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RESEARCH ARTICLE

DNA BARCODING OF MEDICINAL PTERIDOPHYTE SELAGINELLA DELICATULA FROM WESTERN GHATS OF KERALA

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ABSTRACT

Indian traditional medicine has gained a lot of importance in recent times. Owing to rich herbal flora in India, medicinal plants have become a major source of natural drugs. New molecular approaches towards identifying these medicinal plants is becoming inevitable to avoid confusions in identification. In this regard, DNA bar coding usingrbc Lgene, is suggested as a marker for pteridophyte identification. In this study, molecular characterization of *Selaginella sp.* was performed using Polymerase Chain Reaction (PCR) for rbcL gene. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA5) software for the obtained sequences. The evolutionary divergences between its closely related species were also performed to disclose its amendments that occurred during evolution.

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INTRODUCTION

Herbal medicines have been important ingredients in human life since earliest times and it has recently acquired increasing importance due to its supposedly harmless nature and effectiveness (Singh *et al*., 2010). Herbal plant tissues encompass hoard of chemical substances required for treating various diseases (Divya, 2011). Understanding these plants both phenotypically and genotypically is imperative for species recognition and identification. The term ''DNA barcode'' is used here to refer to a DNA sequence-based identification system that may be constructed of one locus or several loci used together as a corresponding unit (Kress and Erickson, 2007). DNA bar coding involves sequencing a standard region of DNA as a tool for species identification as they have flanking regions that are conserved to be serviced by the universal primers (Daniel *et al*., 2006). In animal groups, large-scale species identification was done using COI gene. Due to low substitution rates of mitochondrial DNA in plants, it has led taxonomists to search for an alternative bar coding regions. From preliminary investigations of plastid regions (Kress *et al*., 2005; Chase *et al*., 2007 and Ford *et al*., 2009), 7 leading candidates have emerged (Pennisi, 2007 and Ledford, 2008) out of which four are portions of coding genes (matK, rbcL, rpoB and rpoC1) and 3 are non coding spacers (atpF–atpH, trnH–psbA, and psbK–psbI). Recently, the Consortium for the Barcode of Life (CBOL) approved two plastid loci, rbcL and matK, as the official DNA barcode for all land plants (Hollingsworth 2011). Recently, unknown pteridophyte gametophytes were shown to be identifiable, often to species level, by using plastid DNA sequences (Li *et al*., 2009), suggesting that this DNA-based identification tool has the potential to be applied to large-scale ecological surveys (Li *et al*., 2010). The DNA barcoding approach has also been useful in distinguishing among pteridophyte species in the horticultural trade (Pryer *et al*., 2010) and in Chinese herbal medicine (Ma *et al*., 2010), two areas where species names are frequently confused. Among pteridophytes, Selaginella (family: Selaginellaceae) is an important, fully unexplored genus with regard to their medicinal properties. Some of the species of the genus are used in folk medicine as a guarded secret (Dixit and Bhatt 1974; Manickam and Irudayaraj 1992). *S. involvens* (SW) Spring, *S. delicatula* (Devs.) Alston, and *S wightii* Hieron are the three important Selaginella species found in the Western Ghats of Kerala. *S. delicatula* is used in ethnomedicine for healing external ulcers and it is found throughout the southern parts of Western Ghats partially in shaded and moist areas. The present study is an attempt to barcode the medicinally important Selaginella sp. Pteridophyte found in the western ghats of Kerala using rbcL gene.

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MATERIALS AND METHODS

Plant collection

Healthy, ailment free samples of Selaginella sp. were collected from Pangode, Quilon district, Kerala. Plant sample was authenticated at St. Xaviers college, Tirunelveli, Tamil Nadu (Voucher No:25437). Sponged air-dried samples were then powdered using liquid nitrogen and processed for DNA isolation.

Isolation of Genomic DNA

Plant genomic DNA was extracted using the CTAB based DNA isolation method (Doyle and Doyle, 1987). Isolated nucleic pellet was washed with 70% ethanol and resuspended in TE buffer (10mM TrisHCl, 1mM EDTA, pH 7.4). Quality of the DNA was assessed on a 1.5% Agarose Gel (Tris Acetate EDTA buffer) stained with Ethidium Bromide visualized on a UV transilluminator. Its purity was found using a Spectrophotometer.

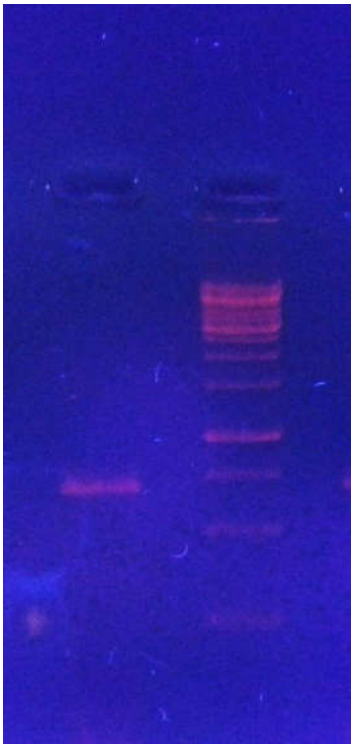

Fig.1. PCR products on 1.5% Agarose gel – Lane 1 : PCR Amplicon Sample: ~ 650bp Lane 2: 1 Kb Ladder

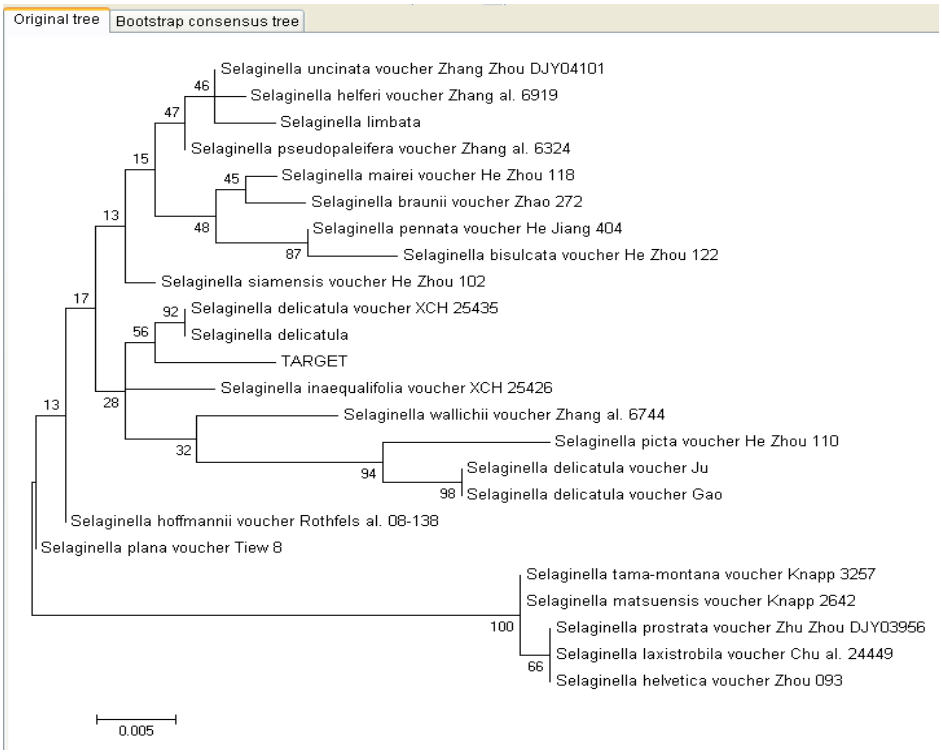

Fig.2. Phylogenetic Tree using MEGA 6 with obtained sequence as TARGET

PCR and DNA sequencing

Internal regions of 650bp of rbcL gene was amplified using gene specific primers. Amplification was carried out in a 20µl reaction set up containing 0.3µM of each primer, 0.2mM deoxynucleotidetriphosphates (dATP, dCTP, dGTP and dTTP), 100ng of template DNA sample and 1U of Prime TaqDNA polymerase (Genetbio, Korea). The reaction tubes were subjected to thermal cycling reactions consisting of an initial denaturation (5 min at 94°C) followed by 32 cycles of denaturation (1 min at 94°C), annealing (1min at 51°C), and extension (1 min at 72°C), with a final extension (10 min at 72°C). The PCR product was purified (QIA quick PCR purification kit, Qiagen, Madrid, Spain) and DNA sequencing was performed by Sanger's method (ABI 3730, Harlow Scientific).

Sequence Analysis

The nucleotide sequences for rbcL obtained was compared with its related species using BLASTn tool. Portable software Molecular Evolutionary Genetics Analysis (MEGA) version 6, was used to construct Maximum Likelihood (ML) tree for the obtained sequences to identify it's inter and intra species relationships.

RESULTS AND DISCUSSION

DNA barcodes has been proposed as a detour to provide new species identification and discovery, as the taxonomists would have spent decades to discovery 10-15 million species using current description and publications (Daniel *et al*., 2006). An ideal DNA barcode should be routinely retrievable with a single primer pair, be amenable to bidirectional sequencing with little requirement for manual editing of sequence traces, and provide maximal discrimination among species. Among plastid regions, rbcL is the best characterized gene. Improvements in primer design make it easily retrievable across land plants (Fazekas *et al*., 2008) and it is well suited for recovery of high-quality bidirectional sequences. Although not the most variable region, it is a frequent component of the best performing multi-locus combinations for species discrimination (CBOL plant working group, 2009)

DNA was isolated from the collected plant samples and its purity (1.8) was found using a Spectrophotometer. Amplicons obtained after PCR were of ~650bp for rbcL gene (Figure1). Obtained sequences from the purified PCR product was compared with nucleotide database (BLASTn) and was found to have maximum identity to *Selaginelladelicatula*, a pteridophyte commonly found in the western ghats and which is widely used in herbal medicine. The Sequence was submitted to NCBI GenBank and their accession numbers generated was KY067592. It is argued that DNA barcoding is the only practical, standardized species level identification tool for the assessment of biodiversity, therefore distance based phylogenetic approaches were not considered (Hebert *et al*., 2003 and Kress *et al*., 2005). However, barcoding currently requires the construction of relationships between sequences using phylogenetic methods that are attributed to identify species and sequences from unknown samples (Hebert *et al*., 2004 and Hogg and Hebert, 2004). Present study was conceded to find out the evolutionary relationship of Selaginella sp. collected from Pangode, Kerala.

Figure 2 exemplifies the Maximum likelihood tree constructedfor the target *Selaginella sp.* (rbcL gene) using MEGA5 software (Tamura *et al*., 2011). Target, *Selaginella sp. a*fter forming cluster with *S. delicatula* branches out to *S.inaequalifolia*, a pteridophyte available in the western ghats and known for its medicinal properties, hinting at its genetic resemblance to the sample. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura, 1983). The tree with the highest log likelihood (-1170.1755) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. 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 Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 532 positions in the final dataset. These evolutionary analyses were conducted in MEGA6 (Tamura 2013).

Conclusion

DNA barcoding will revolutionize our understanding of pteridophyte ecology, most especially because the accurate identification of the independent but cryptic gametophyte phase of the pteridophyte's life history--an endeavor previously impossible--will finally be feasible. DNA bar coding has great potential for enhancing ecological and evolutionary investigations if the right genetic markers are designated. In addition, phylogenetic analysis is also necessary to identify the potential changes in the nucleotides of a species and identify its variations. In this study, molecular characterization of the collected plant was carefully studied and its evolutionary relationship was constructed.

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