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Phylogenetic relationship of selected *Aristolochia* spp. with different generic segregates inferred using rbcL and matK genes

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Abstract

The genus *Aristolochia* comes under the family Aristolochiaceae, a conspicuous family with distinctive and showy flowers positioned among the basal angiosperms. The taxonomic treatment of the genus *Aristolochia* is ambiguous. Several generic segregates have been proposed for the genus and most literatures propose four subgenera within two subtribes. The study focused on the relationship of *A. indica*, *A. tagala*, *A. ringens* and *A. krisagathra* with the different generic segregates. The sequencing was carried out using rbcL, matK, psbA-trnH, ITS and ITS2. However, only rbcL and matK were used to infer the relationship because of the lack of availability of sequences of psbA-trnH, ITS and ITS2 representing the different generic segregates. Both the neighbor joining trees constructed using rbcL and matK placed *A. krisagathra* as the sister taxon of *A. indica* with very high bootstrap values. *A. krisagathra*, *A. indica* and *A. tagala* forms a part of the subsection Podantheum and Diplolobus section along with *A. zollingeriana*, whereas the exotic *A. ringens* formed a part of Gymnolobus section.

Keywords: *A. krisagathra*, matK, rhodantheum, rbcL

Introduction

The genus *Aristolochia* comprises about 120 or 400 to 500^[1, 2] species depending on the differentiation within the subgenera. In India, only 8 species have been reported^[3]. However, the proper documentation of the genus is not yet accomplished in India^[4]. *Aristolochia* in Kerala is represented by three species *A. indica*, *A. tagala* and *A. krisagathra*^[5]. The plants grow as perennial rhizomatous herbs or deciduous or evergreen shrubs and lianas.

Aristolochia has been treated in its broad sense by many authors^[6-14]. The circumscription of the genus has been a significant taxonomic problem and is ambiguous. Several generic segregates have been proposed primarily based on floral and fruit characters such as the morphology of the gynostemium, the gross shape of the perianth, the dehiscence of fruits and the morphology of the seeds. Most literatures propose four subgenera within two sub-tribes^[15-17]. The genus *Aristolochia* s.l. comes under the tribe Aristolochieae with two subtribes Isotrematiinae and Aristolochiinae. The subtribe Isotrematiinae encloses two subgenera *Endodeca* and *Isotrema*; Aristolochiinae subtribe with *Par Aristolochia* and *Aristolochia*.

Subgenus *Endodeca* includes herbaceous group in North America, with reduced subtending leaves, clasping bracts, short internodes in the laterals, and prostrate inflorescence^[1, 15].

Subgenus *Isotrema* (= *Siphisia*) comprises of 70 species, 50 of which grow in Eastern Asia mainly the Sino-Japanese province. *Isotrema* has a primarily intercontinental Asia to North/Central America disjunct distribution. Within the genus *Aristolochia*, shrubs and shrub-like growth forms are limited to a handful of species recorded within *Isotrema*^[13]; characterized by basally dehiscent capsules, a trilobed perianth tube and a gynostemium with three lobes each accompanying two anthers^[18]. Sometimes, *Siphisia* is used for the combination of *Endodeca* and *Isotrema* or for *Isotrema* alone. Subgenus *Aristolochia* contains approximately 350 species of mostly woody tropical climbers, widely distributed and is characterized by an apically dehiscent capsule, lobes of the perianth unilaterally appressed in the bud breaking up into one to three segments, six or fewer lobes of the gynostemium, six or fewer anthers. They are morphologically quite diverse and several lower taxonomic ranks recognized^[19, 20]. The subgenus *ParAristolochia* is the smallest group and proposed as the most primitive group in *Aristolochia* s.l. because of its morphological similarity to *Thottea* s.l.^[10]. It has fleshy indehiscent fruits, a three-lobed perianth, valvate in bud, 6-12 lobes of gynostemium, 6-24 anthers.

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The present study focused on the relationship of four medicinal species of *Aristolochia*, *A. indica* L., *A. tagala* Cham., *A. ringens* Vahl. and *A. krisagathra* Sivarajan and Pradeep within the different generic segregates.

Materials and Methods

Collection of source plants

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.

Isolation of genomic DNA, PCR amplification and sequencing

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). 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.

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

Target	Primer Name	Direction	Sequence (5'-3')
matK	matK xf	Forward	TAATTTACGATCAATTCATTC
	matK MALPRI	Reverse	ACAAGAAAGTCGAAGTAT
rbcL	rbcLa f	Forward	ATGTCACCACAAACAGAGACTAAAGC
	rbcL724 rev	Reverse	GTAATAATCAAGTCCACCRCG
psbA-trnH	psbA-F	Forward	GTTATGCATGAACGTAATGCTC
	trnH2	Reverse	CGCGCATGGTGGATTCACAATCC
ITS2	ITS2-F	Forward	CCGTGAACCATCGAGTCTTT
	ITS-R	Reverse	CTCGCCGTTACTAGGGGAAT

The sequences obtained were submitted in GenBank database using Sequin Application Version 13.05. The accession numbers are given in Table 2. ITS sequencing did not work in *A.*

indica. Other *Aristolochia* sequences representing the various generic segregates were selected from GenBank (Table 3).

Table 2: Accession numbers of the five barcodes

	rbcL	matK	psbA-trnH	ITS	ITS2
<i>A. indica</i>	KF498583	KF498586	KP763859	-	KP763863
<i>A. tagala</i>	KF498584	KF498587	KP763862	KP763869	KP763864
<i>A. ringens</i>	KF498585	KF498588	KP763860	KP763867	KP763865
<i>A. krisagathra</i>	KF498589	KF476063	KP763861	KP763868	KP763866

Table 3: Accession numbers of rbcL and matK sequences retrieved from GenBank

Species	Accession number (rbcL)	Accession number (matK)
<i>A. serpentaria</i>	KJ773281.1	AB211558.1
<i>A. convolvulacea</i>	AB205589.1	AB211557.1
<i>A. tomentosa</i>	AB205598.1	AB071814.1
<i>A. manshuriensis</i>	JX944488.1	AB060748.1
<i>A. californica</i>	GQ248551.1	AB071813.1
<i>A. arborea</i>	AB205586.1	AB060739.1
<i>A. macrophylla</i>	KF724349.1	AB060742.1
<i>A. kaempferi</i>	AB205593.1	AB180166.1
<i>A. promissa</i>	AB205602.1	AB211590.1
<i>A. clematitis</i>	HE963329.1	AB060767.1
<i>A. pistolochia</i>	AF543711.1	AF543724.1
<i>A. debilis</i>	JX944485.1	AB060768.1
<i>A. contorta</i>	JX944486.1	AB211581.1
<i>A. lutea</i>	HE963332.1	HE970672.1
<i>A. fordiana</i>	KP094727.1	KP093790.1
<i>A. zollingeriana</i>	AB205599.1	AB071817.1
<i>A. micrantha</i>	AB205595.1	AB071815.1
<i>A. burelae</i>	AB205587.1	AB060788.1
<i>A. maxima</i>	AB205594.1	AB060784.1
<i>A. pilosa</i>	KJ594107.1	AB060802.1
<i>A. littoralis</i>	GU135225.1	AB211571.1
<i>A. gigantea</i>	AB205591.1	AB060793.1
<i>A. grandiflora</i>	AB205592.1	AB071812.1
<i>A. cordiflora</i>	KJ594102.1	KJ593770.1
<i>A. tonduzii</i>	KJ594108.1	JQ586748.1
<i>A. reniformis</i>	AB205600.1	AB211568.1
<i>T. borneensis</i>	AB205604.1	AB211554.1
<i>T. tomentosa</i>	AB205606.1	AB060738.1
<i>T. macrantha</i>	AB205605.1	AB211555.1

Phylogenetic analysis

The phylogenetic relationships proposed based on molecular data was examined [15, 21]. In order to infer the phylogenetic relationship, the statistics of various barcodes in the NCBI database was searched. The statistics of these five barcodes used in the present study was noted from the NCBI database (Fig. 1).

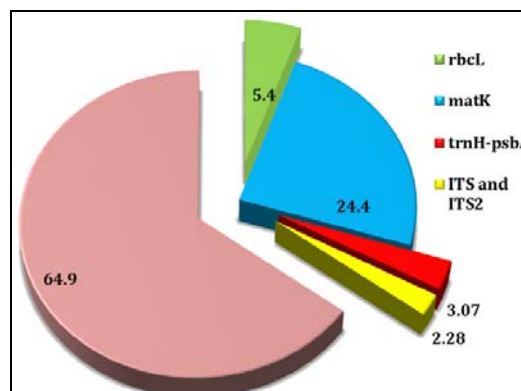


Fig 1: Percentage of various barcode sequences deposited in GenBank

As the number of sequences for psbA-trnH (3), ITS (7 of which 3 sequences were of *A. mollissima*) and ITS2 was very few compared to rbcL and matK and being the “agreed upon –

core barcodes”, these were used further for analyzing the phylogenetic relationship of the four species.

From the available *rbcL* and *matK* sequences of *Aristolochia* in NCBI database, the selection criteria was

- Common species for which sequences were available for both *rbcL* and *matK*
- Unidentified species were avoided
- When more than one sequence was available for a particular species, those which had the longer sequence length was selected.

Thus a total of 26 species were selected and these along with the four species *A. indica*, *A. tagala*, *A. ringens* and *A. krisagathra* were used for phylogenetic analysis. The genus *Aristolochia* is monophyletic and is sister to *Thottea*^[17]. Hence 3 species of *Thottea* (*T. tomentosa*, *T. borneensis* and *T. macrantha*) was selected as outgroup. These made a final set of 33 sequences.

In the phylogenetic analysis, number of conserved sites, variable sites and parsimony-informative sites were determined using Mega 4.0^[22]. The phylogenetic tree construction with the coding nucleotide sequences was done using the neighbour joining, a distance based method. All positions containing gaps and missing data were eliminated

from the dataset (Complete deletion option). Codon positions included were 1st+2nd+3rd+Noncoding. Model adopted was K2P; substitutions to include were d: Transitions + Transversions; homogenous pattern among lineages and uniform rates among sites. The reliability of the inferred tree was evaluated using bootstrap analysis with 1000 bootstrap replicates. Bootstrap values greater than 50% have only been indicated.

Results and Discussion

rbcL phylogeny

The *rbcL* sequences generated for *Aristolochia* were aligned and the total aligned length including the sequences of the outgroup species was 506 forming the final data set. The number of conserved sites was 464. Out of the 45 variable sites 32 were parsimony informative.

Overall average interspecific K2P distance for the 33 sequences of *rbcL* analyzed was 0.0196. The highest interspecific distance was observed for *A. kaempferi* and *A. pilosa* (0.0363). *A. pistolochia* - *A. zollingeriana*, *A. contorta* - *A. debilis* and *A. littoralis* - *A. grandiflora* - *A. cordiflora* - *A. gigantea* - *A. reniformis* formed clusters with zero interspecific distance.

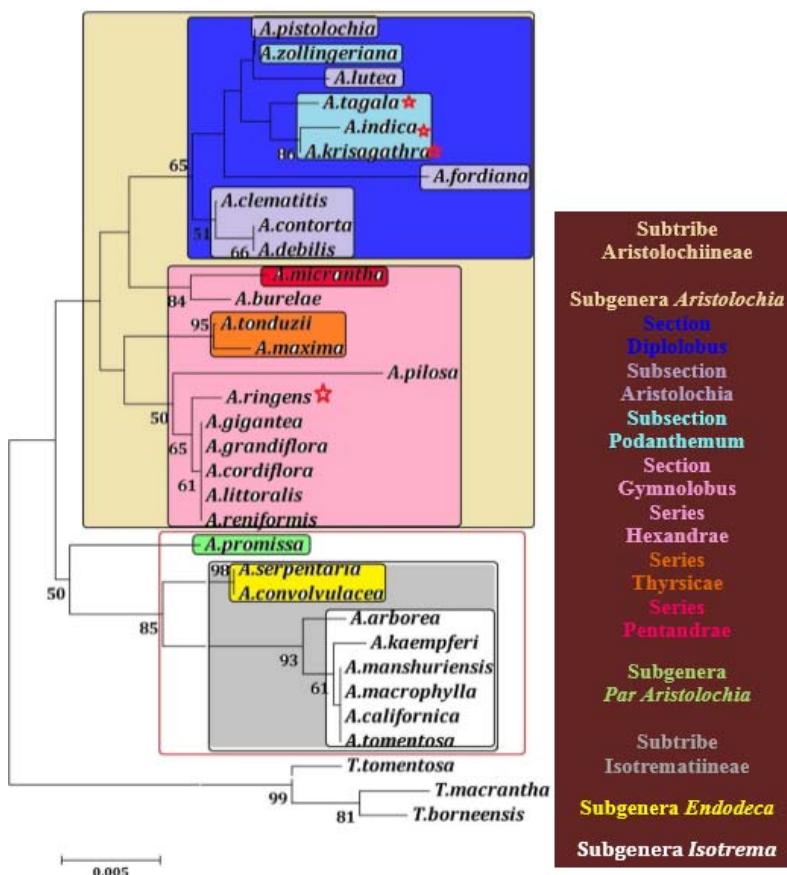


Fig 2: Neighbour joining tree of 33 plant species based on *rbcL* (★sequences generated from the present study)

The phylogenetic tree of *rbcL* showed that *Aristolochia* is monophyletic and embraces two major clades indicating two major lineages (Fig. 2) but without strong support. One of the major clade was *Aristolochiineae* and the other major clade further subdivided with *ParAristolochia* in one branch and *Isotrematiineae* (*Endodeca* and *Isotrema*) as the other. The *Isotrematiineae* with *Endodeca* and *Isotrema* was monophyletic with a strong support as observed in a study^[17]. The placement

of *ParAristolochia* represented by a single species *A. promissa* in the study, within the *Isotrematiineae* was found odd and against the general classifications^[15-17]. This relationship gains no significant support from bootstrapping also. The clade *Endodeca* included *A. serpentaria* and *A. convolvulacea* which showed zero interspecific distance in the distance matrix. *Isotrema* with 6 species - *A. arborea*, *A. kaempferi*, *A. manshuriensis*, *A. macrophylla*, *A. californica* and *A.*

tomentosa of which *A. manshuriensis*, *A. macrophylla*, *A. californica* and *A. tomentosa* formed a single cluster with no interspecific distance.

The other major clade treated as Aristolochiineae showed several subclades. *A. indica*, *A. tagala*, *A. ringens* and *A. krisagathra* were grouped in the Aristolochiineae clade with *A. indica* and *A. krisagathra* forming the sister taxons and sister clade to *A. tagala*. *A. indica*, *A. tagala* and *A. krisagathra* came under the Diplolobus section coming under the Old World and Podanthemum subsection. However, *Aristolochia* subsection showed splitting in the phylogenetic tree and were placed in different clades. *A. ringens* was placed in Haplolobus section coming under New World. *Thottea* was basal to the other taxa and is supported strongly (bootstrap value – 100).

The species pairs which showed zero distance were *A. gigantea* X *A. grandiflora*, *A. littoralis* X *A. grandiflora*, *A. cordiflora* X *A. grandiflora*, *A. littoralis* X *A. gigantea*, *A. reniformis* X *A. gigantea*, *A. cordiflora* X *A. gigantea*, *A. cordiflora* X *A. littoralis*, *A. macrophylla* X *A. manshuriensis*, *A. californica* X *A. manshuriensis*, *A. debilis* X *A. contorta*, *A. californica* X *A. macrophylla*, *A. tomentosa* X *A. macrophylla*, *A. tomentosa* X *A. californica*, *A. zollingeriana* X *A. pistolochia*, *A. cordiflora* X *A. reniformis*. These involved 15 of the 33 species studied and the species discrimination rate was 45.45%.

matK phylogeny

The matK sequences generated were aligned and the total aligned length forming the final data set including those of

outgroup species was 768 bp. The number of conserved sites was found to be 539. 218 variable sites were obtained in the dataset of which 140 were parsimony informative.

The distance matrix obtained using K2P model with 33 matK sequences showed an overall average interspecific distance of 0.0623. All the species were successfully discriminated by matK sequences.

The matK tree generated (Fig. 3) was much strongly supported with higher bootstrap values. The tree obtained was in congruence with the classification [15]. The subfamily Aristolochioideae divided into two tribes Bragantia (with *Thottea*) and Aristolochieae (with *Aristolochia*). *Thottea* treated as outgroup was basal to *Aristolochia* which is strongly supported. *Aristolochia* was found monophyletic and gave rise to two major clades representing two strongly supported lineages Isotrematinae and Aristolochieae as observed by Huber [1]. In matK phylogeny, the Isotrematiinae had two subclades representing *Endodeca* and *Isotrema*; and Aristolochiineae subdivided into *ParAristolochia* and *Aristolochia*. The subgenera *Aristolochia* was further divided into two, one with the series Thyrsciae. The other included the section Diplolobus and other members of *Gymnolobus* which was further divided into three, one with a member of series Pentandrae (*A. micrantha*), *Diplolobus* members and other *Gymnolobus* members. In the *Diplolobus* section the members of the subsections *Podanthemum* and *Aristolochia* was correctly placed forming two major clades of *Diplolobus*.

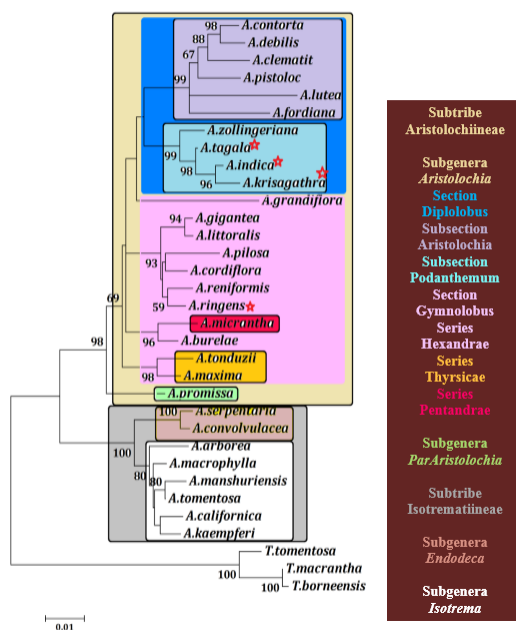


Fig 3: Neighbour joining tree of 33 plant species based on matK sequences generated from the present study)

The results also supported the sister group relationship of *Endodeca* and *Isotrema*; subgenera *ParAristolochia* and *Aristolochia* also showed sister group relationship and were found to be monophyletic. These results were in congruence with the earlier studies [15, 16, 18, 23, 24].

The *Aristolochia* clade was further divided into subclades forming section *Diplolobus* and *Gymnolobus*; the section *Diplolobus* with subsection *Podanthemum* and subsection *Aristolochia*. *Podanthemum* and *Aristolochia* showed sister relationship and found monophyletic as in previous studies [17, 23]. All the species studied were fully resolved using matK

phylogeny.

The phylogenetic analysis using *rbcL* and *matK* core barcodes revealed *matK* to be more useful in resolving species compared to *rbcL* as the natural groups were clustered together mostly. Moreover, all the species were fully resolved using *matK*. *matK* tree was more strongly supported with the bootstrap values. *matK* also showed a higher number of parsimony-informative characters in comparison to *rbcL*. On comparing the phylogenetic trees, placing *ParAristolochia* within the subtribe *Isotrematiinae* by using the *rbcL* phylogeny

was a major drawback. However, matK phylogeny almost resembled the pattern of classification as proposed by Stevenson and Gonzalez [15], than with the classification in which *Aristolochia* s.l. is deemed to be one large genus with three subgenera [10, 15, 19, 20]. Both the rbcL and matK phylogenies made clear distinction between *Endodeca* and *Isotrema*; stated monophyletic origin, with strong support. The subgenus *ParAristolochia* is the most primitive group in *Aristolochia* s.l. because of its morphological similarity to *Thottea* s.l. [10]. The subgenera *Isotrema* was placed as the primitive group. In rbcL and matK phylogenies, *A. krisagathra* was placed as the sister taxon to *A. indica* with strong support.

Conclusion

The analysis showed that *A. indica*, *A. tagala* and *A. krisagathra* formed the Podanthemum subsection under the section Diplolobus. They also showed a monophyletic origin and are also evident from the morphology as *A. krisagathra* is often allied to *A. indica* and *A. tagala*. *A. ringens* formed a part of the Gymnolobus section. All the four species formed a part of *Aristolochia* subgenera under the subtribe Aristolochiineae.

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