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Utility of ITS2 as a specific barcode for *Aristolochia* spp.

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Abstract
The genus *Aristolochia* includes medicinal plants and some are used as ornamentals due to their showy flowers. In many countries including India the plant is commonly used to treat snake bite and has great ethno pharmacological importance. DNA barcoding studies were carried out using the commonly employed marker genes rbcL, matK, psbA-trnH and ITS2 in four species of *Aristolochia*, *Aristolochia indica* L., *Aristolochia tagala* Cham., *Aristolochia ringens* Vahl. and *Aristolochia krisagathra* Sivarajan and Pradeep. Of this *A. indica*, *A. tagala* and *A. krisagathra* are native to India; *A. ringens* is native to South and Central America. *A. krisagathra* is reported to be endemic to India and is found restricted to the southern part of Western Ghats in Kerala and Tamil Nadu. No reports are available on the molecular aspects of this plant which makes the study relevant. It was found that among the four markers used, ITS2 showed higher variation percentage followed by psbA-trnH. The dendrograms obtained from ITS2 also backed the morphological observation.

Keywords: *Aristolochia*, ITS2, specific barcode

1. Introduction
The genus *Aristolochia* with around 500 species comes under the family Aristolochiaceae distributed mostly in the tropical, subtropical and Mediterranean regions. In India, eight species of *Aristolochia* has been reported [1], but the proper documentation of the genus is yet to be accomplished [2]. *Aristolochia* finds wide applications in traditional and folk medicines. However, *Aristolochia* species used in traditional medicines especially in China was withdrawn later due to the potential health hazards reported. Chinese herbal nephropathy was found associated with the intake of *Aristolochia* species containing aristolochic acids [3]. In India, *Aristolochia* species are used for medicinal purposes especially by the tribals. Hence proper identification and documentation of the genus is essential. DNA barcoding provides an easy and rapid way to species identification even by non-taxonomists and to compare the query sequence with the numerous gene sequences available in the database with a single search. DNA barcoding is well established in animals and makes use of the mitochondrial coxI region as the universal barcode, but in plants because of the slower rate of evolution the coxI gene becomes unsuitable. Hence a number of genes and intergenic regions are being tested in which the chloroplast and nuclear regions are most commonly exploited. The search for universal barcode compared to "Holy Grail" [4] is still in progress and a universal barcode in plants is yet to be evolved. Moreover, various approaches from "gene to genome" in barcoding like singe-locus barcode, multi-locus barcodes, super-barcoding etc. have been tried [5]. Discovering a universal barcode locus for land plants is not possible especially in the chloroplast wherein lineage-specific evolution and nonrandom spatial patterns of substitution can occur is not feasible [6]. Specific-barcode which is a fragment DNA sequence has an adequately high mutation rate to facilitate species identification within a given taxonomic group which seems to be more practical [5].
In present study, the discrimination potential of four barcodes rbcL, matK, psbA-trnH and ITS2 in four species of *Aristolochia*, *A. indica*, *A. tagala*, *A. ringens* and *A. krisagathra* was analyzed; and to find out the one which can serve as a specific barcode for *Aristolochia*. Studies on phylogenetic analysis of *Aristolochia* are available; however, there are no previous studies or reports on *A. krisagathra* which is often confused with *A. indica* or *A. tagala* though the plant is easily distinguishable with the unwinged seeds [7]. Hence, the barcoding studies using the commonly used barcodes especially relating to *A. krisagathra* gains significance.

2. Materials and methods

The four *Aristolochia* species were collected from various locations of Kerala, India. *A. indica* was collected from Eroor region of Ernakulam district, *A. tagala* from Thamarassery Churam of Kozhikode district, *A. ringens* from Vythiri, Wayanad and *A. krisagathra* from Aryankavu, Kollam.

The total genomic DNA was isolated from fresh leaf tissues of the four species. DNA isolation was carried out using GenElute Plant Genomic DNA Miniprep Kit (Sigma). PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (150mM Tris HCl, pH-8; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM MgCl2, 20ng DNA, 1 unit of AmpliTaq Gold DNA polymerase enzyme (Applied Biosystems), 0.1 mg/ml BSA and 4% DMSO, 5pM of forward and reverse primers (Table 1).

Table 1: Primers of rbcL, matK, psbA-trnH and ITS2 used

Target	Primer Name	Direction	Sequence (5'-3')
matK	matK_xf	Forward	TAATTTACGATCAATTCATTC
	matK_MALPR1	Reverse	ACAAGAAAGTCGAAGTAT
rbcL	rbcLa_f	Forward	ATGTCACCACAAACAGAGACTAAAGC
	rbcL724_rev	Reverse	GTAAAATCAAGTCCACCRCG
psbA-trnH	psbA-F	Forward	GTTATGCATGAACGTAATGCTC
	trnH2	Reverse	CGCGCATGGTGGATTCACAATCC
ITS2	ITS2-F	Forward	CCGTGAACCATCGAGTCTTT
	ITS-R	Reverse	CTCGCCGTTACTAGGGGAAT

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 [8].

The sequences obtained were submitted in GenBank database using Sequin Application Version 13.05. DNA sequences were compared and multiple sequence alignment was carried out using Clustal W in the BioEdit version 7.0.9.0[9]. Pairwise alignment was done to note the nucleotide variations of rbcL, matK, psbA-trnH and ITS2 between different species.

The dendrogram was constructed using MEGA 4.0[10] by UPGMA method. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Model adopted was p-distance and substitutions to include were d: Transitions + d: Transversions; homogenous pattern among lineages and uniform rates among sites.

3. Results and Discussion

The sequences of rbcl, matK, psbA-trnHand ITS2 obtained were submitted to GenBank and accession numbers were obtained. The accession number and size of each barcode of *A. indica*, *A. tagala*, *A. ringens* and *A. krisagathra* are given in Table 2.

Table 2: The accession numbers and size of rbcL, matK, psbA-trnH and ITS2 in *A. indica, A. tagala, A. ringens* and *A. krisagathra*

	rbcL		matK		psbA-trnH		ITS2	
	Accession no.	Size (bp*)	Accession no.	Size (bp)	Accession no.	Size (bp)	Accession no.	Size (bp)
A. indica	KF498583	697	KF498586	898	KP763859	268	KP763863	364
A. tagala	KF498584	697	KF498587	898	KP763862	280	KP763864	386
A. ringens	KF498585	697	KF498588	922	KP763860	259	KP763865	396
A. krisagathra	KF498589	697	KF476063	880	KP763861	264	KP763866	370

Multiple sequence alignment was performed for rbcL, matK, psbA-trnH and ITS2. For the rbcL gene (Figure 1), *A. krisagathra* did not have any single distinct variable nucleotides to distinguish it from other three species. *A. indica* had a single variable nucleotide at position 421, *A. tagala* with two nucleotides (258 and 398) and *A. ringens* having the maximum of ten. For matK (Figure 2), *A. indica* had only a single nucleotide varying (21st), *A. tagala* did not have any such variable nucleotide, *A. krisagathra* had 6. The nucleotides from 220 to 234 and 637 to 645 in mat K gene can be used a distinct barcode region to differentiate *A. ringens* from the other 3 species. For psbA-trnH (Figure 3) gene 5 nucleotides showed distinct variation in *A. indica* and 7 in *A. krisagathra*. Unique region for *A. tagala* present in psbA-trnH gene were the nucleotides from 199-203 and 226 to 232 and for *A. ringens* they were found at regions from 112 to 118, 204 to 206 and 242 to 244 along with other variable nucleotides. psbA-trnH produced thirteen mononucleotide 'T' repeats at region 204 to 216, in *A. tagala* it showed sixteen 'T' repeats (201 to 216). Presence of such long mononucleotide repeats, in which one DNA base recurs excessively within the spacer was mentioned [11]. For ITS2 (Figure 4), nucleotides from positions 87 to 103 forms a unique identifier of *A. ringens* as in other species these regions have gaps and 283 to 288 forms another distinct region. *A. indica* (6 bases), *A. tagala* (12 bases) and *A. krisagathra* (12 bases) also showed some variable nucleotides.

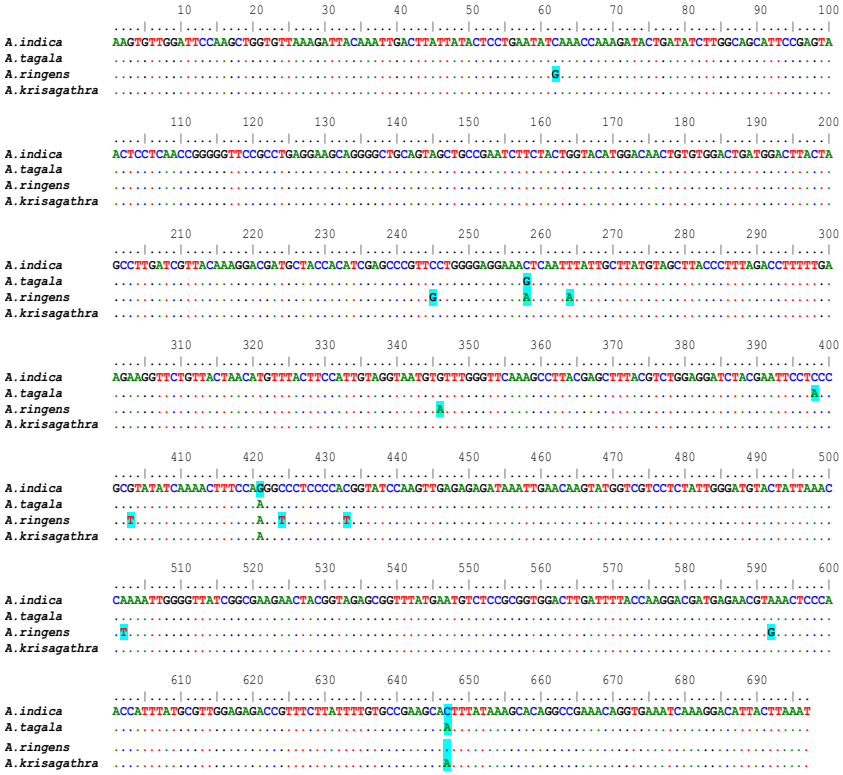


Fig 1: Multiple Sequence Alignment of rbcL sequences of *A. indica*, *A. tagala*, *A. ringens and A. krisagathra* with the highlighted variable sites

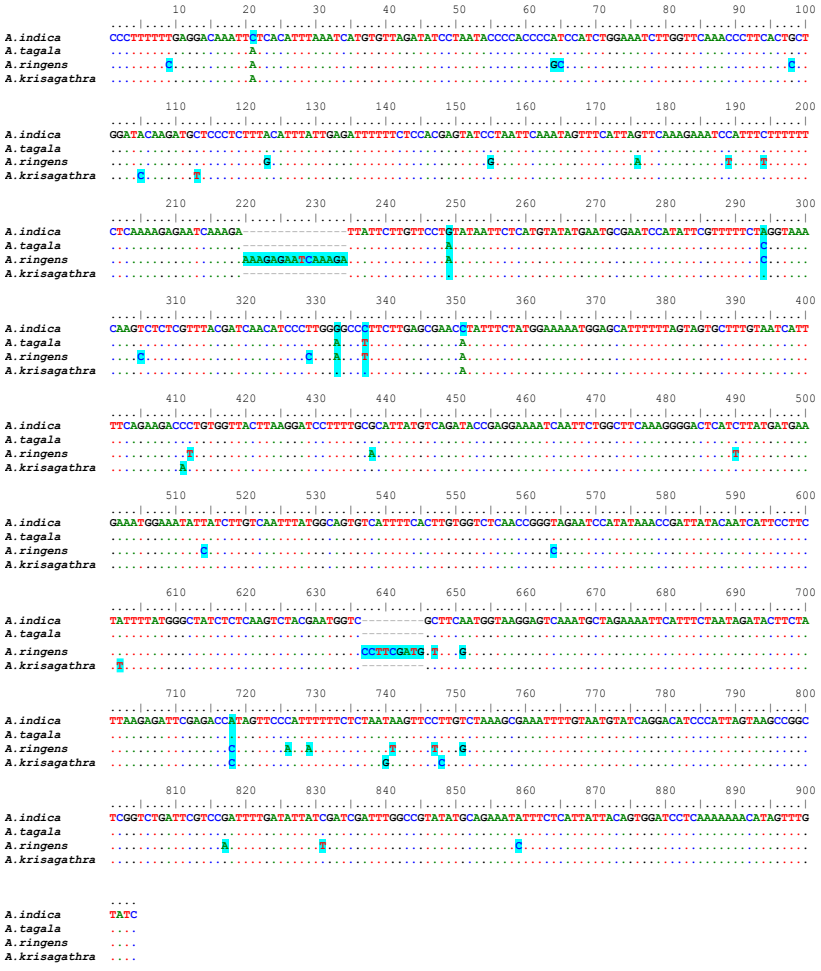


Fig 2: Multiple sequence alignment of matK sequences of *A. indica*, *A. tagala*, *A. ringens and A. krisagathra* with the highlighted variable sites

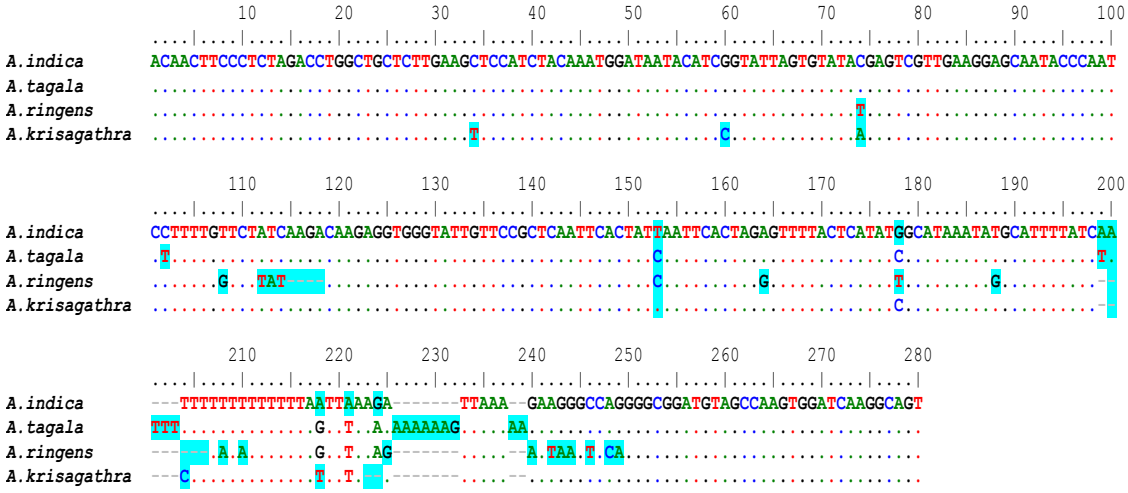


Fig 3: Multiple sequence alignment of psbA-trnH sequences of *A. indica, A. tagala, A. ringens* and *A. krisagathra* with the highlighted variable sites

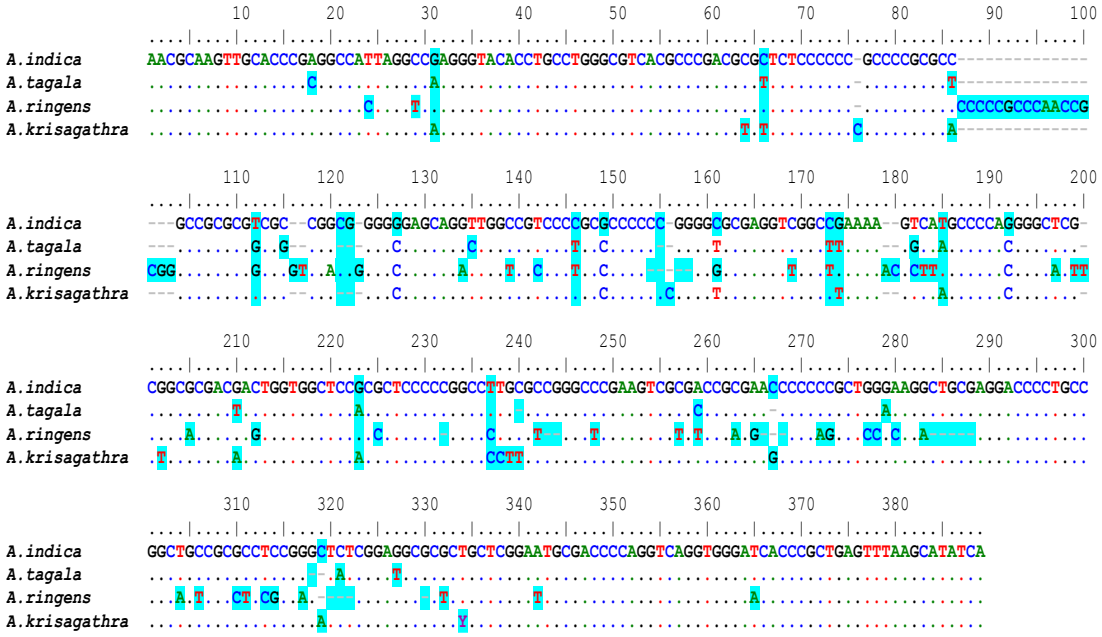
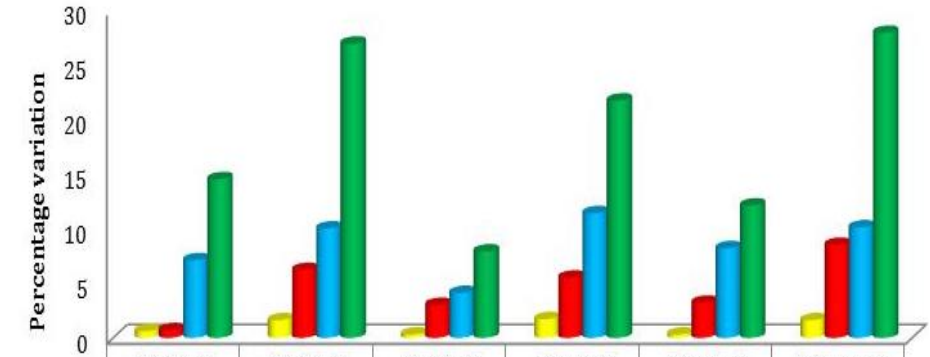


Fig 4: Multiple sequence alignment of ITS2 sequences of *A. indica, A. tagala, A. ringens and A. krisagathra* with the highlighted variable sites

The percentage of nucleotide variation were analyzed by pairwise alignment and given in Figure 5. The order of variation percentage shown by the four barcodes was in the order of ITS2>psbA-trnH>matK>rbcL. Almost a similar result was obtained in a study of herbal medicinal materials confused with *Aristolochia* herbs where the variation percentage was in the order of trnH-psbA>matK>rbcL without ITS2 [12]. Similar results were also observed at the genus level in forensic botany [13]. ITS2 barcode exhibited higher interspecific variations in various studies [14, 15, 16].



	A.indica X A.tagala	A.indica X A.ringens	A.indica X A.krisagathra	A.tagala X A.ringens	A.tagala X A.krisagathra	A.ringens X A.krisagathra
rbcL	0.6	1.6	0.3	1.7	0.3	1.6
matK	0.7	6.2	3	5.5	3.2	8.5
psbA-trnH	7.1	10	4.1	11.4	8.2	10.1
ITS2	14.5	26.9	7.9	21.7	12.1	27.9

Fig 5: Bardiagram showing the nucleotide variation (in percentage) between *A. indica, A. tagala, A. ringens* and *A. krisagathra*

Dendrograms constructed using the four barcodes produced rooted trees (Figure 6). The rooted tree bifurcated into two branches forming a dichotomous tree. One of these main branches (A) was further again divided into two branches and the other (B) culminated in *A. ringens* which remained distinct and distant from the other species. These branching patterns of the dendrogram were similar for all the barcodes analyzed.

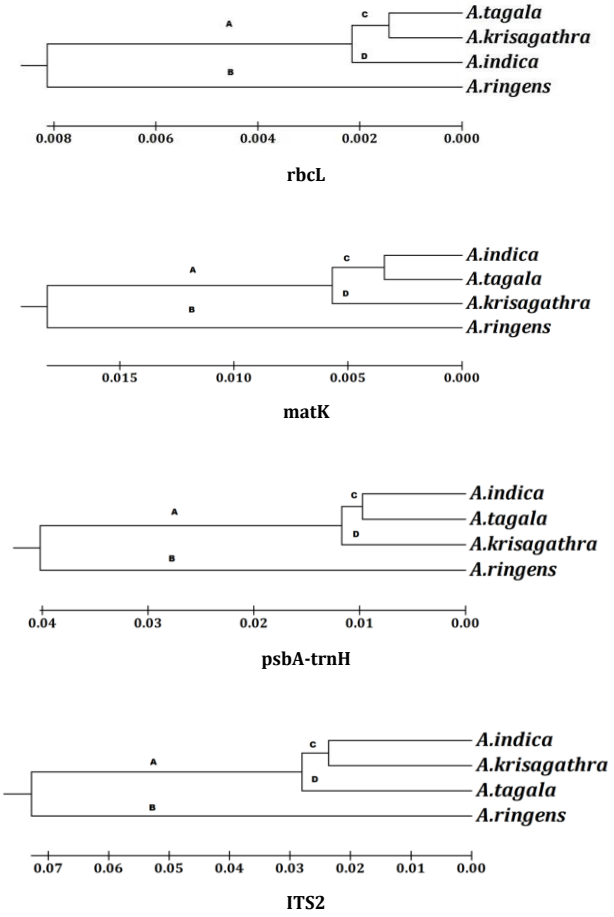


Fig 6: Dendrogram constructed by UPGMA rbcL, matK, psbA-trnH and ITS2

Thus, from all the dendrograms it was observed that three species *A. indica*, *A. tagala* and *A. krisagathra* formed a part of main bifurcation. *A. krisagathra* was found as a sister taxon to *A. indica* in ITS2 and as a sister taxon to *A. tagala* in rbcL. In trnH-psbA and matK it was obtained as a sister clade to the clade of *A. indica* and *A. tagala*. Considering the morphology of *A. krisagathra*, during collections it was found to be more similar to *A. indica* and may be misidentified by non-taxonomists. On considering the dendrograms build by using various barcodes, it was found that, compared to other barcodes; the tree generated by using ITS2 backed the morphology as observed from our field collections. In ITS2 *A. indica* and *A. krisagathra* was best closely placed forming the sister taxons.

On the basis of the sequences and dendrograms generated from the present study, it was found that ITS2 could serve as a reliable barcode for the selected *Aristolochia* species. The applicability of ITS2 in discriminating a wide array of plants from the families of Asteraceae, Rutaceae, Rosaceae etc. have also been discussed [17, 18, 19, 20]. However, its use as a barcode region is less studied in *Aristolochia*. trnH-psbA has been reported as a useful barcode for identifying *Aristolochia* species [21] and ITS2 as a reliable barcode for medicinal plants [22]. In the present study also ITS2 was found effective which included medicinal species of *Aristolochia*.

4. Conclusions

In the present study ITS2 showed higher variations between closely related *Aristolochia* species in comparison to other barcodes. Hence, though the number of species taken and analyzed is limited, the study showcases the suitability of ITS2 as a potential candidate barcode for *Aristolochia*.

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