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SEQUENCE ANALYSIS OF 18S rDNA AMONG FIVE NATIVE SPECIES OF APIACEAE SUBFAMILY APIOIDEAE FROM SOUTH INDIA

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ABSTRACT
Five native species of the family Apiaceae subfamily Apioideae namely *Bupleurum mucronatum*, *Heracleum candolleanum, Peucedanum dhana, Pimpinella leschenaultii & Polyzygus tuberoses* were collected from various regions in South India. The genomic DNA was isolated from the plant leaf sample. The 1.8 kb 18s rDNA fragment was amplified using high fidelity PCR polymerase. The PCR product was sequenced to obtain complete sequence of 18s rDNA region. These molecular data were submitted to NCBI Gene bank. The following are the gene bank accession numbers provided by NCBI. *Polyzygus tuberosus* - JN411079, *Peucedanum dhana var. dalzellii* - JN411080, *Bupleurum mucronatum* - JQ612129, *Heracleum candolleanum* - JQ612130, *Pimpinella leschenaultii* - JQ612131. Sequence comparison by ClusterW2 analysis among these species reveals the guide tree data for *Polyzygus tuberosus* - 0.04872, *Peucedanum dhana var. dalzellii* - 0.01250, *Bupleurum mucronatum* - 0.03256, *Heracleum candolleanum* - 0.02393 & *Pimpinella leschenaultii* - 0.01665. The cladogram for these data was constructed.

KEY WORDS: Apiaceae, 18s rDNA sequencing, *Bupleurum mucronatum, Heracleum candolleanum, Peucedanum dhana var. dalzellii, Pimpinella leschenaultia, Polyzygus tuberosus,* Cladogram, Apioideae.

INTRODUCTION

The plant family Apiaceae comprises about 464 genera and some 4250 species and, although largely confined to temperate regions, is cosmopolitan in distribution (Pimenov and Leonov, 1993). It is one of the best known families of flowering plants, because of its characteristic inflorescences and fruits and the distinctive chemistry, reflected in the odor, flavor, and even toxicity of many of its members (Heywood, 1993). This family is divided into three subfamilies namely Hydrocotyloideae, Saniculoideae, and Apioideae. In the present study, the selected five plant specimens belongs to Apioideae with recognized 41 clades (Stephen R. Downie, 2010). The specimen *Bupleurum mucronatum* Wight and Arn. is included in clade Bupleureae, *Heracleum Candolleanum* Wight & Arn.in clade Tordylieae, *Peucedanum dhana var dalzellii* C.B. Clarke in clade Selineae, *Pimpinella leschenaultii* DC., Prodr. in clade Pimpinelleae and *Polyzygus tuberosus* Dalzell in Cymbocarpum clade.Members of Apioideae, the typical "umbellifers," are distinguished from those in the other two subfamilies by the shared presence of compound umbels, a specialized fruit consisting of two one-seeded mericarps suspended from a common bifurcate carpophore, a soft endocarp that is sometimes hardened by woody subepidermal layers, a terminal style arising from the stylopodium, fruits without scales.

Recently, phylogenetic analyses of chloroplast DNA rbcL sequences (Plunkett, Soltis, and Soltis, 1992) and morphological and anatomical characters (Judd, Sanders, and Donoghue, 1994) reveal that Apiaceae subfamilies Apioideae and Saniculoideae are each monophyletic and are sister taxa. Apioideae are the largest and most taxonomically complex of the three subfamilies of Apiaceae. Existing treatments (e.g., Bentham, 1867; Drude, 1898), constructed largely on the basis of morphological and anatomical characters, give contradictory interpretations of relationship, and the tribal circumscriptions employed in each do not coincide in number or in content. In the present context we have attempted tracing the phylogenetic interrelatedness through 18s rDNA sequence analysis.

MATERIALS AND METHODS

Plant Material

Most of the plants of this family were spotted to inhabit in high altitude regions of South India. Table 2 represents the location of plant collection and flowering season. The plant specimens were expelled from the soil along with rhizome (whole plant). Its morphological and floral characters were studied using several Flora of South India. The specimens collected for planting purpose were transferred in polythene bags and brought as quickly as possible to the selected field and planted there. Few flowering specimens of the species were collected separately for the purpose of identification; few specimens were processed to make herbarium sheets and housed in the department herbarium. The plant identification was done by reference to The Flora of the Presidency of Madras, (Gamble, 1935); The Flora of South Indian hill stations (Fyson, 1932); The Flora of Presidency of Bombay, (Theodore Cooke, 1967) & Umbelliferae (Apiaceae) of India, (P.K.Mukherjee, 1993). The literature survey on these plants by reference of various journals was made, but very less scientific information is available.

DNA Extraction & 18s rDNA sequencing

Total genomic DNA was extracted by CTAB method (Doyle, 1987) which was modified according to the samples. In brief, 100mg of each young leaf sample were

transferred into mortar & pestle. 750µl of suspension buffer were transferred, crushed into fine paste. The extract was pipetted into a 2ml vial by using a tip that is cut at the bottom. 5µl of RNase were added and mixed thoroughly by inverting the vial. It was placed at $65^{O}C$ for 10 min with intermitted mixing. 1ml of lysis buffer was added, thoroughly mixed and kept at $65^{O}C$ for 15 min. The sample was cooled at room temperature and centrifuged at 13000rpm at room temperature. The supernatant was collected and transferred into a 2ml vial and was loaded on the spin column (600 µl each time) and centrifuged at 13000 rpm for a minute at room temperature. The contents of the collection tube were discarded and the spin column was placed back in the same collection tube. 500 µl of wash buffer was added to the column and centrifuged at 13000 rpm for 1 minute at room temperature. The contents of the collection tube were discarded. The spin column was placed back in the same collection tube. The empty column was spin with the collection tube at 13000 rpm for 2 min at room temperature. The spin column was placed in a fresh vial. 50 µl of warm elusion buffer maintained priory at $65^{O}C$ was added into the spin column. The vial was kept along with the spin column at $65^{O}C$ for 1 min and centrifuged at 13000 rpm for a minute at room temperature. The above steps were repeated, eluted and DNA sample were collected in the same vial. The DNA concentration was determined by both UV spectrophotometer and quantitative analysis on agarose gel. The 700bp rDNA fragment was amplified using high –fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward & reverse primers that is as represented in table 1.

TABLE 1: Primer details used for amplifying 18s rDNA for plant specimens

Specimen	Forward primers	Reverse primers
Bupleurum mucronatum	5'-GTAGTCATATGCTTGTCTC- 3'	5'-GAAACCTTGTTACGACTT -3'
Heracleum candolleanum	5'-GTAGTCATATGCTTGTCTC- 3'	5'-GAAACCTTGTTACGACTT -3'
Peucedanum dhana var dalzelii	5'- TAGTCATATGCTTGTCTC- 3'	5'- GAAACCTTGTTACGACTT -3'
Pimpinella leschenaultii	5'-GTAGTCATATGCTTGTCTC- 3'	5'-GAAACCTTGTTACGACTT -3'
Polyzygus tuberosus	5'-TCCGTAGGTGAACCTGCGG-3'	5'-TCCTCCGCTTATTGATATGC-3'

PCR Amplification and Purification

The reactions for PCR amplification were performed with a final volume of 50 μL, containing 27.5 μL ddH_2O, 10 μL 5× Taq Buffer (Mg^{2+}), 6 μL dNTPMix (2.5 mM), 5 μL of the DNA template, 2 μL of each PCR primer (50 mol μL^{-1}), and 0.5 μL Taq DNA polymerase (5 U μL^{-1}). The amplification of 18S rDNA was performed with an initial denaturation at 95°C for 2 min, 35 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 4 min, and a final extension step at 72°C for 6 min. And the products of amplification are preservation in 4°C. The PCR products were confirmed by electrophoresis in 1% agarose gel. The gels were stained with bromophenol blue and photographed by Bio-imaging system.

Identification software details

Phylogenetic Tree Builder uses sequences aligned with System Software aligner. A distance matrix is generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000.

Weighbor Tree

Weighbor is a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix. The weights are based on variances and covariances expected in a simple Jukes-Cantor model.

Jukes-Cantor Correction

The Jukes-Cantor distance correction is a model which considers that as two sequences diverge, the probability of a second substitution at any nucleotide site increases. For distance-based trees such as Weighbor, the difference in nucleotides is considered for the distance, therefore, second substitutions will not be counted and the distance will be underestimated. Jukes and Cantor createad a formula that calculates the distance taking into account more than just the individual differences.

Bootstrap

Bootstrapping is a statistical method for estimating the sampling distribution by resampling with replacement from the original sample. In making phylogenetic trees, the approach is to create a pseudoalignment by taking random positions of the original alignment. Some columns of the alignment could be selected more than once or not selected at all. The pseudoalignment will be as long as the original alignment and will be used to create a distance matrix and a tree. The process is repeated 100 times and a majority consensus tree is displayed showing the number (or percentage) of times a particular group was on each side of a branch without concerning the subgrouping.

Sequence comparition and phylogenetic tree construction

The 18S rDNA sequences were aligned using ClustalW2. The phylogeny analysis through ClustalW2 study revealed the comparative phylogenetic tree relationship among the five apioideae species. For comparative analysis, the sequences as mentioned in the above NCBI GenBank accession numbers were taken.

18s rDNA sequencing: The 18S rDNA Sequence fragments with the primers mentioned above were successfully amplified. The base size of *Bupleurum mucronatum* was 1702 bp. The base size of *Heracleum candolleanum* was 1702 bp. The base size of *Peucedanum dhana var dalzellii* was 1712 bp. The base size of *Pimpinella leschenaultia* was 1712 bp. The base size of *Polyzygus tuberosus* was 672 bp. The base sizes of *Bupleurum mucronatum* & *Heracleum candolleanum* were almost the same and similarly the base sizes of *Peucedanum dhana var dalzellii* & *Pimpinella leschenaultia* were almost the same. After sequencing the 18S rDNA, we got the accession numbers from GenBank. All these data were shown in Table 3.

RESULT AND DISCUSSION

Plant material: The following table 2 provides the list of species used in this study along with the collection details.

TABLE 2: Plant specimens of subfamily Apioideae with location, collection date & Flowering – fruiting season.

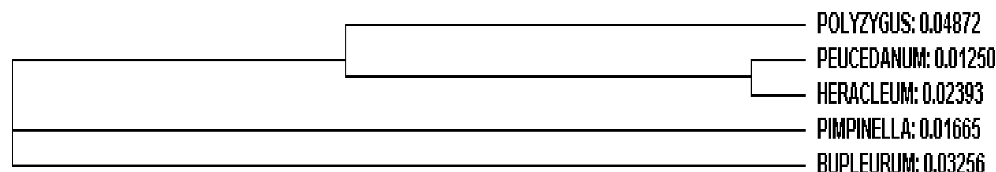
Specimen	Location & Collection date	Flowering & Fruiting
Bupleurum mucronatum Wight and Arn.	Kodanadu view point, Ooty (October 2010); Kalatti shola region on the way from Masangudi to Ooty (November 2010)	October – December
Heracleum Candolleanum Wight & Arn.	Yelanalli hills on the way from Ooty to Coonor (October 2010)	September - November
Peucedanum dhana var dalzellii C.B. Clarke	Karnataka University campus, Dharwad.	June – August
Pimpinella leschenaultia DC., Prodr.	Kodanadu view point, Ooty, (November 2011)	October – December
Polyzygus tuberosus Dalzell	Bangalore University campus, JB campus (Dec 2011)	January – February

TABLE 3: Plant specimens, voucher deposited and NCBI GenBank Accession numbers

Plant specimen	Voucher	GenBank Accession number
Bupleurum mucronatum	BUBH 46/2010	JQ612129
Heracleum candolleanum	BUBH 62/2010	JQ612130
Peucedanum dhana var dalzelii	BUBH 18/2011	JN411080
Pimpinella leschenaultia	BUBH 72/2010	JQ612131
Polyzygus tuberosus	BUBH 12/2011	JN411079

Genetic Distance : Comparing the 18S rDNA sequence obtained in this study, the results of the genetic distance and the ratio of sequence divergence are shown in Table 4. Further between genus *Bupleurum* and genus *Pimpinella* had closer genetic relationship with genetic distance of 0.03256 and between genus Peucedanum and Heracleum had closer genetic relationship with genetic distance of 0.01250.

Phylogenetic Tree Analysis: From the sequences of 18S rDNA, NJ phylogenetic tree was constructed (Figures 1). *Peucedanum dhana var. dalzellii* and *Heracleum candolleanum* belong to one branch. The homology of *Polyzygus tuberosus* with both *Peucedanum dhana var. dalzellii* and *Heracleum candolleanum* was obviously higher than *Pimpinella leschenaultia* and *Bupleurum mucronatum.*



FIGURES 1: Cladogram of five Apioideae plant specimens

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