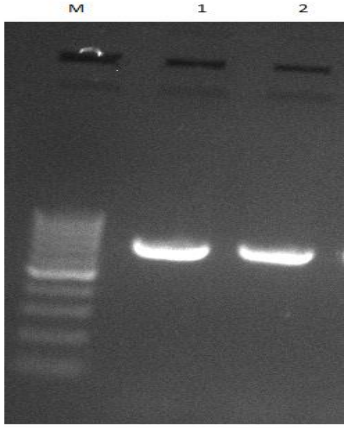


Figure 3: Electrophoresed gel (0.8% agarose) image of total DNA extracted of **M**. DNA ladder **1**. *Ceropegia odorata* Nimmo ex J. Graham **2**. *Ceropegia hirsuta* Wight & Arn

DNA sequence:

***Ceropegia odorata* Nimmo ex J Graham:**

GACTGCATAGGATCATGACGGTGGGTAGA
GCCTGAGTGACTTATTATACTCCTGAATAC
GAAACAAAGACCTGATATCTTGGCCGGTT
GCGAGTAACTCCTCACCCCGGAGTTCCTCC
CGAAGAAGCAGGGGCCGCGGAATCTGCC
GAATCTTCTACTGGTACATGGACACTGACC
GGACCGATGGACTTACCAGACTTGATCGT
TACAAAGGACGATGCTACCATATCTAGGCC
GTTCCTGGAGAAAAAAATCGATATATTGCT
TATGTAGCTTACCCTTTAGACCTTTTTGAA
CAAGGTTCTGTTACTAACATGCTTACCTCC
ATTGTAGGTAATGTATTTGGGCTCAAAGC
CCTACGCGCTCTACGTCTGGAAGATTTGCC
AATCCCGCCGGCTTATACTAAAACCTTCCA
AGGCCCGCCGCATGGCATCCTCGTTGAGA
GGGATAAATTGAACAAATATGGGCTTCCC
CTGTTGGGATGTGCTATTAAACCCAAATTG
GGGTTATCCGCTATAAACTACAGTAAGAG
GCGTTATGAATGTCTTCCCCGGGGACTTCA
TTTTACCCTCCATGATGAAAGAGGTGAACT
CCCAACCGCTTATATGTTAGAGAGATCTTT
TTCTTGTTTTGCGCCGATAATATTTTTATAT
CTCCTGTTGAAACCCGCGGGATCATCGGG
CATTACTTGAATGCTACTGCTGGGGGGCC
CCTAAAAAAA

***Ceropegia hirsuta* Wight & Arn:**

GGGGGATGGGATCGTGCCGCGTGGGTTG
AGTACTGATTGACTTATTATACTCCTGAAT
ACGAAAAAAAGATACTGATATCTTGGCCG
GTTCCGAGTAACTCCTCAACCCGGAGTTCC
GCCCGAAGAAGCAGGGGCCGCGGAATCT
GCCGAATCTTCTACTGGTACTTGGCTGATT
GGACCGATGGACTTACCAGACTTGATCGT
TACAAAGGACCATGCTACCATATCTAGGCC
GTTCCTGGAGAAAAAAATCGATATATTGCT
TATGTAGCTTACCCTTTAGACTTTTTGAAC
AAGGATCTGCTACTAACATGCTTACCTCCA
TTGTAGGTAATGTATTTGGGCCCACAGTG
CTACGCGCTCTGCGTCTGGAGGATTTGCC
AATCCCGCCGGCTTATACTAAAACCTTCCA
AGGCCCGCCGCATTTTCTCCTCGTTGAAAG
GGATAAATTGAACAAATATGGCCTTCCCCT
GTAGGGATGTACTATTAAACCTCAATTGG
GGCTATCCGCTAAAACTACAGTAAGATGG
GTTATCAACGTCTTCCCCGGGTACTTCATT
TTGCCCTCCATGATGAGGAGGTGCACTCCC
AACCGCCTATATATCAGAAACATCTGTTCT
TGATTTGCGCCGATAATATTTTTATATCAC
CTGTTGAACACCGGCGATTTAATCGGGCA
TTACTTGAATGCTACTGCAGGGGGAACCA
AAAAAAA

BLAST Formatting Result

Blast formatting results shows 99% identical of both the species. Red color key for alignment score shows more than 200. It means both species are very closely identical with each other.

Figure 3: Overall Phylogenic tree represents the evolutionary relationship between the plant species of *Ceropegia odorata* Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn and other neighbor joining species.

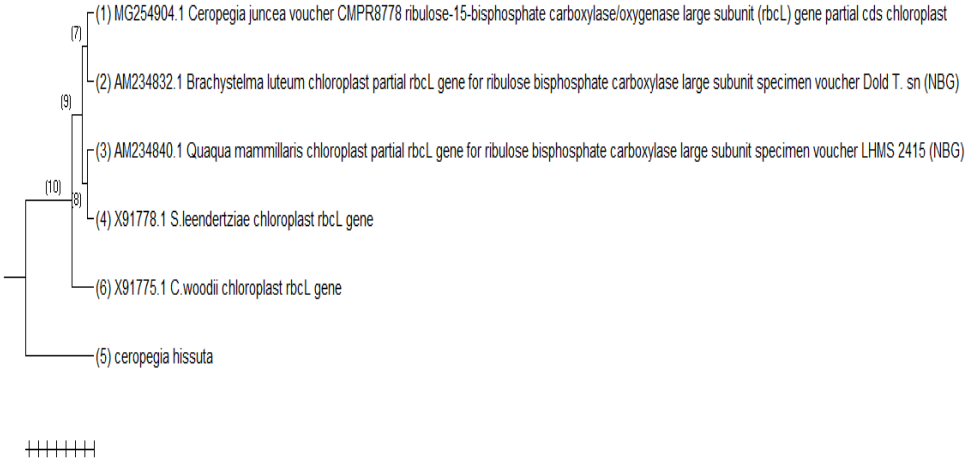
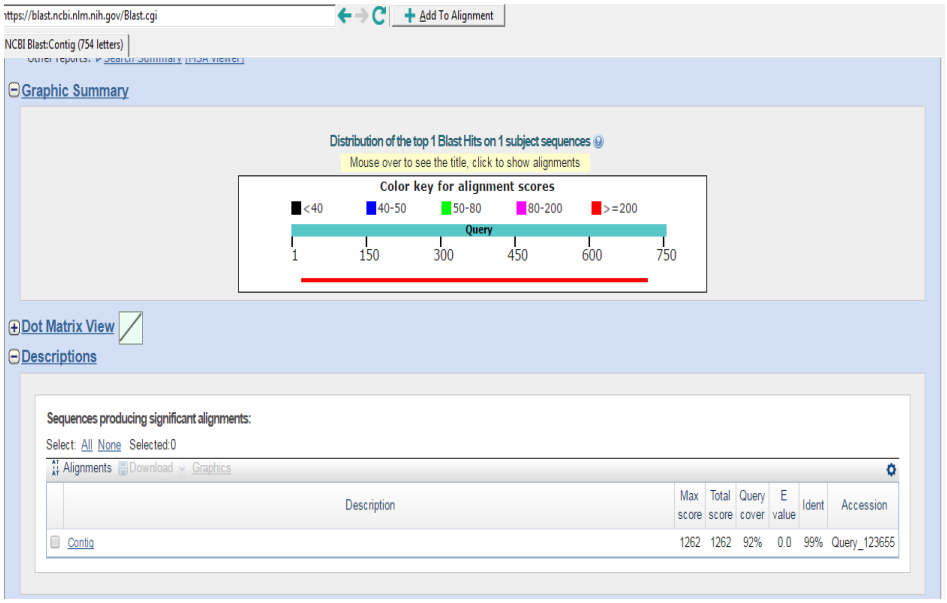


Figure 4: BLAST formatting results of *Ceropegia odorata* Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn



Sequence analysis: It is typically difficult to compare unknown sequences to reference sequences because currently there is no standardized method for it. BLAST algorithm has continuously been used for this purpose in recent years, yet it is not specifically designed for barcoding (Ford *et al.*, 2009) and (Altschul *et al.*, 1997). The major disadvantage of BLAST is there are no statistical methods that can give a measure of the accuracy of identification of sequences. (Munch *et al.,* 2008). However, the E-value and maximum identity are two statistics. Both can be utilized as an informal measure of the possibility of an identification being correct. In overall, one can presume that the closer a hit approach 100% in sequence identity the more probable it is to have been correctly identified to species as easily. Though, there is a possibility that hits, scoring 100% sequence identity may be inappropriate, if there are closely related species in the target geographic region that were not admitted in the character database. In the present study the BLAST algorithm is utilized to identify, compare and discriminate the species analyzed and it shows fare results.

Phylogenetic tree analysis: In the present investigation, the DNA sequences were analyzed using BLAST for pairwise sequence alignment. Multiple sequence alignment used by clustalW. BLAST also used for identification of species using the matK gene. It had greater resolving ability of single region barcode, dominated under NJ method. Only it can't give better results than rbcL. The basic sequence statistics, which include nucleotide frequencies, transition/transversion (NS/NV) ratio and changing in different neighborhoods of the sequences were studied which was computed by MEGA X, which is a cohesive tool for automatic and manual sequence alignment to construct

phylogentic trees Which is used to estimate the rates of molecular evolution and also test evolutionary hypothesis. In the present study, the phylogenetic trees were constructed for the species studied and their related taxa by using Neighbor joining (NJ) method to study the identification, discrimination, closeness and the evolutionary trend among them and the constructed trees.

Morphologically it was identified that *Ceropegia odorata* Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn are from same family. The rbcL gene sequence were used for both species. The present study sequence showed more similarity to the both species, belonged to same family. The other species used for phylogentic relationship in the present study were *C.woodii*, *S.leendertziae*, *Quaqua mammilaris*, *Brachystelma luteum*, *Ceropegia juncea*. Phylogenetic tree shows that *Ceropegia odorata* and *Ceropegia hirsuta* are closely linked to their genus *Ceropegia*. It works out that both the species are from same genus which will be very helpful for future study. It will be help to study their functions for non-taxonomist and for further conservation.

Conclusion

On the basis of morphological characters, both the endangered plant species identified in the main by our taxonomist. The combination of rbcL gene sequence of *Ceropegia odorata* and *Ceropegia hirsuta* Wight & Arn may modify the identification process. This operation will assist in the conservation and tackling illegal trade of this regionally endangered species. As per morphological characters taxonomic methods require the whole plant, preferably in flowering stage for its reliable identification, DNA sequence may offer identification of the species even if a trace of tissue is available. Phylogenetic relationship of both the species with other taxa must be drawn with caution as only few sequences of the related taxa are currently available in the databases. Nevertheless, the presence of rbcL and other plant gene sequences in the databases has been increasing fast. Consequently, future work on this species will offer reliable information regarding the phylogeny of this species.

Acknowledgment

We are thankful to the Forensic Department, Gujarat University to provide lab facility. Besides we are thankful to Xcelris lab providing us necessary materials.

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Cite this article as:
Purohit Nikisha, Qureshimatva Umarfaruq, Gamit Sandip and Solanki Hiteshkumar. DNA Barcoding of endangered Ceropegia odorata Nimmo ex J. Graham and *Ceropegia hirsuta* Wight & Arn. species of Gujarat. Annals of Plant Sciences 8.4 (2019) pp.3541-3546.
http://dx.doi.org/10.21746/aps.2019.8.4.1

Source of support: Nil; **Conflict of interest:** Nil.