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Heliotropium indicum L. and *Heliotropium keralense* Sivar. & Manilal, J.: DNA Barcoding Studies for Species Identity

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Abstract

The family Boraginaceae comprises around 2000 species within 145 genera with a myriad of therapeutic and pharmacological attributes. Kerala holds 26 species of Boraginaceae members coming under 9 genera. The genus Heliotropium comprises 8 species where *Heliotropium indicum* L. and *Heliotropium keralense* Sivar. & Manilal, J. is commonly found in Kerala. *H. keralense* is endemic to Kerala. Only minute morphological differences can be noticed between these two species. The present available data suggests the idea that only morphological characters are used to demarcate these two plants into two species. Hence an authentication is an immediate necessity for confirming the taxonomic identity of these plants. The present study aims to inspect whether these plants can be categorized into different species or not and DNA barcoding is used to clarify the dispute. Here, highly conserved regions of the genome, especially short stretches (~600 bp) are used to differentiate species-level taxonomy. The DNA barcode marker used in this technique is Rbcl and sequence analysis was performed using online tool BLAST of NCBI database and based on maximum identity score E value top most sequences was utilized for multiple sequence alignment (Clustal W2) and dendrogram was also constructed. The differences in DNA sequence and amino acid composition clearly specifies that *Heliotropium indicum* L. and *Heliotropium keralense* Sivar. & Manilal, J. can be categorized into two species.

Keywords: Boraginaceae, *Heliotropium indicum, Heliotropium keralense,* DNA barcoding, Kerala

Introduction

The family Boraginaceae Juss., Gen. Pl. [Jussieu] 128 (1789), nom. cons., commonly called the borage or forget-me-not family holds 145 genera that comprises around 2,000 species with a global distribution ((Luebert *et al*., 2016). As per the APG system of classification (Angiosperm Phylogeny Group, 2009 and 2016), Boraginaceae is treated as a single family of the order Boraginales within the Asterids. Based on the older Cronquist system, Boraginaceae was counted in Lamiales. George Benthem and Joseph Dalton Hooker included the family Boraginaceae in the cohort Polemoniales within the series Bicarpellatae (Subrahmanyam, 2009). In Kerala the family Boraginaceae comprises 9 genera with 26 species (Sasidharan, 2012). Among the 9 genera, Heliotropium holds the most number with 8 species. Among the genera Heliotropium, the species *Heliotropium indicum* L. having a pantropical distribution and *Heliotropium keralense* Sivar. & Manilal, J. is endemic to Kerala. Both of these species are very closely related with an array of similar morphological features and hence the identification of these two is a taxonomic dilemma. The present study is an attempt to evaluate the morphological identity of the two species and its DNA barcoding for improved taxonomic authentication.

Plant description

Heliotropium indicum L.

Erect herbs; branched and pubescent. Leaves 10 x 6 cm, opposite, broadly ovate, chartaceous, base truncate, apex acute, margin undulate, lateral nerves 4-6 pairs; petiole 7 cm long. Inflorescence terminal or leaf-opposed spikes, to 18 cm long. Flowers small, sessile. Calyx-lobes 5, 2.5 mm, linear-lanceolate, acute. Corolla 4 mm across, pale violet, salver-form; tube 3.5 mm, cylindric; lobes 5, ovate, 1mm, obtuse. Stamens 5, free, subsessile; anthers 1.5 mm, lanceolate. Ovary 4-lobed, 0.8 mm, globose; style 0.8 mm, disciform; stigma conical. Nutlets joined to pairs, ribbed, 3-4 mm long.

Figure 1. *Heliotropium indicum* L. (Source-Tojo Jose, 2020)

Heliotropium keralense Sivar. & Manilal, J.

Erect herbs; 20-25 cm tall, pubescent. Leaves to 7.5 x 4.5 cm, ovate, decurrent on petiole at the base, apex acute or obtuse, a flexuous margin, lower surface strigose on veins, upper surface glabrous; petiole 6-8 cm long. Inflorescence terminal, unilateral, scorpioid, cymes, to 14 cm long. Calyx ca. 2 mm; lobes 5, persistent, subequal. Corolla ca. 5 mm, white, pubescent; lobes 5, plicate, triangular. Stamens 5, included. Ovary lobes 4, 4-loculed; ovules one in each locule; stigma hemispheric, lobed. Fruit dividing into 4 pyrenes. Seeds brownish-black.

Figure 2. *Heliotropium keralense* Sivar. & Manilal, J. (Source-Tojo Jose, 2020)

Table 1. Major differences in morphology between the two species

Plant part	*H. indicum*	*H. kealense*
Leaves	10 x 6 cm Sub-deltoid or broadly ovate Margins undulate	7.5 x 4.5 cm Ovate Margins flexuous
Flowers	Pale violet or pink	White

Materials and methods

Rbcl gene sequencing is an amplicon sequencing method for studying phylogeny and taxonomy. Several researchers have exploited the conserved regions of the Rbcl gene for phylogenetic analysis. Here we explored the possibility of rbclF and rbclR primers for amplification. DNA was isolated from the sample provided. Electrophoresed the DNA in 1% agarose and visualized under UV. Rbcl region were PCR amplified with specific primers and amplicon was checked for appropriate size by agarose gel visualization. Amplicon was gel purified using commercial column based purification kit (Invitrogen, USA) and sequencing was performed with forward and reverse primers in ABI 3730 XL cycle Sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. Sequence analysis was performed using online tool BLAST of NCBI database and based on maximum identity score E value top most sequences was utilized for multiple sequence alignment (Clustal W2) and dendrogram was constructed.

Results and Discussion

The family Boraginaceae comprises around 2000 species under 145 genera with an array of medicinal and ethno-pharmacological properties. The state of Kerala has 26 species of Boraginaceae members reported till date which comprises 9 genera. The genus Heliotropium comprises 8 species where *Heliotropium indicum* and *Heliotropium keralense* are commonly found in Kerala. *H. keralense* is endemic to Kerala. The present study inspected whether these plants can be categorized into different species or not and DNA barcoding is used to clarify the dispute. The present study pointed out that both *Heliotropium indicum* and *Heliotropium keralense* are considered as separate species with notable differences in DNA sequence and amino acid composition. The promising vistas of DNA barcoding which is the process of identification of species based on nucleotide diversity of short DNA segments are well illustrated by many workers recently (Vijayan and Tsou, 2010; de Vere *et al.*, 2015 and Dormontt *et al.*, 2018). The results of amplification and sequencing of *Heliotropium indicum* and *Heliotropium keralense* are given in Fig 3.

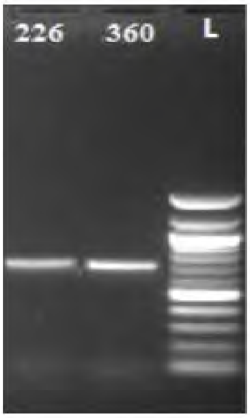

Figure 3. Amplification and sequencing were done with respective primers

Plant code 226 = *Heliotropium indicum* L.

Plant code 360 = *Heliotropium keralense* Sivar. & Manilal, J.

The gel purified product was sequenced. The Rbcl region was sequenced very well and was excellent for prediction of the identity of organisms. Contigs were obtained for Rbcl region after trimming off the low quality bases and good consensus were obtained.

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Contig:

>AACAGAAACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGAGT ACAAATTGACTTATTATACTCCTGAATACGAAACCAAAGATACTGATAT CTTGGCAGCATTCCGAGTAACTCCTCAACCCGGAGTTCCCCCTGAAGAA GCAGGGGCTGCAGTAGCCGCCGAATCTTCTACTGGTACATGGACAACT GTGTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGC TACCACATCGATCCCGTTCCTGGAGAAGAAAATCAATATATTGCTTATG TAGCTTATCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTT ACTTCCATTGTAGGTAATGTATTTGGTTTCAAAGCCCTACGCGCTCTAC GTCTGGAAGATCTGCGAATCCCTGTTGCTTATGTTAAAACTTTCCAGGG CCCGCCTCACGGATCCAGGTTGAGAGAGATAAATTGAACAAGTATGG TCGTCCTTTGTTGGGATGTACTATTAAACCTAAATTGGGGTTATCTGCT AAAAACTACGGTAGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGATT TTACCAAAGATGATGAAAACGTGAACTCCCAACCATTTATGCGTTGGA GAGACCGTTTCTTATTTTGTGCAGAAGCTATTTATAAATCACAGGCTGA AACAGGTGAAATCAAAGGCATTACTTGAATGCTACTGCAGGTACATGCG

The blast annotation shows 98.48% identity with *Tournefortia hirsutissima* with 0 E-value and 99% query coverage according to the nucleotide homology.

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Contig:

>AAACAGAAACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGAG TACAAATTGACTTATTATACTCCTGAATACGAAACCAAAGATACTGATA TCTTGGCAGCATTCCGAGTAACTCCTCAACCCGGAGTTCCCCCTGAAGA AGCAGGGGCTGCAGTAGCCGCCGAATCTTCTACTGGTACATGGACAAC TGTGTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATG CTACCACATCGATCCCGTTCCTGGAGAAGAAAATCAATATATTGCTTAT GTAGCTTATCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTT TACTTCCATTGTAGGTAATGTATTTGGTTTCAAAGCCCTACGCGCTCTA CGTCTGGAAGATCTGCGAATCCCTGTTGCTTATGTTAAAACTTTCCAGG GCCCGCCTCACGGATCCAGGTTGAGAGAGATAAATTGAACAAGTATG GTCGTCCTTTGTTGGGATGTACTATTAAACCTAAATTGGGGTTATCTGC TAAAAACTACGGTAGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGAT TTTACCAAAGATGATGAAAACGTGAACTCCCAACCATTTATGCGTTGGA GAGACCGTTTCTTATTTTGTGCAGAAGCTATTTATAAATCACAGGCTGA AACAGGTGAAATCAAAGGCATTACTTGAATGCTACTGCAGGTACATGCGA

The blast annotation shows 98.62% identity with *Tournefortia hirsutissima* with 0 E-value and 99% query coverage according to the nucleotide homology.

Lineage

Lineage (full): cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliopsida; Mesangiospermae;

eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Boraginales; Heliotro piaceae; Tournefortia.

While decoding the DNA sequences to amino acid sequences using CODONS-GENETIC CODE tool the following results have been obtained;

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>Asn Arg Asn Thr Lys Ala Ala Gly Thr Gly Thr Trp Ala Thr Thr Gln Ser Trp Thr Gly Thr Thr Lys Glu Thr Thr Ala Ala Thr Thr Asp Thr Thr Ala Thr Thr Ala Thr Ala Cys Thr Cys Cys Thr Glu Thr Thr Lys Pro Lys Ala Thr Ala Cys Thr Gly Ala Thr Ala Thr Cys Thr Trp Gln Cys Ala Thr Thr Pro Ala Gly Thr Asn Thr Cys Cys Thr Gln Pro Gly Gly Thr Thr Pro Cys Cys Thr Glu Glu Ala Gly Gly Cys Thr Ala Gly Thr Ser Arg Arg Ala Thr Cys Thr Thr Cys Thr Ala Cys Trp Thr Thr Trp Thr Ala Cys Thr Gly Thr Gly Trp Thr Gly Met Asp Thr Thr Thr Ser Cys Thr Thr Gly Ala Thr Cys Gly Thr Thr Thr Lys Asp Gly Met Cys Thr Thr Thr Thr Arg Thr Pro Gly Thr Thr Cys Cys Trp Arg Arg Lys Thr Gln Thr Ala Thr Ala Thr Thr Gly Cys Thr Thr Met Thr Ser Thr Thr Ala Thr Cys Cys Thr Thr Thr Arg Cys Cys Thr Thr Thr Thr Thr Glu Glu Gly Gly Thr Thr Cys Thr Gly Thr Thr Ala Cys Thr Asn Met Thr Thr Thr Ala Cys Thr Thr Pro Thr Thr Gly Thr Arg Thr Ala Met Thr Ala Thr Thr Trp Thr Thr Thr Gln Ser Cys Cys Thr Thr Arg Thr Cys Thr Ala Cys Gly Thr Cys Trp Lys Ala Thr Cys Thr Ala Ala Ala Thr Pro Thr Gly Thr Thr Gly Cys Thr Thr Met Thr Thr Lys Ala Cys Thr Thr Thr Pro Gly Pro Ala Thr His Gly Ala Thr Pro Gly Gly Thr Thr Glu Arg Gly Ala Thr Lys Thr Thr Glu Gln Gly Thr Met Gly Thr Cys Gly Thr Cys Cys Thr Thr Thr Gly Thr Trp Gly Met Thr Ala Cys Thr Ala Thr Thr Lys Cys Cys Thr Lys Thr Trp Gly Gly Thr Thr Ala Thr Cys Thr Gly Cys Thr Lys Asn Thr Thr Gly Thr Arg Ala Gly Thr Thr Thr Met Ala Met Thr Cys Thr Thr Arg Gly Gly Trp Ala Cys Thr Thr Gly Ala Thr Thr Thr Thr Thr Lys Gly Met Met Lys Thr Thr Glu Cys Thr Pro Asn Cys Ala Thr Thr Thr Met Cys Gly Thr Trp Ala Glu Thr Gly Thr Thr Thr Cys Thr Thr Ala Thr Thr Thr Thr Gly Thr Ala Glu Gly Cys Thr Ala Thr Thr Thr Ala Thr Lys Thr His Arg Cys Thr Gly Ala Asn Arg Thr Glu Ala Thr Gln Arg Ala Thr Thr Ala Cys Thr Thr Glu Thr Gly Cys Thr Ala Cys Thr Ala Gly Gly Thr Thr Thr Ala

360

>Lys Gln Lys Cys Thr Lys Ala Ala Gly Thr Gly Thr Trp Ala Thr Thr Gln Ser Trp Thr Gly Thr Thr Lys Glu Thr Thr Ala Ala Thr Thr Asp Thr Thr Ala Thr Thr Ala Thr Ala Cys Thr Cys Cys Thr Glu Thr Thr Lys Pro Lys Ala Thr Ala Cys Thr Gly Ala Thr Ala Thr Cys Thr Trp Gln Cys Ala Thr Thr Pro Ala Gly Thr Asn Thr Cys Cys Thr Gln Pro Gly Gly Thr Thr Pro Cys Cys Thr Glu Gly Ala Ser Arg Gly Thr Ala Gly Thr Ser Arg Arg Ala Thr Cys Thr Thr Cys Thr Ala Cys Trp Thr Thr Trp Thr Ala Cys Thr Gly Thr Gly Trp Thr Gly Met Asp Thr Thr Thr Ser Cys Thr Thr Gly Ala Thr Cys Gly Thr Thr Thr Lys Asp Gly Met Cys Thr Thr Thr Thr Arg Thr Pro Gly Thr Thr Cys Cys Trp Arg Arg Lys Thr Gln Thr Ala Thr Ala Thr Thr Gly Cys Thr Thr Ala Thr Gly Thr Ser Thr Thr Ala Thr Cys Cys Thr Thr Thr Arg Cys Cys Thr Thr Thr Thr Thr Glu Glu Gly Gly Thr Thr Cys Thr Gly Thr Thr Ala Cys Thr Asn Met Thr Thr Thr Ala Cys Thr Thr Pro Thr Thr Gly Thr Arg Thr Ala Met Thr Ala Thr Thr Trp Thr Thr Thr Gln Ser Cys Cys Thr Thr Arg Thr Cys Thr Ala Cys Gly Thr Cys Trp Lys Ala Thr Cys Thr Ala Ala Ala Thr Pro Thr Gly Thr Thr Gly Cys Thr Thr Met Thr Thr Lys Ala Cys Thr Thr Thr Pro Gly Gly Ala Arg Cys Thr His Gly Ala Thr Pro Gly Gly Thr Thr Glu Arg Gly Ala Thr Lys Thr Thr Glu Gln Gly Thr Met Gly Thr Cys Gly Thr Cys Cys Thr Thr Thr Gly Thr Trp Gly Met Thr Ala Cys Thr Ala Thr Thr Lys Cys Cys Thr Lys Thr Trp Gly Gly Thr Thr Ala Thr Cys Thr Gly Cys Thr Lys Asn Thr Thr Gly Thr Arg Ala Gly Thr Thr Thr Met Ala Met Thr Cys Thr Thr Arg Gly Gly Trp Ala Cys Thr Thr Gly Ala Thr Thr Thr Thr Thr Lys Gly Met Met Lys Thr Thr Glu Cys Thr Pro Asn Cys Ala Thr Thr Thr Met Cys Gly Thr Trp Ala Glu Thr Gly Thr Thr Thr Cys Thr Thr Ala Thr Thr Thr Thr Gly Thr Ala Glu Gly Cys Thr Ala Thr Thr Thr Ala Thr Lys Thr His

Arg Cys Thr Gly Ala Asn Arg Thr Glu Ala Thr Gln Arg Ala Thr Thr Ala Cys Thr Thr Glu Thr Gly Cys Thr Ala Cys Thr Ala Gly Gly Thr Thr Thr Ala Ala

The sequential analysis of DNA and amino acids of *Heliotropium indicum* and *Heliotropium keralense* indicates that there are differences in the sequences and amino acid composition. These differences give the correct authentication that *Heliotropium indicum* and *Heliotropium keralense* can be considered as two species. The phylogenetic tree analysis also attested the same.

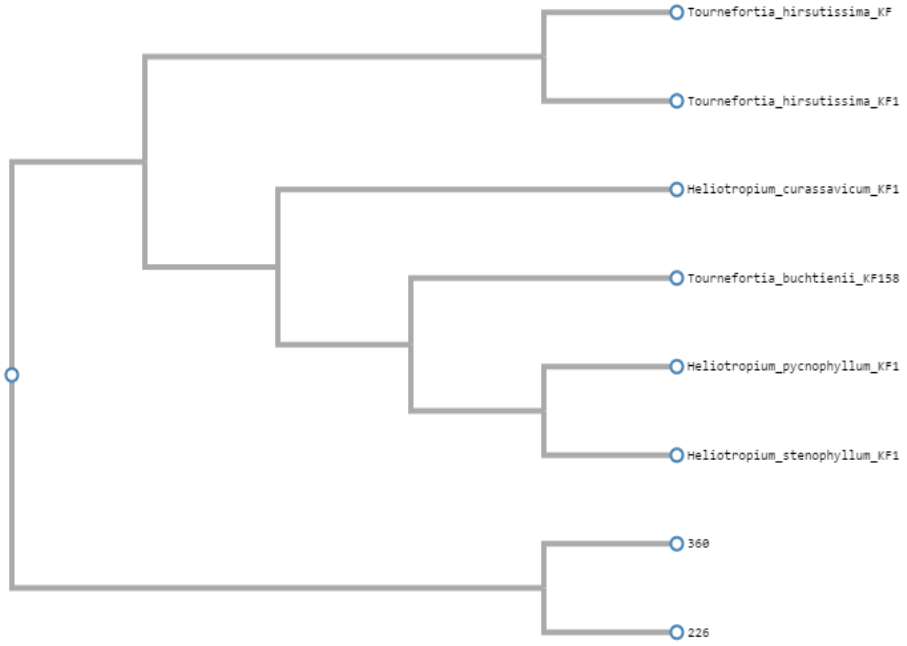

Figure 4: Phylogenetic tree

The phylogenetic tree depicted that *Heliotropium indicum* and *Heliotropium keralense* can be categorised under two separate species. The tree also projects the information that the two species are very closely related and they have an immediate common ancestor. Hilgern and Diane (2003) systematically analyzed the trnL and ITS1 sequence data of Heliotropiaceae (Boraginales). Kumar and Kumar (2016) also portrayed the biosystematics of three species of Boraginaceae *viz*., of *Heliotropium indicum*, *Trichodesma indicum* and *Trichodesma zeylanicum* with 70.96% resemblances. Thus it can be concluded that *Heliotropium indicum* and *Heliotropium keralense* are two species not only with morphological and floristic characteristics but also with DNA barcoding studies.

Conclusion

The present study clearly depicts the taxonomic identity of *Heliotropium indicum* and *Heliotropium keralense* which has wide applications in taxonomic ambiguities between the two species. To the best of our knowledge, the present study is a pioneer attempt in this line.

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