

# An exploration of the phylogeny and phylogeographic relationships of the subfamily Salacioideae

Shrisha Naik Bajpe<sup>1</sup>, Kuppuru Mallikarjunaiah Marulasiddaswamy<sup>2</sup>, Manu G<sup>3</sup>, Abhijeeth S Badiger<sup>1</sup>, Ramith Ramu<sup>4</sup>, Maruthi Katenahally Rudrappa<sup>5</sup>, Kukkundoor Ramachandra Kini<sup>2\*</sup>

<sup>1</sup>Department of Biotechnology, Sri Dharmasthala Manjunatheshwara College (Autonomous), Ujire, Karnataka, India.

<sup>2</sup>Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysuru, Karnataka, India.

<sup>3</sup>Department of Pharmacology, JSS Medical College, JSS Academy of Higher Education and Research, Mysuru, Karnataka, India.

<sup>4</sup>Department of Biotechnology and Bioinformatics School of Life Sciences JSS Academy of Higher Education and Research (Deemed to be University) Sri Shivarathreshwara Nagara, Mysuru, Karnataka, India.

<sup>5</sup>Department of Botany, Sri Dharmasthala Manjunatheshwara College (Autonomous), Ujire, Karnataka, India.

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## ABSTRACT

Salacioideae is one of the subfamilies of the family Celastraceae. *Salacia* is a rare, endangered, and threatened genus found in India. *Salacia* is one of the Western Ghats' endemic species. *Salacia* species segregation and delimitation are difficult to achieve just based on taxonomic identification. The current research is an attempt to conduct a complete investigation into molecular phylogeny, estimation of age, and geographical implications of *Salacia* species. *Salacia* sp. Internal transcribed spacers (ITS), *matK*, and *rbcL* sequences from the Western Ghats of Karnataka, Kerala, and sequences from GenBank were utilized for the present study. Bayesian inference analysis (BI) and maximum likelihood (ML) to construct a phylogenetic tree using MrBayes and RAxML software. BI tree was used to calculate the divergence age of the *Salacia* species. Utilizing RASP v.2.0b geographic inferences were analyzed. A Well resolved phylogenetic tree was observed in the ITS and ITS + *matK* + *rbcL* concatenated sequence of the old world (OWC) and new world (NWC) samples forming two major clades. However, a poorly resolved phylogenetic tree was observed in the *matK* + *rbcL* sequences. The crown age of OWC and NWC was 9.82 Ma and 17.4 Ma, respectively. Madagascar and East Africa *Salacia* species had common tip node age of 3.13 Ma. Phylogeographic analysis suggests a total of 50 dispersals and 25 vicariance events. Phylogeographic analysis reveals the *Salacia* species could have originated from Southeast Asia. The endemism and diversity of *Salacia* species in the Western Ghats are explained by the fact that *Salacia* species from various geographic regions were positioned discordantly in the phylogenetic trees, demonstrating a high level of sequence similarity and congruence.

## 1. INTRODUCTION

The six genera *Cheiloclinium*, *Peritassa*, *Salacia*, *Salacighia*, and *Thyrosalacia Tontelea* make up the Salacioideae family. Salacioideae have bisexual, three stamens, pentamerous flowers with an extrastaminal disc, and fruits which are berries with arillate mucilaginous pulp. [1]. *Salacia chinensis* (SC) is commonly known as Sapta chakra and *S. reticulata* (SR) as Ekanayakam in Kannada. It is distributed in Dry Deciduous, Evergreen Forests of the Western Ghats of Kerala, Karnataka, Tamil Nadu, and also found in forests of Andhra Pradesh, Northeast India [2]. *Salacia* sp. is used in Ayurvedic medicine to treat diabetes. SR, *S. macrosperma* (SM), *S. oblonga* (SO) along with other

plants such as *Gymnema sylvestre*, *Curcuma longa*, *Azadirachta indica*, *Aegle marmelos*, *Emblica officinalis*, and natural sweetener *Stevia rebaudiana* is used in the preparation of the polyherbal medicines such as Diajith churna [3], Rajanyamalakadi [4], and Madhujeevan Churna [5]. *Salacia* species has anti-inflammatory, antimutagenic, nephroprotective, hypolipidemic, antimicrobial activity [6] antitumor, antiviral (H1N1, HIV) [7,8], and immunomodulatory activities. The Western Ghats have a wide range of climatic and topographic variability, which supports a broad range of plant types and animals. The endemism of the Western Ghats' flora and fauna has been widely documented. The moist zones of the Western Ghats have a greater level of endemism than the dry zones [9].

The previous studies of the Salacioideae, *Salacia* supported monophyly [10]. Salacioideae have an Old-World origin and within New World, Salacioideae have undergone single successful radiation. In the previous study, *Salacia* samples from Central-South East Asia and other parts of Asia were excluded except for *Kokoona* which is

\*Corresponding Author:

Kukkundoor Ramachandra Kini,

Department of Studies in Biotechnology, University of Mysore,  
Manasagangotri, Mysore – 570006, Karnataka, India.

Email: [krk@appbot.uni-mysore.ac.in](mailto:krk@appbot.uni-mysore.ac.in); [shrishanaik@gmail.com](mailto:shrishanaik@gmail.com)

sampled from Sri Lanka. The morphological characters and sequences from *phyB*, 26S, 18S, internal transcribed spacers (ITS), *matK*, *trnL-F*, *atpB*, and *rbcL* have been used to construct the phylogeny of the Celastraceae [11,12] and Salacioideae, Dev, Anoop [13] have used *rbcL*, *matK*, *trnH-psbA* and ITS2 region for species discriminate of *Salacia* samples Kerala. Genetic diversity of the four *Salacia* sp. SM, SC, SO, and *S. fruticosa* (SF) samples from the Karnataka region was analyzed using ISSR, RAPD, and ITS marker [14]. Using ITS2 sequence secondary structure the variation within the *Salacia* sp. sampled with the Western Ghats was assessed by Anaz, Sasidharan [15]. The current research focuses on the phylogeny and phylogeography of the subfamily Salacioideae.

## 2. MATERIALS AND METHODS

### 2.1. Sampling, DNA Extraction

SM, SC, SO, and SF samples were collected from the Western Ghats of Karnataka. The modified Stange, Prehn [16,17] method of DNA extraction was used. In this method, lithium chloride (LiCl<sub>2</sub>) and high concentration of sodium chloride (NaCl) selectively precipitates DNA eliminating the use of RNase in the extraction procedure. Furthermore, the polyphenolic complex is removed using Polyvinylpyrrolidone (PVP). DNA extraction buffer contains two solutions A and B. Solution A (100 mM Tris-HCL [pH 8.0], 20 mM EDTA [pH 8.0], 1.4 M NaCl, 4% PVP, 2% [w/v] CTAB, 2% - mercaptoethanol/DTT) and Solution B (4M LiCl<sub>2</sub>) which are mixed in a 1:1 ratio just before the extraction. 100-200 mg of sample was weighed, thoroughly washed and rinsed with alcohol to remove any adhering dirt.

Using liquid nitrogen, samples are crushed in a pestle and mortar. The crushed samples were transferred to a 1.5 mL microfuge tube containing 700 uL of extraction buffer and shaken well. Sample tubes were incubated for 30 min at 65°C while shaking intermittently in a heating block. After cooling, 700 uL of chloroform and isoamyl alcohol (24:1) were added to the samples. The tubes were thoroughly shaken and were then centrifuged at 12,000 rpm for 10 min at room temperature. The above step is repeated once more. To sample containing tubes, an equal volume of chilled isopropyl alcohol was added and incubated in -20 refrigerators for 1 h or was kept overnight. Tubes were centrifuged at 12,000 rpm for 10 min to obtain the DNA pellet. The supernatant was decanted, and the DNA pellet was washed with 70% alcohol. The pellet was air-dried and then the DNA pellet dissolved in 20–50 ul Tris<sub>10</sub>-EDTA<sub>1</sub> buffer.

ITS, *matK*, and *rbcL* region polymerase chain reaction (PCR), sequencing, and Homology Search were conducted as the method described by Bajpe *et al.* [18]. For PCR amplification ITS1-5.8s-ITS2 region, ITS4, and ITS5 primers, were used by White *et al.* [19], for the *matK* region, the primers 3FKIM and 1RKIM developed by Ki-Joong Kim were used, and for the *rbcL* region, the primers *rbcLa-F* and *rbcLa-R* were used Kress and Erickson [20]. ITS, *matK*, and *rbcL* sequences of Salacioideae genera *Cheilochlinium*, *Peritassa*, *Salacia*, and *Tontelea* were retrieved from the NCBI GenBank database. SM, SC, SO, and SF sequences of the Karnataka Western Ghats deposited in the GenBank repository are listed in Table 1.

### 2.2. Phylogenetic Analyses

MEGA 5 was used to build the sequences. Using the Mega 5 software's Model function, the model with the lowest Bayesian information criterion (BIC) was generated [21]. Bayesian inference analysis (BI) [22] and maximum likelihood (ML) [23] approaches were used to perform

phylogenetic studies. A Markov chain Monte Carlo (MCMC) technique, as implemented in Mr.Bayes, version 3.2.2, was used to perform the Bayesian analysis [24]. With a sampling frequency of 1000 iterations, four parallel MCMC runs were generated for a total of 10,000,000 iterations. The first 25% of samples were eliminated due to burn-in. The graphical interface in RAxML version 1.3 was utilized to do ML tree searches and bootstrapping (BS) of the data with 1000 repetitions [25].

Using the program Sequence Matrix, ITS, *matK*, and *rbcL* sequences were concatenated (Vaidya *et al.* 2011). MEGA 5 was used to determine genetic divergences for the three markers using the P-distance approach. The samples that exhibited n/c in the distance analysis for concatenated sequences were eliminated. To determine whether the ITS, *matK* and *rbcL* sequences could be combined into one dataset, a partition homogeneity test (PHT) with a thousand repetitions of ITS, *matK*, and *rbcL* sequences were examined to see if the partitions were homogenous in PAUP\* v4.0b1. FigTree v. 1.3 was used to view and modify the trees. The BI analysis consensus tree and the best tree of ML analysis with BS replicates were combined using TreeGraph 2 [26]. The combined tree for user-specified tree topology was analyzed using the Maximum Parsimony (MP) method to calculate tree length (TL), consistency index (CI), retention index (RI), homoplasy index (HI), and combined index (RCI) in MEGA X.

### 2.3. Estimation of Age

BEAST 2.1.3 was used to calculate divergence times (Drummond *et al.*, 2012). The BEAST analysis was conducted using the GTR + G + I model with the best-fit clock model and tree prior. The most recent common ancestor (MRCA) of *Salacia* species was calculated using a concatenated data barcode dataset. A taxonomic set has been created. The divergence was calculated using an uncorrelated relaxed lognormal clock model. The mean previous distribution UCLD was set to normal with an initial value of 50 million years (Ma) and a standard deviation of 5 Ma prior to the analysis. The MCMC chains were run for 50 million generations, with 5000 generations being sampled. Tracer 1.6 was used to check for convergence and sufficient effective sample size (ESS) values (>200) (Rambaut *et al.*, 2014). TreeAnnotator 2.1.2 software (available in the BEAST v.2.1.3 package; <http://beast.bio.ed.ac.uk/treeannotator>) was then used to create maximum clade credibility tree with median branch lengths and 95% greatest posterior density intervals on nodes, with the first 25% of trees removed as burn-in. The maximum-credibility tree and divergence dates were displayed using FigTree.

### 2.4. Phylogeographic Inference

Utilizing RASP v.2.0b (Reconstruct Ancestral State in Phylogenies) and Bayesian binary MCMC (BBM) analysis for the geographic inferences. The F81 model of evolution was used, and various rates of change across ancestral states were permitted to minimize any limitations on ancestral state reconstruction. For all other MCMC parameters, default values were chosen. In all analyses, the MCMC chains were run for 5x10<sup>6</sup> generations. Six regions were assigned according to the sampling and distribution ranges. A=central and South America (CSA), B= Western Ghats of India, C = Central and South-East Asia (SEA), D = Madagascar/East Africa, E=West Africa, and F=Australia.

## 3. RESULTS

### 3.1. ITS Phylogeny

To build the ITS sequences phylogenetic tree a total of 134 sequences were used. Multiple sequence alignment (MSA) produced a consensus

**Table 1:** Sample code, and accession number of ITS, *matK*, *rbcL* *S. chinensis*, *S. macrosperma*, *S. fruticosa*, *S. oblonga* samples sequences deposited in NCBI GenBank database.

S. No.	Name	Sample code	Sampling area	Accession number		
				ITS	<i>matK</i>	<i>rbcL</i>
1	<i>S. chinensis</i>	SC1	Udupi District	SC1-KF986225	SC1-KX573059	SC1-KX581076
		SC2		SC2-KF986226	SC4-KX573061	SC5-KX581078
		SC3		SC3-KF986227	SC5-KX573062	SC10-KX581077
		SC4		SC4-KF986228	SC6-KX573063	
		SC5		SC5-KF986229	SC11-KX573060	
	<i>S. macrosperma</i>	SM2	Shanivarsanthe, Kodagu District.	SM2-KF860877	SM3-KX573069	SM3-KX581083
		SM3		SM3-KF881918		
		SM4	Abbi falls, Madikeri District	SM4-KF881919		
		SM5	Madikeri town, Madikeri District	SM5-KF881920	SM5-KX573070	SM5-KX581084
		SM6	Bisle ghat, Hassan District	SM6-KF881921	SM6-KX573071	
		SM7	Kigga, Chikkamagaluru District	SM7-KF881922		SM7-KX581085
		SM8	Somwarpet, Madikeri District	SM8-KF881923	SM8-KX573074	
		SM9		SM9-KX573072		
		SM10	Kigga, Chikkamagaluru District	SM10-KF894421		
SM11	Makuta, Madikeri District	SM11-KF894422	SM12-KX573067	SM12-KX581082		
SM12		SM12-KF894423	SM13-KX573068			
SM13		SM13-KF881925				
SM14						
3	<i>S. fruticosa</i>	SF1	Makuta, Madikeri District	SF1-KF894424	SF2-KX573064	SF1-KX581081
		SF2		SF2-KF894425	SF3-KX573065	SF2-KX581080
		SF3		SF3-KF894426	SF-4KX573066	SF6-KX581079
		SF4		SF4-KF894427		
		SF5		SF5-KF894428		
		SF6		SF6-KF894429		
4	<i>S. oblonga</i>	SO1	Kigga, Chikkamagaluru District	SO1-KF881913	SO1-KX573074	SO1-KX581088
		SO2		SO2-KF881914	SO2-KX573075	SO2-KX581087
		SO3		SO3-KF881915	SO3-KX573076	SO3-KX581086
		SO4		SO4-KF881916		SO7-KX581089
		SO5				
		SO6				
		SO7				

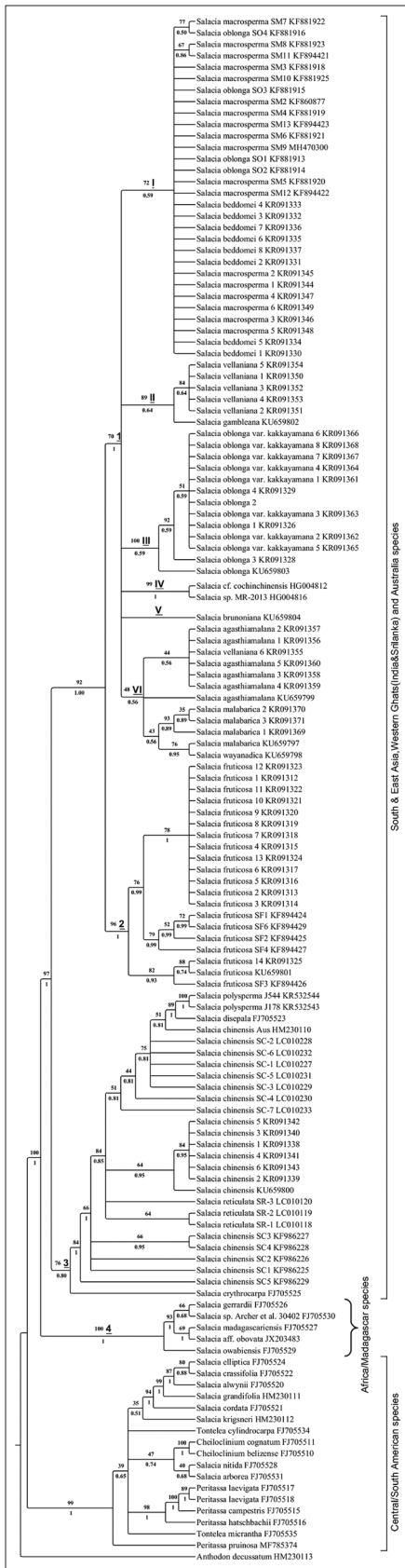
ITS: Internal transcribed spacers

sequence of 1029 bp, with 435 bp, 539 bp, and 452 bp conserved, variable, and parsimony informative sites, respectively. Tamura-Nei model with gamma distribution and invariable sites (T92 + G + I) had the lowest BIC values of 19637.527 and tree log-likelihood of -22756.75. The tree topology of the combined tree had a log-likelihood of -8140.22. Likewise, tree topology was analyzed using the Maximum Parsimony method had TL, CI, RI, and RCI to be 1462, 0.58 (0.55), 0.85 (0.85), and 0.49 (0.47) for all sites and parsimony-informative sites (in parentheses).

The Salacioideae subfamily was separated into two major clades in combined BI and ML trees. The phylogram was clear and free of polytomies. Clade I-old world clade (OWC) comprises *Salacia* samples from the Western Ghats, Africa, Madagascar, Australia, and Southeast Asia. Six groups made up the first subclade [Figure 1]. Within the OWC, subclades 1 (Western Ghats), 2 (*S. Chinensis*), 3 (Madagascar), and 4 (Africa) were observed. Along with SM, *S. agathamaliana* (SA) from Western Ghats, the cladogram contained *S. beddomei* (SB), SO, and *S. oblonga* var *kakayamana* (SOVK), as well as *S. vellianiana* (SV), *S. malabarica* (SMa), *S. brunoniana* (SBr), and *S. wayanadica* (SW) from Karnataka and Kerala.

SO samples from the Western Ghats of Karnataka were nested within the SM samples from Karnataka rather than being grouped with samples from Kerala in Group 1. In Group 1, SM samples from Karnataka were merged with those from Kerala. There was also a polytomic group in SM and SB samples from Karnataka and Kerala. SM and SV from Kerala's Western Ghats, both of which showed strong posterior probability (PP) support values at the node. There was also a polytomic group of SM samples from Karnataka and Kerala that included SB. There were two species of SM and SV from the Western Ghats of Kerala in Group 2, both of which had high PP support values at the node. SO samples from Karnataka's Western Ghats were nested within the SM. Kerala's SO was placed in Group 3 with node support values of 100% (BS) and 0.59 (PP). This group included *S. cf. cochinchinensis*, *Salacia* sp. (MR-2013), and SBr. Group 4 included SA, SV, SBr, and SW, all of which exhibited excellent support values. SA and SV were nested inside group 4, while SMa and SW formed a separate group with high node and tip support values. SF samples from Karnataka and Kerala formed a robust monophyletic group in Group 5.

Subclade 2 consisted of SR, SC, *S. erythrocarpa*, *S. polysperma*, and *S. disepala*. This clade included all *S. chinensis* from Karnataka,



**Figure 1:** Combined cladogenetic tree of maximum likelihood bootstrap consensus (above branches boot-strap values >50%) and Bayesian inference (below branches posterior probability values >0.5) of the ITS sequences of Salacioideae.

Kerala, and Australia. *S. erythrocarpa*, *S. disepala*, and SR were members of the clade. *Salacia* samples from Madagascar were discovered in Subclade 3, a clade with a strong node support value. Subclade 4 included East African samples.

Clade II - New world clade (NWC) includes samples from Central and South America, as well as a disjunct sample from the Ivory Coast, *S. nitida*. The phylogenetic distribution matched that of the Salacioideae analysis of Coughenour, Simmons [10]. From ITS analysis, it was clear that the principal nodes have a high PP value (>0.9) and a BS of >90%. However, an average and weak support value of 50% was recorded for the internal node, as well as a strong PP support value of >0.5.

### 3.2. matK Phylogeny

To build the *matK* sequences phylogenetic tree, a total of 80 sequences were used. MSA produced a consensus sequence of 1383 bp, with 801 bp, 565 bp, and 514 bp conserved, variable, and parsimony informative sites, respectively. T92+G provided the substitution model with the lowest BIC values of 10488.262 and tree log-likelihood of -22756.75. The tree topology of the combined tree had a log-likelihood of -8140.22. Likewise, tree topology was analyzed using the Maximum Parsimony method had TL, CI, RI, and RCI to be 656, 0.93 (0.92), 0.95 (0.95), and 0.89 (0.88) for all sites and parsimony-informative sites (in parentheses).

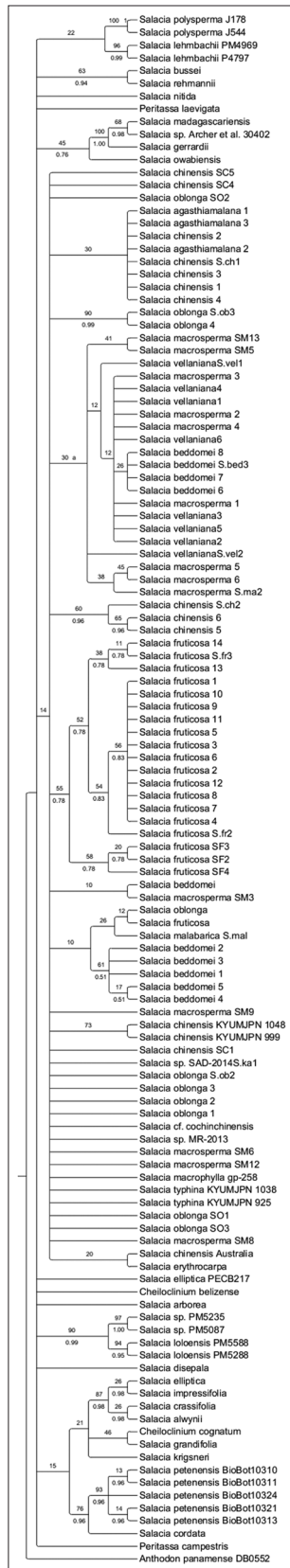
In the *matK* sequencing phylogeny, there were no noteworthy clades including NWC *Salacia* and OWC *Salacia* species. Samples from several geographical zones were grouped ambiguously. The BS and PP support levels for nodes were low [Figure 2]. The samples from the Western Ghats, as well as those from Africa and Australia, were found to be discordantly positioned throughout the phylogeny. The Western Ghats samples of SM, SO, SC, SA, and SB formed polytomies and were disjunct and distributed within the tree. Only SF formed a monophyletic group with strong nodal BS and PP support values. Madagascar (*S. madagascariensis*, *Salacia* sp., *S. gerrardii*, *S. owabiensis*), Africa (*Salacia* species and *S. loloensis*-SL), and central American samples (*S. elliptica*, *S. impressifolia*, *S. crassifolia*, *S. alwynii*, *Cheiloclinium cognatum*, *S. grandifolia*, *S. krigneri*, *S. petenensis*, and *S. cordata*) were grouped to form a paraphyletic clade with weak and good BS and PP support values. *S. lehmbachii* and *S. polysperma* were sister taxa *Peritassa laevigata* and *Salacia* samples from Madagascar (*S. bussei*, *S. rehmannii*, and *S. nitida*). The samples from China and South-East Asia (SEA) were nested with those from the Western Ghats, with no different clades visible in the cladogram.

### 3.3. rbcL Phylogeny

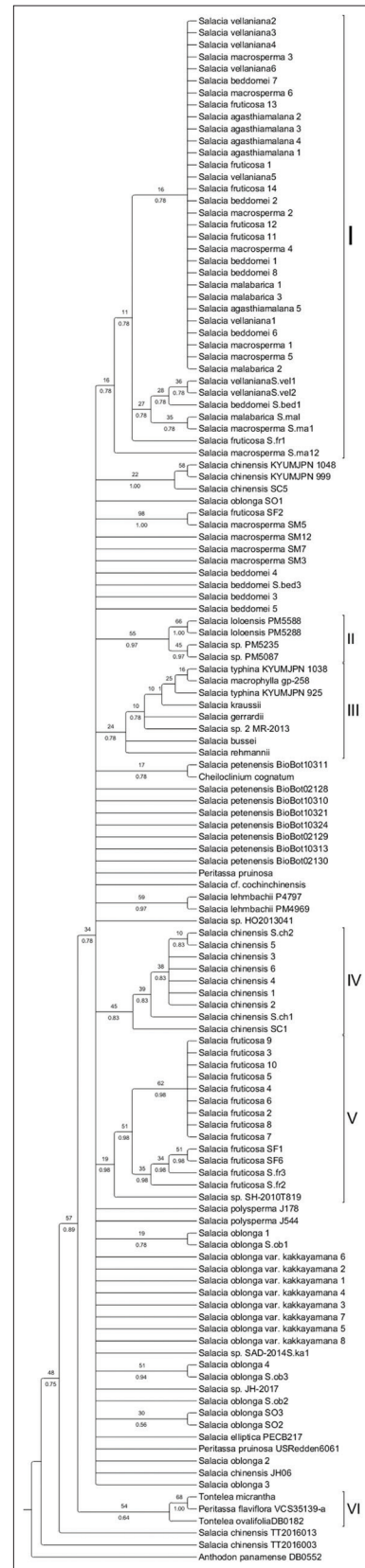
To build *rbcL* sequences phylogenetic tree, a total of 73 sequences were used. MSA produced a consensus sequence of 733 bp, with 639 bp, 93 bp, and 30 bp conserved, variable, and parsimony informative sites, respectively. Kimura 2-parameter model with gamma distribution (K2+G) provided the best fit substitution model, with the lowest BIC values of 4968.493 and log-likelihood of - 1708.209. Likewise, tree topology was analyzed using the Maximum Parsimony method had TL, CI, RI, and RCI to be 115, 0.83 (0.62), 0.81 (0.81), and 0.68 (0.50) for all sites and parsimony-informative sites (in parentheses).

In the *rbcL* phylogeny, species resolution was low with very weak BS, and PP support was seen within the cladogram. The samples from Karnataka and Kerala were significantly discordantly dispersed in Clade I. SM, SA, SB, SO, SV, and SMA were assigned a low BS, but





**Figure 2:** Combined cladogenetic tree of maximum likelihood bootstrap consensus (above branches boot-strap values >50%) and Bayesian inference (below branches posterior probability values >0.5) of the *matK* sequences of Salacioideae.



**Figure 3:** Combined cladogenetic tree of maximum likelihood bootstrap consensus (above branches boot-strap values >50%) and Bayesian inference (below branches posterior probability values >0.5) of the *rbcL* sequences of Salacioideae.

a high PP. Karnataka's Western Ghats samples *Salacia* species from Kerala and Africa were sister taxa to SC, SM, SO, and SF. *Salacia* species from Africa formed two different clades in Clades II and III, with *S. macrophylla* and *Salacia* species from Thailand and China, nested within one of the clades [Figure 3]. Clades IV and V contained samples from Kerala and were different clades of SC and SF. *Tontelea micrantha*, *Peritassa flaviflora*, and *Tontelea ovalifolia* formed a distinct clade (Clade VI) with good support values.

### 3.4. ITS, *matK*, *rbcL* Concatenated Sequences and BI, ML Combined Tree Phylogeny

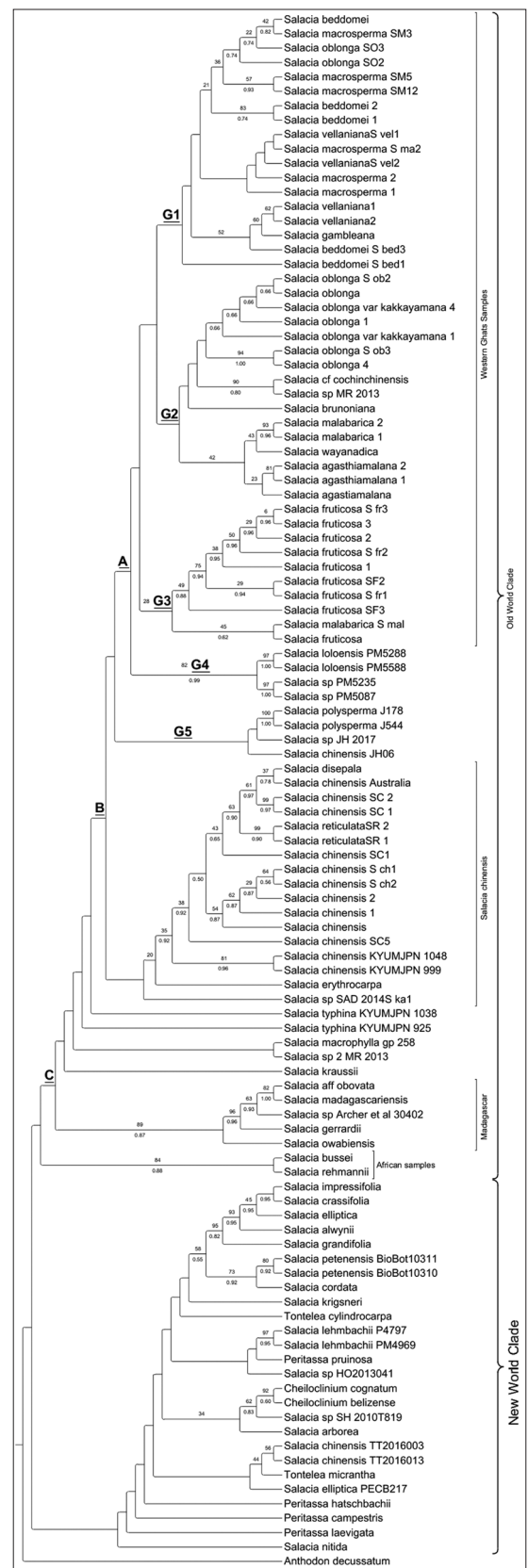
Before constructing a phylogenetic tree, genetic divergences for the concatenated ITS + *matK* + *rbcL* were examined in Mega 5 software using the P-distance technique. In the distance analysis for concatenated sequences, samples with n/c were excluded from the dataset. Partition homogeneity test (PTH) results were  $p = 0.03$  indicating that the DNA sequences have a consistent phylogenetic tree topology and no conflicts in the genes' sequence dataset for the bootstrap trees. To build concatenated sequences phylogenetic tree, a total of 106 sequences were used. The total length of the concatenated sequences was 3166, with 1931 bp, 1187 bp, and 988 bp being conserved, variable, and parsimony informative sites, respectively. The best fit substitution model was T92 + G having the lowest BIC values of 34956.694 and log-likelihood of -16176.919.

The cladogram was separated into two major clades in the combined analysis, namely the OWC and the NWC, with no or very poor support at the branch nodes. Three subclades of the OWC were identified: A, B, and C [Figure 4]. Subclade A nested within the clade comprised samples from Karnataka and Kerala, as well as samples from China and South-East Asia. G1, G2, G3, and G4 are the sub-clade A groupings. G1 included samples from Karnataka's Western Ghats, SO, and SM. SM, SB, *S. gambleana*, and SV from Kerala constituted a paraphyletic group. *Salacia* species nested inside G2 included SO, SOVK, *S. brunoniana*, SMA, SW, SA from Kerala and *S. cf cochinchinensis* from China. G3 contains samples of SF as well as SMA. Samples from Africa, SL, and *Salacia* sp. (PM5235) were found in the clade G4, which had high node branch support values. Both of these samples were taken in Cameroon and other geographically adjacent regions. *S. polysperma* (China), *Salacia* sp. (JH-2017-24-4063) from Borneo, and SC-JH06 (Australia) formed a paraphyletic clade in clade G5, and these species were sister taxa to Western Ghats species.

SC from Karnataka, Kerala, and Australia was found in Subclade B, as were SR, *S. disepala*, and *S. erythrocarpa*. *Salacia* sp. (SAD2014S. ka1), *S. macrophylla*, *Salacia* sp. (MR2013), and *S. kraussii* create polytomies at the clade's base. *S. aff obovata*, *S. madagascariensis*, *Salacia* sp. (Archer et al. 30402), *S. gerrardii*, *S. owabiensis*, and east Africa (Zambia and Mozambique) species *S. rehmannii* and *S. bussei* have high support values in sub-clade C. The NWC formed into a paraphyletic group that included all of the species from Central and South America. Within this primary clade, however, samples from West Africa Cameroon-*S. lehmbachii*, Ivory Coast-*S. nitida*, and SEA SC (TT2016003 and TT2016013), Thailand, *Salacia* sp. (HO2013041), *Salacia* sp. (SH-2010), and *S. arborea* from Indonesia were nested.

### 3.5. Divergence Time Analysis -Age Estimation *Salacia* Species

BEAST analysis had an ESS of more than 500 for all parameters. According to age estimates, the node age of old-world *Salacia* species and new-world *Salacia* species was 50.35 M.Y. All *Salacia* species have stem ages of 32.58 Ma. The OWC and NWC crown



**Figure 4:** Concatenated, combined cladogenetic tree of maximum likelihood bootstrap consensus (above branches boot-strap values >50%) and Bayesian inference (below branches posterior probability values >0.5) of the ITS, *matK* and *rbcL* sequences of Salacioideae.

ages were 9.82 Ma and 17.4 Ma, respectively. A paraphyletic cluster of *Salacia* species from Madagascar and East Africa has a shared tip node age of 3.13 Ma within OWC. The SC (Kerala, SEA, and Australia) and SR share the same tip node age of 3.57 Ma. SC from Karnataka and *S. erythrocarpa*, on the other hand, shared the same tip node age of 4.29, demonstrating a divergent rate of SC divergence throughout the Western Ghats. SF had common tip node age of 2.11 Ma indicating a similar rate of divergence within species. The NWC *Salacia* species has the youngest tip node age of 0.28 million years and the oldest tip node age of 11.3 million years. With a tip node age of 1.69 Ma, two China species, *S. cf. cochinchinensis* and *Salacia* sp. (MR2013) were nested among new world species at the base of the clade. This basal distribution of *S. cf. cochinchinensis* and *Salacia* sp. (MR2013) was not seen in the ITS, *matK*, *rbcL*, or concatenated sequences [Figure 5].

### 3.6. Geographic Inference

Based on BEAST combined DNA barcode phylogeny and the BBM technique, geographic analysis reveals 50 dispersals and 25 vicariance episodes. According to node 216, the root node of all *Salacia* species evaluated in the current geographic study had an ancestral area of C-81.84: A-11.46: AC-2.36 with a probability of 0.4623, indicating that *Salacia* species might have originated in Asia.

The observation that node 208, which contains the majority of the NWC *Salacia* species represented, had the ancestral state of C-56.74: A-19.98: F-9.57: E-7.60 but a very low probability of 0.0931 in a route of C->FE->F|E with three Dispersal, a Vicariance, and an extinction event, strengthened this observation. *S. bussei* (Zambia), *S. rehmannii* (Mozambique) had ancestral state C-40.47: E-37.31: D-9.77 with two Dispersal, a Vicariance event in the route C->EC->E|C but a low probability of 0.1631 in OWC, Madagascar, and East Africa.

Most *Salacia* sp. of Western Ghats of Kerala and Karnataka have the origin note 174, which has ancestral state B 66.05 C 21.44 BC 6.36 with a route of B->B^B->B|B and Probability of 0.3116. The clade containing most of SC from the Western Ghats and Australia, along with SR, *S. typhina*, and *S. macrophylla* has Indian, and Asia origin, and 16.13% of unknown origin, with a probability of 0.4196 [Figure 6].

Some of the disjunct geographic distributions were seen in east Africa *Salacia* species SL, *S. kraussii* west Africa *Salacia* species – *S. lehmbachii*, and *Salacia* species-PM5288, 5588 which were nested along with Western Ghats *Salacia* species. In the *Salacia* species from Asia disjunct distribution of *Salacia* species SH\_2010T819, *Salacia* sp.- HO2013041, SC-JH06 was observed which was nested within the NWC. Details are listed in Table 2.

## 4. DISCUSSION

Following the previous study, the monophyly of Salacioideae has been convincingly established (Coughenour *et al.* 2010). *Salacia* species from Central and SEA, the Western Ghats, and East Africa were included in this study to present a more complete overview of species diversity. Even though the Salacioideae have been determined to be monophyletic, the disjunct distribution of *Salacia* sp. throughout new and OWCs indicates that the genera have undergone substantial divergence. ITS sequences generated a well-resolved, high-support value phylogenetic tree than *matK* and *rbcL* sequences among the three DNA barcodes evaluated. Furthermore, ITS sequences have 59.59% efficiency in comparison *matK* and *rbcL* sequences have

a substantially lower potential for species discrimination [27]. Furthermore, the combination of the ITS + *matK* and ITS + *rbcL* has stronger species discrimination capacity, although the ITS sequence alone was able to identify between *Salacia* sp. when the TaxonDNA tool was used. Anaz, Sasidharan [15] confirm the current finding by analyzing delimited ITS2 sequences utilizing a neighbor-joining tree and ITS2 secondary structure.

The distribution of species in the Western Ghats demonstrates the relic character of the once-continuous distribution of flora and fauna [9]. Various authors have proposed three theories to explain the distribution and dissemination of flora and wildlife in the Western Ghats: convergence, dispersal, and vicariance [28]. The vicariance theories explain the disjunct distribution of SEA samples and monophyly among *Salacia* samples from the Western Ghats. The geographical distributions of SF and SC have not been changed, as samples from diverse locations have formed a monophyletic group. We hypothesise that sympatric speciation resulted in the evolution of the SM, SO, SB, SMa, SA, SOVK, and SV of Karnataka and Kerala. Based on the evolutionary study and physical characteristics, it is clear that SF is endemic to the Western Ghats. According to Karanth [29], spurious disjuncts are a result of incorrect identification and are not actual sister taxa. Because ambiguous samples were deleted from the sequence matrix, incorrect identification can be ruled out. The samples from Madagascar and Africa are indisputably well-resolved in the OWC. *S. rehmannii* is the sister species of the NWC and African/Madagascar samples. *Salacia* sp. from East Africa is related to Madagascar, SC, and Western Ghats samples. *S. cf. cochinchinensis* and *Salacia* sp. (China) form polytomies nested inside SEA and Western Ghats samples, implying that disjunction arose owing to convergence.

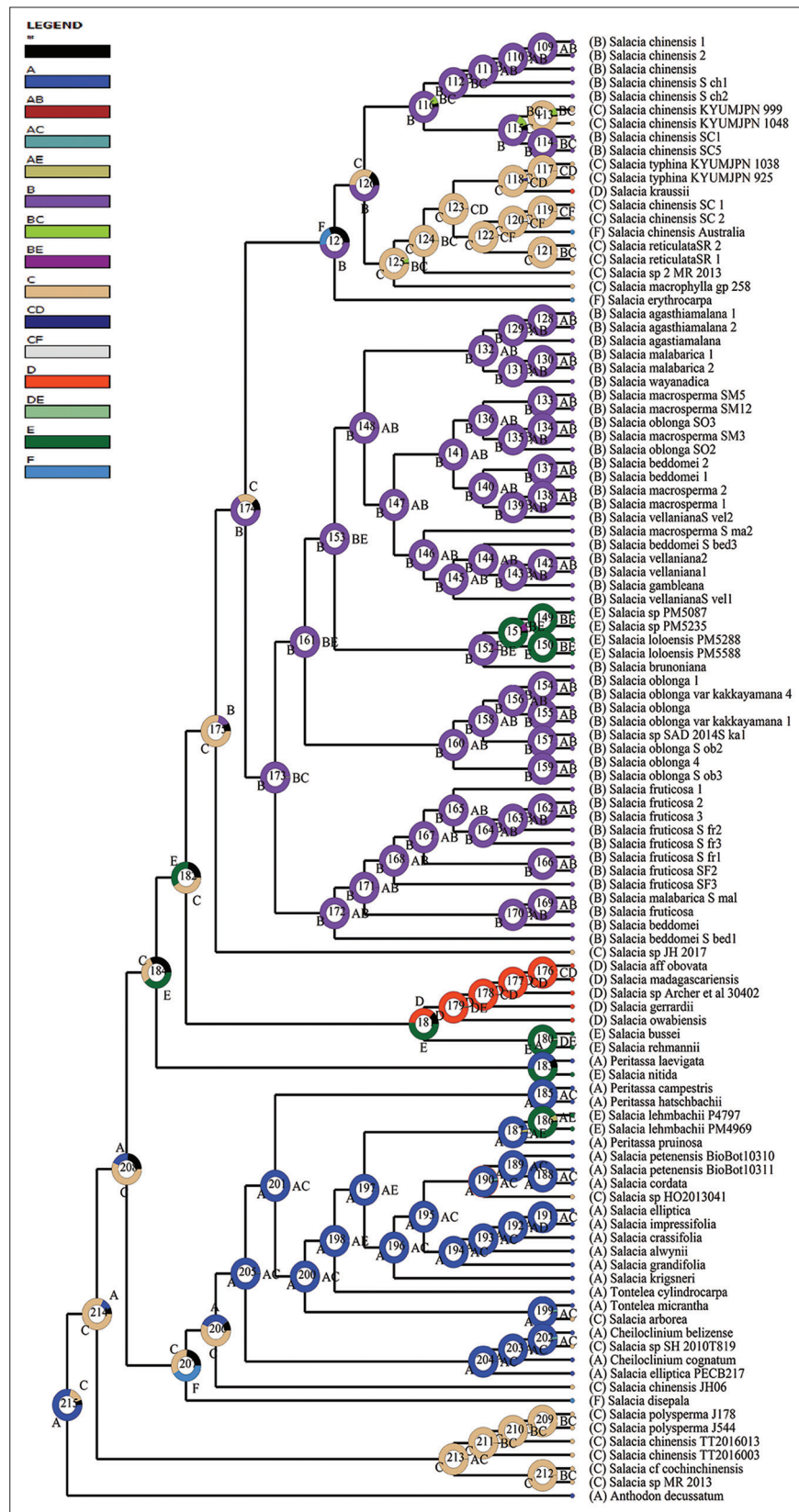
The estimated age of the Salacioideae is based on a geographic examination of the Celastraceae samples from Madagascar (Bacon, Simmons [30] and the crown age of Celastral as inferred by Magallón and Castillo [31] which is 71.6 Ma. Yoder and Nowak [32] estimated Celastraceae Madagascar lineages to be 69.6–1.7 Ma. Madagascar *Salacia* sp. has stem node age and crown node age to be 4.7 (7.1-2.8) Ma and 3.7 (5.7-1.9) Ma, respectively. However, in the current study, we estimated the crown age of the Madagascar clade to be 4.63 Ma which is higher than the latter studies. *S. rehmannii* and *S. nitida*, which were previously nested inside the NWC *Salacia* sp. were nested alongside the Madagascar *Salacia* sp. and had tip ages of 1.81 and 1.12 Ma, respectively. *Salacia* sp. (HO2013041, JH-2017, and SH2010T819) and *S. lehmbachii*, together with *S. arborea*, were still nested with *Salacia* samples from NWC, as previously found in the combined barcode sequence analysis. Furthermore, the Salacioideae divergence period coincides to the estimated divergence time of Asian-North American disjunct lineages, which is between 3 and 25 Ma (Wen *et al.* 2010).

Salacioideae's ancestral state shows that *Salacia* sp. spread is due to long-distance dispersion episodes followed by vicariance between Asia and America. The presence of a significant ancestral state of SEA in the NWC suggests a long-distance dispersion event of *Salacia* sp., followed by allopathic species and radiation among *Salacia* sp. in Central and South America. Long-distance dispersion might result in the distribution and dissemination of samples *S. arborea*, *Salacia* sp. HO2013041, JH-2017, SH2010T819, and *S. lehmbachii* from West Africa. The disjunct distribution of SEA *Salacia* sp. among NWC species might be owing to geographic disjunctions between Eastern Asia and North America [33], and *S. lehmbachii* and *S. nitida* nesting among NWC species could be due to a vicariance event.



**Figure 5:** Chronogram for the sub-family Salacioideae based on ITS sequence obtained with BEAST analysis. The numbers at nodes refer to the mean age of the nodes blue bars represent 95% confidence intervals for the estimated mean dates.





**Figure 6:** The output of the BBM analysis from the RASP program showing ancestral geographic reconstructions at each node of the phylogenetic tree of Salacioideae. The proportion of colours in circles at each node represents possible ancestral ranges, letter close to the color are ancestral state and letters in the centers of the circles show the node number. Biogeographical regions: A: Central and South America, B: Western Ghats of India, C: Central and South/East Asia (SEA), D: Madagascar/East Africa, E: West Africa, and F: Australia.

**Table 2:** Details of ancestral state, route, probability of some of the *Salacia* species.

Node	Species	Ancestral state	Route	Probability	Event
Node-214	<i>S. polysperma</i> J178&J544 <i>S. chinensis</i> TT2016003 & 13 <i>S. cf cochinchinensis</i> <i>Salacia</i> sp MR2013	C=81.84, A=11.46	C->C^C->C C	0.4623	N/A
NODE 207	<i>S. disepala</i>	F=41.3, C=34.75, A=15.09	F->FC->F C	0.2332	D: 2, V: 1, E: 0
NODE 206	<i>S. chinensis</i> JH06	C=56.46, A=33.14	C->CA->C A	0.5582	D: 2, V: 1, E: 0
NODE 183	<i>Peritassa laevigata</i> , <i>Salacia nitida</i>	E 49.21 A 41.15	E->AE->A E	0.4921	D: 2, V: 1, E: 0
NODE 186	<i>S. lehmbachii</i> P4797, <i>S. lehmbachii</i> PM4969	E=92.46, AE=5.87	E->E^E->E E	0.9246	N/A
NODE 199	<i>Tontelea micrantha</i> <i>S. arborea</i>	A=97.13	A->AC->A C	0.9713	D: 2, V: 1, E: 0
NODE 202	<i>Cheiloclinium belizense</i> , <i>Salacia</i> sp SH2010T819,	A=96.98	A->AC->A C	0.9698	D: 2, V: 1, E: 0
NODE 190	<i>Salacia</i> sp HO2013041	A=96.77	A->CA->C A	0.9655	D: 2, V: 1, E: 0
NODE 151	<i>S. loloensis</i> PM5288 <i>S. loloensis</i> PM5588 <i>Salacia</i> sp PM5087 <i>Salacia</i> sp PM5235	E=89.47, BE=7.80	E->E^E->E E	0.9957	
NODE 127	<i>S. erythrocarpa</i> ,	B=47.57, F=21.12, un=31.31	B->FB->F B	0.2402	D: 2, V: 1, E: 0
NODE 120	<i>S. chinensis</i> Australia	C=96.88, CF=2.49	C->FC->F C	0.9669	D: 2, V: 1, E: 0

## 5. CONCLUSION

*Salacia* species can be misidentified with the genera of sub-family Hippocretoideae. The current study is an attempt to understand the endemism of *Salacia* sp. of Western Ghats using DNA barcodes. *Salacia* sp. from the distinct geographic area were discordantly distributed within the phylogenetic tree indicating a high level of sequence similarity. The convergence of the Western Ghats indicated homoplastic speciation of *Salacia* species. SOVB and SO could be Plesiomorphic species and SC and SR are the same species. Age estimation is a crucial aspect to understand the endemism of a particular geographic region. *Salacia* species serve as Paleoendemic species of Western Ghats which in turn give clues to the dynamics of the habitat of Western Ghats and its fragile ecosystems. Furthermore, the geographic analysis reveals the origin of *Salacia* species in Asia. Western Ghats and the forest of SEA are relict for several important flora. Therefore, it is essential to conserve the habitat of the Western Ghats which is one of the significant biodiversity hot spots of the world.

## 6. AUTHORS' CONTRIBUTIONS

SNB and KKM carried out the molecular studies, sequence alignment, and analysis and drafted the manuscript. ASB, MG, RR and KRM helped in reviewing and corrections of Manuscript. KRK conceived the study, participated in its design, coordination, and supervised the current research work.

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## 8. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## 9. ETHICAL APPROVALS

This article does not contain any studies conducted on human or animal subjects.

## 10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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