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Research Article

MOLECULAR CHARACTERISATION OF *ALPINIA* SPECIES FROM WESTERN GHATS

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A R T I C L E I N F O

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A B S T R A C T

The present study highlights the phylogenetic relationship between the *Alpinia* species collected from Western ghats. Maximum phylogenetic relationship is seen between *A.calcarata* and *A.mutica*. In this study we propose the *trn*L (UAA) intron - *trn*F (GAA) exon as marker for barcoding *Alpinia* species. Chloroplast DNA sequencing resulted in an unambiguous 396bp for *Alpinia calcarata* 399bp for *A. malaccensis*, 404bpfor *A. purpurata,* 383bp for *A. galanga,* 402bp for *A. vittata,* 393bp for *A. mutica*, 401bp for *A.abundiflora.*

INTRODUCTION

DNA taxonomy has become a beneficial tool for the foreseeable future. It is a tool for biodiversity research and it will help for the detection of commercial products such as drugs prepared from plants. DNA barcoding is also a powerful diagnostic tool in the hands of the enforcement agencies for checking the illegal trade of endangered species of both animals and plants ie., biopiracy[1,2] and is an investigative tool for forensic specialists[3]. It uses a genetic marker in the plant DNA to identify its species[4,5]. In recent years the practical utility of DNA barcodes proved to be an appealing tool to help resolve taxonomic ambiguity in screening biodiversity and to support applications in conservation biology[6,7,8,9]. DNA barcoding is a method of identifying previously described taxa. Reference sequences lie at the very heart of the DNA barcoding initiative[10,11]. DNA barcoding mirrors the distribution of intra and intra- specific variation that is separated by a distance called DNA barcoding gap[12,13]. The Consortium of Barcode of Life coordinates DNA barcoding development and implementation universally. DNA barcoding is very essential for the molecular identification of already described species and the discovery of new species[14].

cpDNA is an extremely valuable molecule for studying phylogenetic relationships between closely related species[15,16].The chloroplast genomes of land plants have highly conserved structures and organization of content; they comprise a single circular molecule with a quadripartite structure that includes two copies of an IR region that separate large and small single-copy[17].The chloroplast *trn*L (UAA) intron have been widely used for reconstructing phylogenies between closely related species[18,19] or for identifying plant species[20]. The *trn*L (UAA) – *trn*F (GAA) locus contains the *trn*L (UAA) gene, its intron and intergenic region between *trn*L (UAA) and *trn*F (GAA). In this study we propose the *trn*L (UAA) intron - *trn*F (GAA) exon as marker for barcoding *Alpinia* species.

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MATERIALS AND METHODS

Plant material

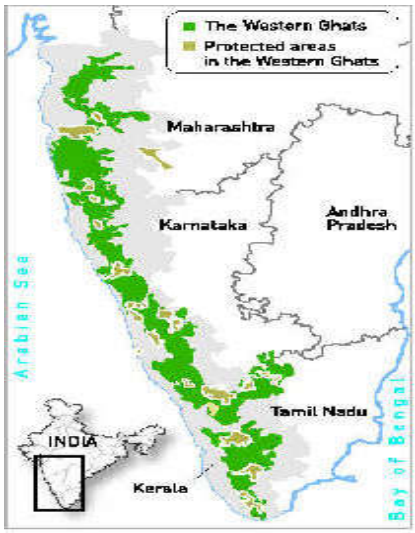
Alpinia is a wide spread genus, and taxonomically complex with 230 species occurring throughout tropical and subtropical Asia[21].The Latin generic name '*Alpinia*' was given to commemorate Prospero Alpini (1553-1617), an Italian botanist who catalogued and described exotic plants. *Alpinia* species have been extensively studied for their chemical and biological properties[22]. *A.calcarata* is a widely distributed aromatic medicinal plant native to India. It is extensively grown in gardens for its showy flowers and aromatic leaves[23]. *A. calcarata* is a valuable medicinal plant, widely used in ayurveda. It has become a threatened species, because of the threat to its natural habitat and pattern of distribution. The rhizome of this plant is used as medicine. It is said that approximately 1.70 tons of dried rhizomes are required annually[24].

The collected species were *A. calcarata* (RHT Garden) RHT65267, *A.malaccensis* (Kumily) RHT65142, *A. purpurata* (Kappadu) RHT65144, *A. galanga* (Vagamon) RHT65145, *A. vittata* (RHT Garden) RHT65143, *A. mutica* (Kodaikanal) RHT56228 and *A. abundiflora* (Western Ghats) RHT58628. All plants were identified morphologically and anatomically

by Director, Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College, Tiruchirappalli and voucher specimens were kept for future reference.

Study area

In the present work, we apply DNA barcoding for seven medicinally important *Alpinia* species of the Western Ghats, runs approximately 1,600 km through the states of Maharashtra, Goa, Karnataka, Kerala and Tamil Nadu. The seven species of *Alpinia* were collected from Kerala and Tamilnadu.



DNA Extraction Protocol

Day 1: The modified Cetytrimethyl Ammonium Bromide (CTAB) procedure was used as described by Aitchitt *et al*., 1993.

Quantification, visualization and amplification of DNA

Before the PCR amplification, the concentration of DNA was quantified spectrophotometrically at 260 nm and 280 nm, then the purity of DNA was determined by calculating the ratio of absorbance at 260/280 nm. Samples were subjected to electrophoresis in 1x TBE buffer for 1 hr at 80 V. 5 µL of the isolated genomic DNA was loaded on 0.8% agarose gel stained with EtBr to check DNA quantity. The gels were photographed under a gel documentation system. The PCR amplification was carried out using the universal PCR primers Tabe *trn*L (UAA) with forward sequence ggttcaagtccctctatccc and Tabf *trn*F (GAA) with reverse sequence atttgaactggtgacacgag. PCR reactions were prepared in 25 µL of the total volume, containing the following reagent concentrations: 2.5 ngµL^{-1} DNA template, 5 µL (forward and reverse primers), 17.5 µl mastermix.

PCR was controlled on 1% agarose gel in 0.5x TBE (10x stock contained 1 M Tris, 0.8 M boric acid, 0.5 M EDTA) stained with EtBr and visualised under UV. The gel image was documented with KODAK Gel Logic 100gel documentation system (Kodak, New Haven, USA) and analysed with UVITEC^{TM} analysis package (Cambridge, UK). Sanger dideoxy technology[14] were used for the sequencing. All sequences of *Alpinia* species have been deposited in Genbank using the SEQUIN 12.30 (Accession numbers KJ609028.1 to KJ609034.1).

Species identification from DNA Sequences

Sequences were assembled, trimmed and edited using DNA Baser v. 3.5.4. software. The largest and most well - known source of DNA sequences is Genbank, maintained by the NCBI[26]. So, the sequences obtained were compared with those existing in the public database Genbank using the BLAST tool of the NCBI. The system retrieves matching sequences with the corresponding % similarity (matching nucleotides) and gives the most likely species for the query sequence. If matching sequences from more than one species are retrieved with a similar probability, then the system displays all the possible putative species. Thus species were identified based on maximum BLAST scores with matching sequences, corresponding to 100% coverage and 100% identity.

Phylogenetic tree construction methods

Comparative analysis of molecular sequence data is essential for reconstructing the evolutionary histories of species and inferring the nature and extent of selective forces shaping the evolution of genes and species. Here we used user - friendly software MEGA6 for building sequence alignments and phylogenetic trees.

Maximum likelihood method

The evolutionary history was inferred by using the maximum likelihood method based on the Tamura - Nei model[27]. The tree with the highest log likelihood (-790. 2759) 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 by using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. The analysis involved 7 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 366 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[28].

Neighbor joining method

The evolutionary history was inferred using the neighbor-joining method[29]. The optimal tree with the sum of branch length = 0.12314825 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method[30]and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 366 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Minimum evolution method

The evolutionary history was inferred using the minimum evolution method[31]. The optimal tree with the sum of branch length = 0.12314825 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base

substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1[32]. The neighbor-joining algorithm was used to generate the initial tree. The analysis involved 7 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 366 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

UPGMA method

The evolutionary history was inferred using the UPGMA method[33]. The optimal tree with the sum of branch length = 0.12319445 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 366 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Maximum parsimony method

The evolutionary history was inferred using the maximum parsimony method[34]. Tree #1 out of 3 most parsimonious trees (length = 55) is shown. The consistency index is 0.981818 (0.923077), the retention index is 0.933333 (0.933333), and the composite index is 0.916364 (0.861538) for all sites and parsimony - informative sites (in parentheses). The MP tree was obtained using the Subtree – Pruning - Regrafting algorithm with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 7 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 366 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

RESULTS AND DISCUSSION

Molecular barcoding based on cpDNA is a useful technique for species identification and assessing genetic diversity. The resulted sequences were given genbank accession numbers (GAN). The sequencing resulted in an unambiguous 396bp for *A. calcarata* (GAN. KJ609031.1), 399bp for *A. malaccensis* (GAN. KJ609033.1), 404bp for *A. purpurata* (GAN. KJ609034.1), 383bp for *A. galanga* (GAN. KJ609030.1), 402bp for *A. vittata* (GAN. KJ609028.1), 393bp for *A. mutica* (GAN. KJ609029.1), 401bp for *A.abundiflora* (GAN. KJ609032.1). The gel image of these species are given in fig I. As described in methodology, multiple sequence alignment (fig. III) and phylogenetic tree (Fig II. A - E) was constructed with MEGA6 software. Seven sequences of *Alpinia* were compared thoroughly by molecular phylogenetic analysis like maximum likelihood method, neighbor - joining method, minimum evolution method, UPGMA method and maximum parsimony analysis to assess the performance in species discrimination. The nucleotide frequencies of KJ609031 have A and T = 32.95%, C and D = 17.05%, KJ609029 have A and T = 32.55%, C and D = 17.45%, KJ609032 consisted of A and T = 32.8%, C and D = 17.2%, KJ609028 with A and T = 32.95%, C and D = 17%, KJ609030 have A and T = 32.75%, C and D = 17.25%, KJ609034 with A and T = 32.45%, C and D = 17.6%, KJ609033 with A and T = 31.8%, C and D = 18.2% (Table. 2). From the above results based on all the methods employed, it has been confirmed that, the species *A.calcarata* was having a high affinity towards *A.mutica* based on *trn*L-*trn*F sequences.

The present study also reveals the similarity between the *A.purpurata* and *A. vittata* according to the Maximum likelihood method**,** Neighbor - joining method, Minimum evolution method and Maximum parsimony analysis. The tRNA Leu (*trn*L) intron is well suited for inferring plant phylogenies between closely related species for various reasons. Such regions of cpDNA can be used to resolve phylogenetic relationships at the intra - generic level. Furthermore, the primers are universal enough to work on a wide taxonomic range.

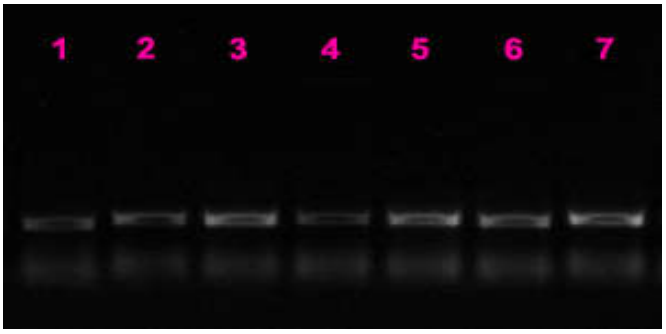


Fig I Gel image of DNA extracted from *Alpinia* species

Lane -1-7: *A. calcarata, A. malaccensis, A. purpurata, A. galanga, A. vittata, A. mutica, A. Abundiflora*

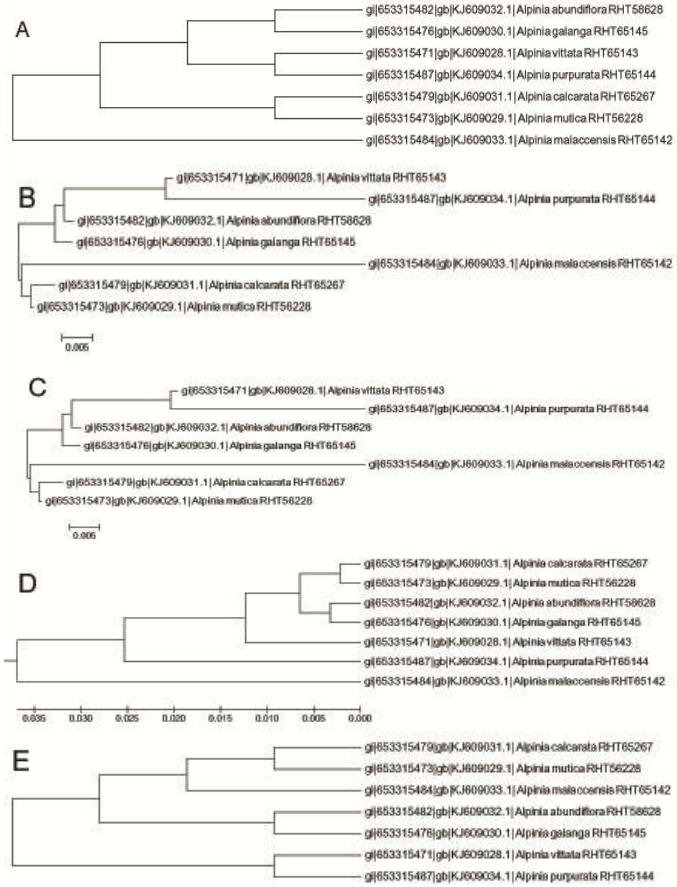


Fig II Molecular Phylogenetic analysis of *Alpinia* species

A. Maximum likelihood method **B.** Neighbor - joining method **C.** Minimum evolution method **D.** UPGMA method **E.** Maximum parsimony analysis.

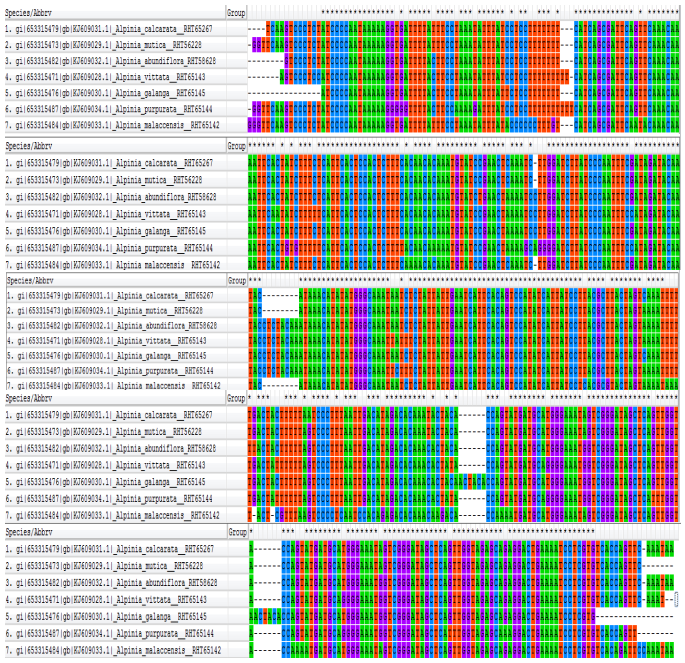


Fig III Multiple sequence alignment of *Alpinia* species

tool for the identification of the original species of *Alpinia.* However, within a taxonomic group 100% species resolution could possibly be obtained by taxa specific barcodes.

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Table I Nucleotide composition in *Alpinia*

Domain : Data	**T**	**C**	**A**	**G**	**Total**	**T-1**	**C-1**	**A-1**	**G-1**	**Pos#1**
gi|653315479|gb|KJ609031.1| *A. calcarata* RHT65267	33.1	22.5	32.8	11.6	396.0	32	23.7	34.4	9.9	131.0
gi|653315473|gb|KJ609029.1| *A. mutica* RHT56228	33.3	22.4	31.8	12.5	393.0	33	23.8	33.1	10.0	130.0
gi|653315482|gb|KJ609032.1| *A. abundiflora* RHT58628	32.9	22.7	32.7	11.7	401.0	32	23.1	35.1	9.7	134.0
gi|653315471|gb|KJ609028.1| *A. vittata* RHT65143	33.6	22.1	32.3	11.9	402.0	34	23.1	33.6	9.0	134.0
gi|653315476|gb|KJ609030.1| *A. galanga* RHT65145	32.6	22.5	32.9	12.0	383.0	31	22.8	36.2	9.4	127.0
gi|653315487|gb|KJ609034.1| *A. purpurata* RHT65144	33.2	21.3	31.7	13.9	404.0	35	20.9	34.3	9.7	134.0
gi|653315484|gb|KJ609033.1| *A. malaccensis* RHT65142	28.3	23.6	35.3	12.8	399.0	27	25.6	36.8	10.5	133.0
Avg.	2.4	22.4	2.8	2.3	96.9	2	3.3	4.8	.8	131.9
Domain: Data	**T-2**	**C-2**	**A-2**	**G-2**	**Pos#2**	**T-3**	**C-3**	**A-3**	**G-3**	**Pos#3**
gi|653315479|gb|KJ609031.1| *A. calcarata* RHT65267	30	17.3	37.6	15.0	133.0	37	26.5	26.5	9.8	132.0
gi|653315473|gb|KJ609029.1| *A. mutica* RHT56228	30	16.7	36.4	16.7	132.0	37	26.7	26.0	10.7	131.0
gi|653315482|gb|KJ609032.1| *A. abundiflora* RHT58628	29	18.8	36.1	15.8	133.0	37	26.1	26.9	9.7	134.0
gi|653315471|gb|KJ609028.1| *A. vittata* RHT65143	29	18.0	36.8	16.5	133.0	38	25.2	26.7	10.4	135.0
gi|653315476|gb|KJ609030.1| *A.galanga* RHT65145	28	18.0	36.7	17.2	128.0	38	26.6	25.8	9.4	128.0
gi|653315487|gb|KJ609034.1| *A.purpurata* RHT65144	29	18.5	34.1	18.5	135.0	36	24.4	26.7	13.3	135.0
gi|653315484|gb|KJ609033.1| *A. malaccensis* RHT65142	27	18.7	39.6	14.9	134.0	31	26.5	29.5	12.9	132.0
Avg.	29	18.0	36.7	16.4	132.6	36	26.0	26.9	10.9	132.4

CONCLUSION

From the present study, it is indicated that intergenic spacer between the *trn*L - *trn*F gene seems to be well suited for inferring plant phylogenies between closely related taxa. From the phylogenetic trees it is also clear that *A.calcarata* has high affinity to *A.mutica*. The results clearly indicate that DNA barcoding system has the potential to resolve some of the taxonomic problems which cannot be resolved by morphology- based taxonomy alone. The analysis of cpDNA sequences in the *Alpinia* species opens up additional interpretation of relationships among the selected species. In conclusion we have shown that DNA profiling is a powerful

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