ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Molecular Systematics of Indian *Salacia* based on ITS Sequences of nrDNA

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Manuscript Info

Manuscript History:

Received: 15 May 2015
Final Accepted: 22 June 2015
Published Online: July 2015

Key words:

Salacia, Internal Transcribed Spacer, Hippocrateaceae, Species, Generic status

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Abstract

The nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) region was characterized in eight species of *Salacia* L. from Kerala, South India employing ITS 1 and ITS 2 primers. The divergence of the New World group of *Salacia* might imply the necessity of a separate generic status. Specific delimitations between the GenBank accessions and the Indian sampling of *Salacia chinensis* were ambiguous. Resolution inside the Indian clade of *Salacia* was not strong and the morphological species demarcation did not corroborate with the ITS phylogeny.

INTRODUCTION

Salacia L., is a genus of tropical climbing shrubs comprising about 200 species world over (Dandy, 1969; Mabberly, 2005), of which 21 have been reported from India and eleven from Kerala (Singh *et al.*, 2000; Sasidharan, 2004; Ramamurthy and Venu, 2005; Udayan *et al.*2012,2013; Sujana *et al.* 2015). Ding Hou (1964) noted that the number is difficult to estimate because of different opinions on generic delimitation, and gave the rough estimates of 29 in New World, 90 in Africa, and 29 in Malesia. The paraphyletic genus was formerly placed under the Hippocrateaceae, but is considered under the major family Celastraceae at present, as the former Hippocrateaceae is now considered to be nested within the present Celastraceae (Simmons *et al.* 2001a,b; Stevens, 2001 onwards). The genus includes small trees or lianas with opposite/subopposite leaves, fascicled/panicled flowers with intrastaminal conical disk and drupaceous and subglobose fruits. The bark of root is golden yellow in colour.

Salacia is distributed mostly in the tropical areas of the world including India, Sri Lanka, Southern China and other Southeast Asian countries such as Thailand, Indonesia, and also in South American Torrid Zone areas such as Brazil (Jayaweera, 1981; Matsuda *et al.* 2005; Wang *et al.* 2011).In India the members are confined mostly to the forests of Eastern and Western Peninsula, Meghalaya and in the Central India up to the 700 m above sea level. In South India, it is distributed in Karnataka (rare in semi-evergreen forests of Western Ghats) and Kerala (coastal forests of Kollam and the Southern Western Ghats of several districts).

S. reticulata, commonly known as *Ekanayaka* (Sanskrit) or *Ponkoranti* (Malayalam), is widely used in Ayurvedic system of Medicine as an important medicinal plant. The roots are utilized in traditional medicine for the treatment of inflammations, leprosy, skin diseases, colic, rheumatism and also for liver disorders. The plant is also reported to have anti-diabetic, antimicrobial and antioxidant activities. The primary constituent responsible for the antihyperglycemic properties of *Salacia* is salacinol, an α-glucosidase inhibitor. In Japan, the plant has been used as a supplementary food to prevent obesity and diabetes.

The eleven species of the genus reported from Kerala include *S. agasthiamalana* Udayan, Regy Yohannan & Pradeep *sp. nov.*, *S. beddomei* Gamble, *S. brunoniana* Wight & Arn., *S. chinensis* L., *S. fruticosa* Heyne ex Lawson, *S. macrosperma* Wight, *S. malabarica* Gamble, *S. oblonga* Wall. ex Wight & Arn., *S. reticulata* Wight, *S. vellaniana* Udayan, Yohannan & Pradeep *sp.nov.*, and *S. wayanadica* Sujana, Nagaraju, Ratheesh & Anil Kumar (Singh *et al.*, 2000; Sasidharan, 2004; Ramamurthy and Venu, 2005;Udayan *et al.* 2012,2013; Shareef and Santhosh Kumar, 2013; Sujana *et al.* 2015). Of these, the occurrence of *S. reticulata* in India is currently doubtful (Udayan and Pradeep, 2012). The Foundation for Revitalization of Local Health Traditions (FRLHT), Bengaluru, have listed *S. oblonga* as vulnerable and *S. reticulata* as endangered in Karnataka, but have shown that data from Kerala was deficient (Ravikumar and Ved, 2000).

Although the chloroplast gene *rbcL* has been utilized for tackling plant phylogenetic controversies at higher taxonomic levels (Chase *et al.*1993; Qui *et al.* 1998), the *nr*DNA ITS region is being increasingly depended upon to resolve inter-generic and inter-specific phylogenetic concerns (Baldwin, 1992; Soltis *et al.* 1996). The internal transcribed spacers (ITS) of the ribosomal DNA repeats, ITS 1 and ITS 2 regions have been successfully engaged in the phylogenetic studies of several genera including *Peonia* (Sang *et al.* 1995), *Kalmia, Leiophyllum, Loiseleuria*etc. (Kronand King, 1996). This region is fast-evolving and serves as a method of direct DNA sequence analysis for detecting reticulate phenomena which would be appropriate for subgeneric or subspecific cladistic studies (Gaut *et al.* 2000).

Simmons *et al.* (2001b) studied the phylogeny of the Celastraceae using 26S nuclear ribosomal DNA, phytochrome B, *rbcl*, *atpB* and morphological markers. They have opined that "*Salacia*, as currently defined, is not supported as a natural group and needs to be broken up into additional segregate genera, or the segregate genera need to be reduced to *Salacia*". Although eleven species of *Salacia* have been reported from Kerala, only eight are correctly identified – the status of the remaining three as well as the genetic relationship between all the eight species remains to be addressed using molecular tools. It is in this context that the present study is initiated to gather information regarding the systematic and phylogenetic relationships between the members of *Salacia* from Kerala using ITS markers and to compare these observations with the molecular data on taxa belonging to same or related genera from other parts of the world.

Materials and Methods

Taxon sampling

Eight species of *Salacia* L.from Kerala, South India, corresponding to 4% of the total 200 species of the genus reported globally, were included in the present study. Author citations are given in Table-1 and are from here onwards not included in the text. These eight species of *Salacia* obtained by the complete sampling of the Kerala state, together represent one-third of the Indian members and reflect almost the entire morphological diversity present within the genus. Majority of them are endemic to the Western Ghats of Kerala except *S. chinensis* and *S. oblonga*. The voucher specimens of the taxa collected have been deposited in SNCH and MH herbaria. Species of the genera *Pristimera, Apodostigma, Hippocratea, Helictonema, Tontelea, Peritassa* and *Cheiloclinium* were also included in the analyses, since according to previous works (Simmons *et al.* 2001a,b; Coughenour *et al*. 2010,2011), the genus *Salacia* is non-monophyletic and hence splits into several clades leading to the necessity of including all the sister genera belonging to Salacioideae and Hippocrateoideae. *Lophopetalum* and *Kokoona* species are included as outgroups following the work of Robson (1965), Savolainen *et al.* (2000) and Simmons *et al.* (2001b). A total of 43 DNA sequences representing the main Salacioideae and Hippocrateoideae were included in the analyses.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Amsterdam, The Netherlands). DNA amplification and sequencing of the ITS region was performed using the primers ITS 1 and ITS 2 (White *et al.* 1990). The polymerase chain reaction (PCR) was performed with standard methods using AccuPower HF PCR premix (Bioneer, Daejeon, Korea) in 20 µl volumes containing 2 µl of 10X buffer, 300 µMdNTPs and 1 unit of HF DNA polymerase. To this 1 µl of a 10 pM solution of each primer, 1 µl of genomic DNA and distilled water were added to make a total volume of 20µl. PCR amplification was performed with 40 cycles (denaturation for 1 min at 94°C, annealing for 1 min at 49°C and 1 min of extension at 72°C followed by a last cycle of final extension for 5 min at 72 °C). PCR products were checked for the presence of appropriate bands on a 0.8 % agarose gel, purified,

and sequenced at Ocimum Biosolutions, Hyderabad, India and Macrogen, Korea. Sequences comprised of ITS 1, 5.8S and ITS 2 regions. Forward and reverse sequences were edited and assembled using the computer program Codon-Code Aligner vers. 3.7.1 (2002–2009, Codon code corp.) and DNA Baser (vers. 3) (2011). All sequences will be deposited in GenBank (Table 2). The experimental work related, with the DNA extraction, amplification and sequencing were carried out in the Plant Systematics Laboratory, Department of Botany, University of Delhi.

Phylogenetic analyses

A total of 43 nucleotide sequences (including all outgroups) were aligned using Clustal X vers. 2.0.11 (Thompson *et al.* 1997) followed by manual adjustments in Clustal W (Thompson *et al.*1994) and Mesquite (v. 2.72). All positions containing gaps and missing data were eliminated. Phylogenetic analyses were done using Bayesian (Maximum Posterior Probability, MPP), Maximum Parsimony (MP), and Neighbor-Joining (NJ) methods. Bayesian analyses were done using Mr Bayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The best-fit model was determined using Model Test vers. 0.1.1 (Posada, 2008; Guindon and Gascuel, 2003) by the Akaike Information Criterion (AIC) and the maximum likelihood method. It was found to be GTR+G with the lowest AIC score and highest log-likelihood score. Parameters for the evolutionary model were set to default and the state frequency parameter for stationary nucleotide frequency of the rate matrix was fixed. The number of chains was set to four with three heated and one cold chain. Two runs were executed in parallel. Analyses were run for 500,000 generations until stationarity (Standard deviation below 0.01). In each run, trees were sampled every 100 generation with a sample frequency of 100. The parameters were summarized after excluding 25% of the samples (sump burnin command) based on the inspection of log-likelihoods of sampled trees after stationarity. The Potential Scale Reduction Factor (a convergence diagnostic) approached 1.0 for all the parameters suggesting good sampling from the posterior probability distribution with no spread. Trees were summarized by the sumtburnin command yielding a cladogram showing posterior probabilities and clade credibility for each split and a phylogram with mean branch lengths. The following criteria were used to evaluate the pp's: 0.50–0.80, low; 0.81–0.94, moderate; 0.95–1.0, strong.

Phylogenetic analyses by MP (Fitch, 1971; Swofford, 1996) and NJ (Saitou and Nei, 1987) methods were conducted using MEGA vers. 5 (Tamura *et al*. 2011). The best-fit molecular model was GTR+G, but because the parameters required to set this model were not available in MEGA5, the nearest model, i.e., Kimura-2-parameter was used to compute pairwise distances for NJ analyses. The MP analyses were done using the close-neighbor-inter-change algorithm with search level one in which ten initial trees were obtained with the random addition of sequences (10 replicates). Positions containing gaps and missing data were eliminated from the dataset (complete deletion option) in both MP and NJ analyses. Relative support for the clades recovered was assessed via bootstrap analyses using 10,000 replicates in both analyses. The following criteria were used to assess bootstrap support percentages (BP): 50–70 %, low; 71–84 %, moderate; 85–100 %, strong. The results of MP and NJ analyses are congruent with those from Bayesian analyses.

Maximum likelihood (Felsenstein, 1973) analyses of nucleotide characters from each of the molecular data matrices were performed as not infallible (Gautand Lewis, 1995; Siddall, 1998; Sanderson and Kim, 2000) tests for long-branch attraction (Felsenstein, 1978). Likelihood analyses were conducted using RaxMl v. 3.1 (Silvestro and Michalak, 2012). Optimal likelihood trees were searched for using 1000 independent searches starting from randomized parsimony trees with the GTR+GAMMA model and four discrete rate categories and a 1000 bootstrap replicates. Likelihood BS analyses were conducted with at least 1000 replicates with ten searches per replicate and the bipartitions tree was analysed in Figtree v 1.3.1 (Rambaut, 2010).

Observations

Characteristics of the ITS region in Salacioideae and Hippocrateoideae

The multiple alignment of the ITS region (with 5.8S) comprised a total of 883 sites including INDELS. Of the total aligned sites, the ITS1 region contained 183 variable and 194 conserved sites, while the ITS2 region contained 209 variable and 111 conserved sites; 152 and 173 sites were potentially informative in ITS1 and ITS2 regions respectively. The 5.8S region is similar in size (~160bp) in all the taxa with no gaps involved to align the sequences. A high proportion of sites are conserved in 5.8S region (77.5 %). The overall mean pairwise distance is 0.105.

In the Bayesian analyses, a total of 11,000 trees were obtained from both the runs with 5,500 in each run. The 50% majority rule consensus trees resulting from the two searches are similar in topology (MPP tree) (Text

Fig.1). The MP analyses recovered 248 equally most parsimonious trees with a tree length (TL) of 656, a consistency index (CI) of 0.59 and a retention index (RI) of 0.87. The bootstrap support for each clade is shown behind each branch in the ML tree, respectively (Text Fig.1). Similar results were obtained from the NJ analyses and MP and Bayesian analyses. The results from Maximum Likelihood analysis are described below, with remarks regarding parsimony, Bayesian and Neighbor joining analyses wherever necessary.

Salacia as a genus is non-monophyletic and is split into two major clades: A (41BS) and B (100 BS) (indicated in Text Fig.1). Clade B consists of all *Salacia* and clade A consists of *Salacia* with sister genera*Tontelea*, *Peritassa* and *Cheiloclinium*. The Indian members (55 BS) form a part of Clade B.

The species of *Salacia*, along with *Tontelea cylindrocarpa* were unambiguously resolved as a polyphyletic group (48 BS, Text Fig.1), with a poorly supported sister clade consisting of the species of *Peritassa* (100 BS) and which is in turn sister to *Cheiliclinium* (100 BS). The whole clade is sister to *S. arborea* (33 BS) and *S. nitida* (45 BS). *S. arborea* is a Brazilian species with crenate-serrate lamina, contracted or very short 2-4-flowered inflorescences with large flowers, measuring 1-1.5 cm across. The limited number of flowers on a very short inflorescence distinguishes this species from other members of the genus (Smith, 1940). The Indian members (except one) of *Salacia* form a moderately supported clade (55 BS) with one member viz., *S. agasthiamalana* sister to *S. chinensis* (98BS) and nesting out of the Indian clade.

In clade A, *S. elliptica* forms a sister group to *S.crassifolia* (94 BS). These two species form a sister group to *S. alwynii* and *S. grandiflora* (100 BS).

In clade B, there are three moderately supported sub-clades, one of them (pink colored), representing entirely the Indian collections, rest two sub-clades representing the GenBank collections and the non-Indian members (green and blue color). *S. chinensis* sampled from India forms a clade with other members viz., *S. oblonga*, *S. beddomei*, *S. vellaniana*, *S. macrosperma* etc. *S. chinensis* retrieved from GenBank forms a clade with *S. agasthiamalana* (80 BS).

The outgroups (*Kokoona* and *Lophopetalum*) make the subfamily Salacioideae and Hippocrateoideae strongly monophyletic. Some species of *Salacia* form a part of Salacioideae – clade A and others a part of clade B. The Hippocrateoideae form a well-supported sister group to the Salacioideae subfamily.

Table: 1 - Plant accessions, along with author citations used for the molecular systematic study of Indian *Salacia*, related genera and the out-groups along with their distributional status, collection area and GenBank sequence

S.No.	Taxa	Voucher number	Collection locality	Distribution/Status	GenBank sequence
1	*Salacia agasthiamalana* Udayan, Regy Yohannan & Pradeep sp. nov.	S009 SNCH	Pongalppara, Agasthiamala	Endemic to Western Ghats of Kerala	We will deposit sequences in GenBank
2	*Salacia beddomei* Gamble	S001 SNCH 171688 MH	Aralam	Endemic to Western Ghats of Kerala	
3	*Salacia chinensis* L.	S006 SNCH	Vandanam , Alappuzha	Indo-Malesia	
4	*Salacia fruticosa* Heyne ex Lawson	S002 SNCH 171689 MH	Nedumangad, Thiruvananthapuram	Endemic to Western Ghats of Kerala	
5	*Salacia macrosperma* Wight.	S003 SNCH 171690 MH	Kakayam	Endemic to Western Ghats of Kerala	
6	*Salacia malabarica* Gamble	S004 SNCH 171691 MH	Kuruva Island, Wyanad	Endemic to Western Ghats of Kerala	
7	*Salacia oblonga* Wall.ex. Wight & Arn.	S005 SNCH 171692 MH	Kakkayam	India and Sri Lanka	
8	*Salacia vellaniana* Udayan, Yohannan & Pradeep sp. nov.	S008 SNCH	Vellanipacha, Thrissur	Endemic to Western Ghats of Kerala	

Sequences retrieved from Gen Bank					
9	*Salacia krigsneri*	JA Lombardi 6687(HRCB)	Linhares,Brazil	South America	HM230112
10	*Salacia cordata*	JA Lombardi 6352 (HRCB)	Brazil	South America	FJ705521
11	*Salacia grandiflora*	JA Lombardi 6851 (HRCB)	Brazil	South America	HM230111
12	*Salacia elliptica*	JA Lombardi 6121 (HRCB)	Brazil	South America	FJ705524
13	*Salacia crassifolia*	JA Lombardi 6206 (HRCB)	Brazil	South America	FJ705522
14	*Salacia alwynii*	JA Lombardi 6333 (HRCB)	Brazil	South America	FJ705520
15	*Salacia arborea*	MW Chase 2096 (K)	Bogor, Indonesia	South East Asia	FJ705531
16	*Salacia nitida*	J Munzinger & Karomoko 14 (BH)	Ivory Coast	West Africa	FJ705528
17	*Salacia chinensis*	AJ Ford 5550 (BRI)	Australia	Australia	HM230110
18	*Salacia erythrocarpa*	AJ Ford 5000 (BRI)	Australia	Australia	FJ705525
19	*Salacia disepala*	WWC 1896 (BRI)	Australia	Australia	FJ705523
20	*Salacia gerrardii*	RH Archer 2121 (PRE)	South Africa	South Africa	FJ705526
21	*Salacia madagascariensis*	RH Archer et al. 2919 (CS)	Madagascar	South East Africa	FJ705527
22	*Salacia* aff *obovata*	RH Archer et al. 3791	Madagascar	South East Africa	JX203483
23	*Salacia* sp.	RH Archer et al. 3040 (CS)	Madagascar	South East Africa	FJ705530
24	*Salacia owabiensis*	J Munzinger & Karomoko 17 (BH)	Ivory Coast	West Africa	FJ705529
Related genera					
25	*Cheiloclinium belizense*	J Meave & A Howe 1435 (MO)	Belize	Central America	FJ705510
26	*Cheiloclinium cognatum*	JA Lombardi 6238 (HRCB)	Brazil	South America	FJ705511
27	*Tontelea cylindrocarpa*	C Feuillet et al 10191 (F)	French Guiana, France	South America	FJ705534
28	*Peritassa hatschbachii*	JA Lombardi 7015 (HRCB)	Brazil	South America	FJ705516
29	*Peritassa laevigata*	JA Lombardi 6532 (HRCB)	Brazil	South America	FJ705518
30	*Peritassa campestris*	JA Lombardi 6210 (HRCB)	Brazil	South America	FJ705515
31	*Tontelea micrantha*	JA Lombardi 6442 (HRCB)	Brazil	South America	FJ705535
32	*Helictonema velutinum*	TB Hart 1580 (MO)	Zaire, Congo	Central Africa	HM230126
33	*Pristimera nervosa*	J Schunke 4073 (MO)	Peru	Western South America	HM230144
34	*Hippocratea volubilis*	JF Castrejon et al	Mexico	North America	HM230127

		949 (MO)			
35	*Pristimera preussii*	D Harris 4969 (E)	Central African Republic	Central Africa	HM230145
36	*Apodostigma pallens*	Sango et al. ML-168 (K)	Mali	West Africa	HM230115
Outgroups					
37	*Kokoona* sp.	MW Chase 2092 (K)	Bogor, Indonesia	South East Asia	FJ705512
38	*Lophopetalum arnhemicum*	W Price s.n. (BRI)	Australia	Australia	FJ705514

Discussion

The Celastraceae *sensu lato*, (including Hippocrateaceae *de Jussieu*, 1811), commonly referred to as the bitter-sweet family or staff-tree family, are largely a pantropical family of herbs, woody lianas, shrubs, and trees with a Gondwanian distribution. The wide variation observed in stamen, fruit, and seed characters of the members has been used to subdivide the family taxonomically. The family includes several economically important taxa such as *Catha edulis* (*Kat* or *Khat*) which contains cathinone in its tender leaves which upon chewing induces amphetamine-like effects in humans (Hassan *et al.* 2002). They are also drunk as tea for their stimulant properties in northeastern Africa, the Arabian Peninsula, and Madagascar (Getahun and Krikorian, 1973). *Euonymus*, *Celastrus* and *Paxistima*, are widely cultivated as ornamentals. Members of the family exhibit substantial variation in stamen, fruit, and seed characters that have been used as the main bases for subdividing the family taxonomically.

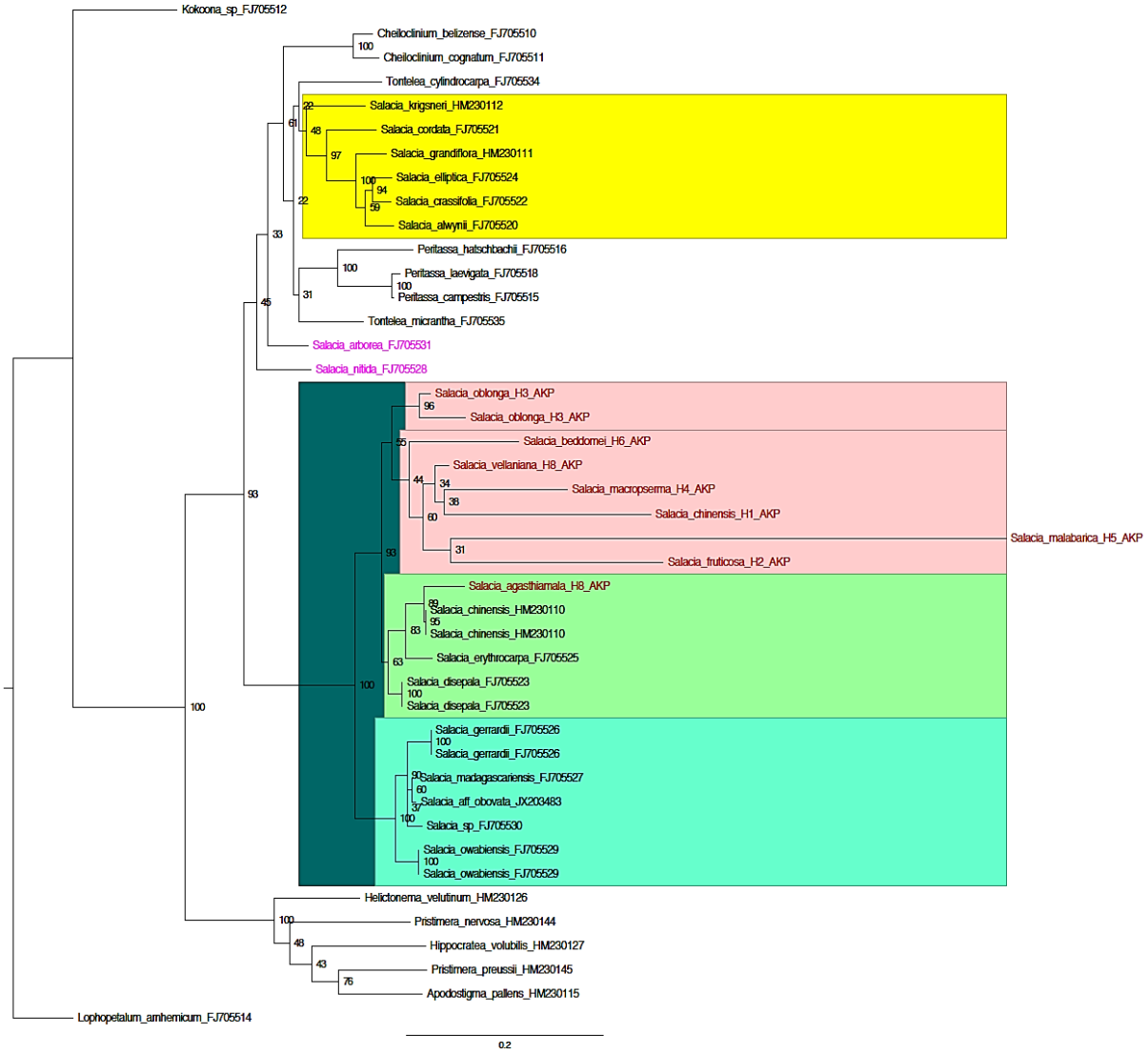
The paraphyletic ex-family Hippocrateaceae differs from the Celastraceae on account of its three stamens (rarely 2-5) *vs.* four or five (rarely 10) in the latter, filaments inserted inside the disk *vs.* at or below the margin of the disk, filaments often basally fused and recurved *vs* .free and incurved, and seeds exalbuminous *vs.* albuminous (Bentham and Hooker, 1862; Cronquist, 1981).Several others argued that the two families need to be merged as they differed from each other only in the place of insertion and number of the stamens (Smith and Bailey, 1941; Losener, 1942a,b). The Hippocrateaceae is now included in the Celastraceae as two sub-families: Salacioideae and Hippocrateoideae (Simmons and Hedin, 1999; Simmons *et al*., 2001a,b).Recent studies employing morphological and molecular markers corroborate this inclusion (Savolainen *et al.* 1997; Simmons and Hedin, 1999; Chase *et al.* 2000; Soltis *et al.* 2000; Simmons *et al.* 2001a,b; Simmons *et al.* 2009a,b; Coughenour *et al*. 2010,2011).

Simmons *et al.* (2001a,b) based on molecular cladistic analyses of the Celastraceae, came to a conclusion similar to the findings of Simmons and Hedin (1999) based on morphological data. In all these studies, the Salacioideae and the Hippocrateoideae were resolved as monophyletic groups independently. But the two subfamilies of the ex-Hippocrateaceae when considered together, did not resolve as a monophyletic group, and instead nested separately within Celastraceae. Thus the Hippocrateoideae and Salacioideae are a polyphyletic group derived from independent lineages of Celastraceae *sensu stricto*. This also exposes the artificial nature of Halle (1986, 1990)'s tribes. Halle had reduced the Salacioideae to a single tribe – the Salacieae and included it under the Hippocrateoideae. So at present, the Salacioideae exist as a separate subfamily in the Celastraceae (although not formally recognized yet as per Simmons and Hedin, 1999) and includes a single tribe - the Salacieae with six genera and 265 species (Coughenour *et al.* 2010, 2011).

Most species in Salacioideae are lianas with revolute stems or, more rarely, bushes or trees with opposite (less commonly, alternate) leaves; small, dichlamydeous, tetrameric or seldom pentameric and monoclonal flowers; bacciform fruits; and non-endospermic seeds (Smith, 1940). Salacioideae and Lophopetaleae, together with *Brassiantha* and *Sarawakodendron*, have also been inferred to be closely related in recent phylogenetic analyses. Using morphological characters, Simmons and Hedin (1999) inferred the relationships ((*Kokoona, Lophopetalum*) (*Brassiantha*((*Dicarpellum, Sarawakodendron*) ((Hippocrateoideae), (Salacioideae))))). Savolainen *et al.* (2000) inferred ((Hippocrateoideae) (*Lophopetalum, Salacia*)), but this inference of *Lophopetalum* and *Salacia* as more closely related than either is to Hippocrateoideae. It is confounded by problematic identification of the *Lophopetalum* voucher specimen (see methods section below) and is therefore doubtful. This problematic identification also underlies the inference by Simmons *et al.* (2001 b) that *Lophopetalum* is more closely related to Salacioideae than it is to *Kokoona*. Within Salacioideae, Simmons and Hedin (1999) inferred the relationships ((*Salacia*) ((*Cheiloclinium*), *Peritassa, Tontelea*)), albeit with Bremer (1988) support of only one for each clade. Simmons *et al.* (2001a,b) did not infer any relationships within Salacioideae with ≥50% bootstrap support.

Text Fig.1- Maximum Likelihood tree showing the bootstrap support along the branches with 1000 replicates and using GTR+G model of substitution

(Colors in the clade indicate the geographic distribution of the personal Indian collections and those of the Gen Bank accessions. Colors of the taxa (red) indicate the Indian sampling from personal collections with their voucher numbers)



The Hippocrateoideae form a recognizably supported clade (100 BS) for which synapomorphies include presence of an annulus in the form of an internal margin around the pollen aperture (Lobreau-Callen, 1977), capsular fruits that part into mericarps and presence of a non-arillate basal seed wing. Potential wood-anatomy synapomorphies for Hippocrateoideae are thick bark often extending into the central wood, wide rayswith some greater than ten cells wide, absence of included phloem and loss of parenchyma like bands of thin- walled septate wood fibers (Mennega, 1997). This resolution of Hippocrateoideae has been supported in most phylogenetic studies which followed, thereby confirming the monophyly of Hippocrateoideae (Simmons and Hedin, 1999; Savolainen *et al.*, 2000; Simmons *et al.*, 2001a,b; Coughenour *et al.* 2011). Fitch (1971) reported that the Hippocrateoideae is of Old World origin and this was followed by 3–5 successful radiations within the New World. This inference was confirmed by Coughenour *et al.* (2011) who did not observe any reversal from the New World to the Old World.

Coughenour *et al.* (2010) had suggested that the Old World origin of Hippocrateoideae is consistent with the Old World origin of Salacioideae. The strongly supported monophyly of Salacioideae observed in the present study (Text Fig.1) corroborates results of previous phylogenetic analyses (Simmons and Hedin 1999; Simmons *et al.* 2001a,b; Coughenour *et al.* 2010) with the greater character and taxon sampling. Synapomorphies for Salacioideae include indehiscent drupaceous fruits with putative arils (or sarcotesta) in the form of mucilagenous pulp, thin bark, narrow rays, parenchyma-like bands of septate fibres and included phloem as concentric bands or isolated strands (Mennega, 1972,1997). Despite this monophyly, the generic delimitation of Salacioideae is at present a matter of much debateas there are conflicting reports ranging from one genus, *Salacia* (Peyritisch, 1878; Robson, 1965), to the current six genera (Mennega, 1997).

The Old World species of *Salacia* are known only from Asia and Africa. So, Miers (1872) omitted the term *Salacia* in his study on the New World American species of *Salacia* and included them under *Raddia*. But Smith (1940) failed to notice any dissimilarity between the 29 American species of *Raddia* studied by him and the Old World species of *Salacia*. Hence, he merged Mier's new world *Raddia* with the traditional Old World *Salacia.* But Ding Hou (1963) noted that the segregate genera of *Salacia* recognized by Smith (1940) were not ". . . based on fundamental characters." For example, *Dicarpellum*, originally described as a subgenus of *Salacia* (Loesener, 1907), was raised to the generic level by Smith (1941) who noted that the genus is not closely related to *Salacia.* Simmons and Hedin (1999)also made a similar observation in a cladistic analysis on 82 taxa belonging to Celastraceae*s.l.*, based on 69 characters from gross morphology, seed anatomy, seedling development, leaf anatomy, wood anatomy, pollen morphology and karyotype. This was supported by the 26S nrDNA tree and the *phyB* tree obtained from the molecular analyses conducted by Simmons *et al*. (2001a,b). Consequently, they observed that "*Salacia*, as currently defined, is not supported as a natural group and needs to be broken up into additional segregate genera".

The genus *Salacia* is non-monophyletic and the position of clade A members, the New World ones, seems to be uncertain and hence the genus needs a taxonomic revision and reinstating certain members to a new genus/sub-genera, as done by Smith (1940) or adding them to the existing ones. The two polytomies within Salacioideae and our under sampling of *Salacia* (8 of about 200 species) complicate any efforts to recircumscribe *Salacia* into natural segregate genera.

The results from the current study corroborate with the previous works of Coughenour *et al.* (2010) on Salacioideae, with two distinct New world and Old world groups. The Indian members of the genus form a part of Old World, as expected. The morphological similarities which retain the Old World as a strong supported clade are:opposite coriaceous glabrous leaves, canaliculated petioles, small, fascicled or cymose flowers, three stamens with reflexed filaments often broadened towards the base, inserted on the intrastaminal, fleshy disk, 3-celled ovary partly or fully sunken in the disk with 2-8 axile ovules, simple umbonate stigma, baccate, sub-globose or elliptical fruits with seeds embedded in mucilaginous pulp.

None of the Indian members seem to share a strong support among itself, indicating a common origin and diversification of the common ancestor of the old world. The Indian clade is sister to the clade consisting of *S. agasthiamalana*, *S. chinensis*, *S. erythrocarpa* and *S. disepala* all of them sharing synapomorphies *viz*. woody shrubby or scandent habit, tendril-tipped branches, grooved petioles, inconspicuous stipules, fleshy intrastaminal disk and baccate fruits. But these characters including others are shared by most other members of the genus. *S. agasthiamalana* and *S. chinensis* are sisters and share several characters, again in common with many other members of the group. The divergence observed between Indian clade and its sister clade might be attributed largely to differences at the molecular level, since they share most of the major characteristics of the group. *S. chinensis* sampled from Kerala state forms a non-monophyletic group with *S. chinensis* retrieved from GenBank. Possibly, either the identification of the GenBank accession is ambiguous or the Indian *S. chinensis* has to be reinstated to a new species.

Conclusions

It is evident from the present study that *Salacia* is non-monophyletic and the New World group needs to be segregated into a separate genus. GenBank identity of *S. chinensis* is ambiguous or the Indian sampling of *S. chinensis* is supposedly a different species. Resolution inside the Indian clade of *Salacia* is not strong and hence nothing concrete can be concluded about the relationships within the Indian clade. The morphological characters used for delimiting the species are not corroborating with the ITS phylogeny. Analysis using more markers and sampling at a broader scale is needed to achieve a clearer resolution in the Indian *Salacia* clade.

Acknowledgements

The authors are thankful to the University of Kerala for financial support and the Principals, Sree Narayana Colleges, Kollam and Chempazhanthy for facilities provided.

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