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RESEARCH ARTICLE

Molecular Taxonomic Identification of *Peristylus densus* (Lindl.) Santapau and Kapadia

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Abstract

It is well known that the Western Ghats of India is a major biological hotspot having a huge diversity in flora and fauna. Orchidaceae are among them which are most evolutionarily and ecologically significant plant species. They are known for a myriad variety of epiphytic and terrestrial growth forms and hardiness. Plants belonging to Orchidaceae found to successfully colonize almost every habitat on earth, including soil (terrestrial), rock surfaces (lithophytic) and other plants (epiphytic). *Peristylus densus*, a typical species of Orchidaceae is well known for its medicinal use. Taxonomic identification and authentication of any diversified organism is a gigantic task to accomplish. Though there are many classical methods which are being exploited for such purposes, but such identification with the help of advanced molecular techniques is proving to be the most authentic DNA sequencing. In the present investigation, an attempt of molecular phylogeny of *Peristylus densus* by amplifying RNA polymerase β' (*rpoC)* subunit region of chloroplast genes was carried out.

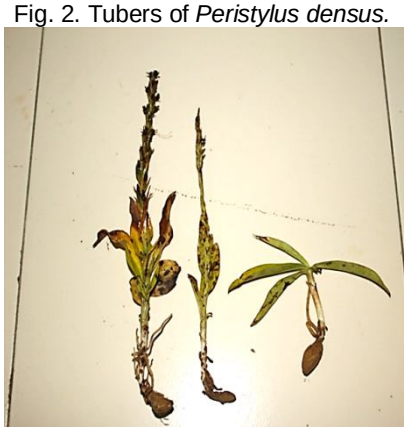
Keywords: Western Ghats, biodiversity, DNA sequencing, *Peristylus densus*, molecular phylogeny.

Introduction

Western Ghats of India are known to be a major biological hotspot that supports the plant diversity and endemism. Orchidaceae are among the most evolutionarily and ecologically significant plants and are known for a wide variety of epiphytic and terrestrial growth forms and hardiness; they successfully colonize almost every habitat on earth, including soil (terrestrial), rock surfaces (lithophytic) and other plants (epiphytic). The *Peristylus densus,* representative species of orchidaceae is famous for its use as medicinal herbs. It is well distributed in India, Myanmar, Hon Kong, Bangladesh, Cambodia, Japan, South Korea, Vietnam, Thailand to Indo-China and China. As far as India is concerned, Kerala, Assam and Maharashtra are natural habitat of those herbs (Yadav and Sardesai, 2010). With other medicinal formulations, the plant is used to treat the ailment resulting due to neurological disorder. Tuber paste is used to treat the inflammation resulting due to insect bite (Oudhia, 1990-2012). Furthermore, the tubers are used with turmeric and the extract can be applied for treating skin diseases. As seen in Fig. 1 and 2, morphologically *Peristylus densus* is oblong-ovoid plant with the stem having 2 or 3 tubular sheaths at base and having 4 to 6 leaves (Santapau and Kapadia, 1960). But morphological description of plants can't be a completely authentic method for their identification and taxonomy. Moreover, still there is a poor understanding related to their taxonomy. Therefore, there is a need of studying their taxonomy with the advanced molecular methods. The molecular identification methods mostly rely on studying the conserved sequences.

Fig. 1. Habit of *Peristylus densus.*

Fig. 2. Tubers of *Peristylus densus.*



As little mutation occurs in that sequence, they are the most authentic markers for identifying plants by using molecular tool. In this context, we have attempted to perform the molecular phylogeny of Orchid by amplifying RNA polymerase β' (*rpoC)* subunit region of chloroplast genes (Michael and Richard, 1988). In the present investigation, two primers specific to *rpoC* region used were 2f and 4r in order to amplify approximately 500 bp sequence of Orchid *rpoC* gene.

Materials and methods

Sampling: Fresh samples of *Peristylus densus* (Lindl.) Santapau and Kapadia were collected during monsoon in the first week of September 2012 from plateau of table land, Panchgani District, Satara regions of Western Ghats of Maharashtra (Fig. 1 and 2). These plants were identified and authenticated using herbarium collection at Botany research laboratory, DST-FIST School of Life Science, SRTM University, Nanded (MS) and Dept. of Botany, Dr. Babasaheb Ambedkar Marathwada, University, Aurangabad (MS), India. Fresh tubers were washed thoroughly under running tap water followed by sterile distilled water and dried under shade. The shade dried material was ground into coarse powder using mechanical grinder and this coarse powder was sieved by 1 mm pore size sieve. The powder was stored in airtight containers at room temperature till further molecular analysis.

DNA extraction and quantification: DNA Extraction was carried out using HiPurA Plant Genomic DNA Miniprep Purification Spin kit (Himedia, Mumbai). About 100 mg of powdered tissue was ground in mortar and pestle and transferred to 2 mL microcentrifuge tube, 400 µL of Lysis Buffer (PL) containing Additive-I was added and mixed thoroughly. The mixture was incubated at 95°C for 10 min with intermittent inversions of the contents, 130 µL of precipitation buffer was added and incubated on ice for 5 min. The sample was loaded on Hishredder placed in 2 mL collection tube and centrifuged for 5 min at 12000 rpm. Transfer flow through to another 2 mL collection tube and 20 µL of RNase A solution was added. The mixture was then incubated at room temperature for 30 min, 1.5 volumes of binding buffer was added to the clear lysate and mixed by pipetting. The lysate was then loaded on HiElute Miniprep Spin Column (Himedia, Mumbai) placed in a 2 mL collection tube. After centrifugation at 10000 rpm for 1 min, the flow through was discarded and entire sample was passed through the column in similar manner. The column was washed with 500 µL wash buffer and centrifugation at 10000 rpm for 1 min. This step was repeated twice with additional centrifugation. The DNA was eluted by incubating the column with 100 µL elution buffer and centrifugation at 10000 rpm for 1 min. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation, Kyoto, Japan). The DNA was stored at -20°C for further use.

Table 1. Primers used for *rpoC*region amplification.

Primers	Primer Sequence (5'-3')
2f (Forward)	GGCAAAGAGGGAAGATTTCG
4r (Reverse)	CCATAAGCATATCTTGAGTTGG

PCR amplification: The DNA isolated from sample was subjected to polymerase chain reaction (PCR) amplification using T-Personal 48 thermal cycler (Biometra, Goettingen, Germany). The PCR reaction mix contained 2.5 µL of 10X buffer, 1 µL of each primer (Table 1), 2.5 µL of 2.5 mM of each dNTP, 2.5 units of Taq DNA polymerase and 1 µL Template DNA and 8.5 µL nuclease free water. The PCR amplification cycle consist of a cycle of 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C and additionally 1 cycle of 7 min at 72°C. The reagents used are procured from GeNei, Bangalore.

Gel electrophoresis: Gel electrophoresis of the amplified product was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplicons.

DNA sequencing: The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). About 100 µL of PCR-A buffer was added to 25 µL of reaction. The sample was mixed and transferred to column placed in 2 mL collection tube and centrifuged at 10,000 rpm for 1 min. The filtrate was discarded. About 700 µL of W2 buffer was added to the column and centrifuged at 10,000 rpm for 2 min. This step was repeated twice. The column was transferred to a new tube. About 25 µL of eluent was added into the column and incubated at room temperature for 2 min. Then it was centrifuged at 10,000 rpm for 5 min and further sequenced using Applied Biosystems 3730xl DNA Analyzer (USA) and chromatogram was obtained. For sequencing of PCR product, 2f-5'GGCAAAGAGGGAAGATTTCG3' sequencing primer was used.

Bioinformatics analysis: The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of orchid. Altogether 19 sequences, including sample were used to generate phylogenetic tree. The tree was constructed using MEGA 5 software (Saitou and Nei, 1987; Felsenstein, 1985; Tamura *et al.,* 2011).

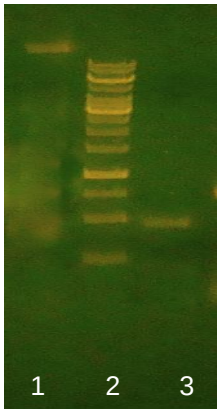
Results and discussion

DNA extraction, amplification and electrophoresis: A relatively quick, inexpensive and consistent procedure for DNA extraction from plants with medicinal value is described herewith. After extracting the DNA, its amplification and electrophoresis, the band of 500 bp for *rpoC* region amplicons were found distinguishably in the gel (Fig. 3).

Table 2. Phylogenetic neighbors of *Peristylus densus* based on sequence *of rpoC* region.

Description	Max score	Query cover	Identity	Accession
Habenaria hexaptera RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	841	100%	99%	GQ917009.1
Habenaria hieronymi RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	835	100%	99%	GQ917001.1
Gymnadeni anigra plastid partial rpoC1 gene for RNA polymerase beta subunit, isolate 5561	832	100%	99%	AM883676.1
Gavilea venosa voucher Morrone & Giussani 5927 (SI) RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	JQ045445.1
Gavilea trullata voucher Morrone *et al.* 6113 (SI) RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	JQ045444.1
Chloraea grandiflora voucher Morrone *et al.* 6059 (SI) RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	JQ045442.1
Chloraea lamellata voucher Morrone *et al.* 6024 (SI) RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	JQ045439.1
Chloraea bidentata voucher Morrone *et al.* 6031 (SI) RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	JQ045438.1
Chloraea chrysantha voucher Morrone *et al.* 6079 (SI) RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	JQ045437.1
Chloraea crispa voucher Morrone *et al.* 5997 (SI) RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	JQ045433.1
Chloraea multiflora voucher Morrone *et al.* 6066 (SI) RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	JQ045431.1
Gavilea longibracteata voucher Morrone *et al.* 6017 (SI) RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	JQ045430.1
Chloraea longipetala voucher Zapata 455 (SI) RNA polymerase beta' chain subunit-1 (rpoC1) gene, partial cds; chloroplast	824	100%	99%	GQ916962.1
Chloraea cylindrostachya voucher Morrone 5742 (SI) RNA polymerase beta' chain subunit-1 (rpoC1) gene, partial cds; chloroplast	824	100%	99%	GQ916958.1
Gavilea glandulifera voucher Morrone 5779 (SI) RNA polymerase beta' chain subunit-1 (rpoC1) gene, partial cds; chloroplast	824	100%	99%	GQ916956.1
Chloraea virescens voucher Morrone 5838 (SI) RNA polymerase beta' chain subunit-1 (rpoC1) gene, partial cds; chloroplast	824	100%	99%	GQ916954.1
Gavilea araucana voucher "Morrone 5677 (SI)" RNA polymerase beta' chain subunit-1 (rpoC1) gene, partial cds; chloroplast	824	100%	99%	GQ916953.1
Chlorae aphilippii RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	GQ917006.1
Gavilea araucana RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	GQ917004.1
Chlorae acylindrostachya RNA polymerase beta' chain (rpoC1) gene, partial cds; chloroplast	824	100%	99%	GQ917016.1

Fig. 3. Amplification of *rpoC* region in orchid *Peristylus densus.*



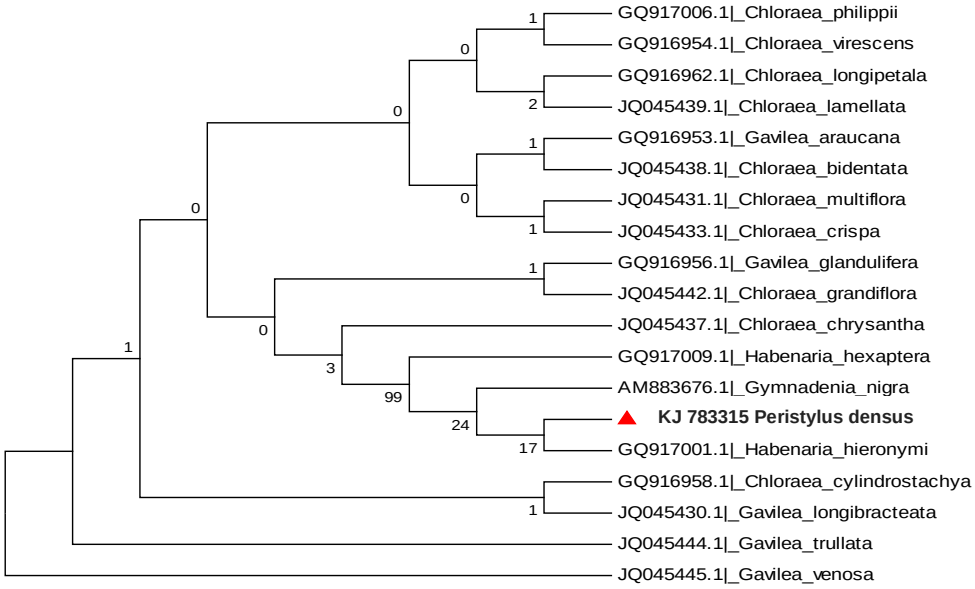
Lane 1: Genomic DNA of *Peristylus densus*
Lane 2: 1 kb DNA marker (Fermentas, #SM0311)
Lane 3: Amplified *rpoC* region of *Peristylus densus*

1 kb DNA marker (Top to bottom): 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 700, 500 and 250 bp.

The present investigation and the obtained result is the best example to use PCR and sequencing for studying chloroplast DNA sequence that are of evolutionary significance. Moreover, the obtained band demonstrates that this technique will contribute a lot for understanding and authenticating the genetic diversity of uncharacterized plant populations.

DNA sequencing: Phylogenetic neighbors of *Peristylus densus* based on sequence *of rpoC* region is shown in Table 2. Sequence of *rpoC* region of *Peristylus densus* was obtained and the sequence was found to contain 465 bp. The said sequence was submitted to NCBI and was provided with the accession number KJ783315.

Fig. 4. Phylogenetic tree for *Peristylus densus* using sequence of *rpoC* region.



ATTATTCGGGGCGTTCCGTCATTGTCGTGGGTCCTT
CTGCTTTCATTACATCAATGCGGATTACCTCGAGAAA
TAGCAATAGAGCTATTCCAAACATTTGTAATTCGTGG
TCTAATCAGGCAAGATGTTGCTTCTAACACAGGGATT
GCTAAAAGCAAAATTCGGGAAAAAGAACCCATTGTA
TGGGAAATCCTTCAAGAAGTTATGCAGGGGCATCCT
GTATTGTTGAATAGAGCGCCTACCCTGCATAGATTA
GGCATACAGGCGTTCCAACCCATTTTAGTGGAGGGA
CGTGCTATTTGTTTACACCCATTAGTTTGTAAGGGCT
TCAACGCAGACTTTGATGGGGATCAAATGGCTGTTC
ACGTACCTTTATCTTTGGAAGCTCAAGCAGAAGCTC
GTTTACTTATGTTTTCTCATATGAATCTCTTGTCTCCA
GCTATAGGGGATCCCGTTTCCGTACCA

Sequence length: 465 bp.

Maximum parsimony analysis of taxa: The evolutionary history was inferred using the Maximum Parsimony (MP) method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 19 nucleotide sequences. All positions with less than 0% site coverage were eliminated. That is, fewer than 100% alignment gaps, missing data and ambiguous bases were allowed at any position. There were a total of 463 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 and the phylogenetic tree was constructed (Fig. 4).

Conclusion

The molecular phylogeny of *Peristylus densus* was determined by analyzing plastid *rpoC* region sequences. On the basis of position of sequence of the given plant in the phylogenetic tree, the plant showed closest similarity with *Habenaria hieronymi*. It proved that, it belongs to family orchidaceae. The present sequence of 465 bp is of *Peristylus densus* (Lindl.) Santapau and Kapadia confirmed morphologically and authenticated by taxonomist. DNA sequencing is an advanced tool for identification and authentication of diversified plant genera and species. DNA sequencing is useful in preservation of endemic as well as endangered plant species for future study. There is an urgent need of alternative method of conservation and reintroduction of such biologically important orchid species.

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