

## Molecular phylogeny of angiospermic plant families using *RBCL* gene sequences

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**Abstract:** The present study was undertaken to assess the role of plastid-*rbcl* (ribulose-1, 5-bisphosphate carboxylase large-subunit) gene sequences in addressing the evolutionary relationships within the angiosperms at inter and intra-familial levels using computational experiment. In order to elucidate the relationships, a set of 92 chloroplast *rbcl* sequences representing from 90 taxa of 12 genera and 10 angiospermic plant families (dicot and monocots) were withdrawn from the GenBank database. The multiple sequence alignment was performed using GeneBee-ClustalW service to find out the regions of conserved or indels among the sequences. The phylogenetic tree was inferred from these sequences by employing Bootstrap method of UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using MEGA (Molecular Evolutionary Genetics Analysis) software. The consistency of these generic-wise groupings was further confirmed by the MP (Maximum-Parsimony), ME (Minimum-Evolution) and NJ (Neighbor-Joining) methods. The analysis of these studies strongly indicates that, out of the 12 selected genera *Trichosanthes* (Cucurbitaceae), *Phyllanthus* (Phyllanthaceae), *Austrobryonia* (Cucurbitaceae), *Solanum* (Solanaceae), *Piper* (Piperaceae) and *Saxifraga* (Saxifragaceae) are grouped into separate clusters and exhibiting monophyletic conditions. Where as, *Drypetes* (Putranjivaceae), *Asparagus* (Asparagaceae), *Cassia* (Caesalpinioideae, sub-family), *Canna* (Cannaceae), *Mentha* (Lamiaceae), are paraphyletic and the members of the *Salvia* (Lamiaceae) are distributed throughout these hiraecoid clades, confirming the polyphyly of this large genus. Similar observations were noticed in all four methods. Thus, chloroplast *rbcl* gene sequences can unambiguously resolve the relationships, as well as provided a good indication of major supra-generic groupings among the selected angiospermic plant families and also gave many clues for future studies.

**Key Words:** *rbcl* gene, monophyletic, paraphyletic, polyphyletic, Phylogenetic relationship, MEGA

### INTRODUCTION

The use of DNA nucleotide sequence data has awakened a quickly growing interest in molecular systematic analyses. Since last decade molecular systematics in plants has progressed rapidly with *in-vitro* DNA amplification and direct sequencing methods. In angiosperm systematics, this molecular approach has been effective in addressing many phylogenetic questions that had not been solved only by using phenotypic characters. The gene for the large subunit of the ribulose-bisphosphate carboxylase/oxygenase (*rbcl*), located on the chloroplast genome, is an appropriate choice for inference of phylogenetic relationships at higher taxonomic levels [1–3]. Because of its slow synonymous nucleotide substitution rate in comparison with nuclear genes and its functional constraint that reduces the evolutionary rate of non-synonymous substitution [4]. The substitution rate of *rbcl* appears appropriate for studies involving taxa that diverged from 10's of million years [5]. In recent years, there has been a growing interest in using sequences of the gene coding for the large subunit of ribulose-1, 5-bisphosphate carboxylase/ oxygenase (*rbcl*) to estimate the plant phylogenies [6]. Several research groups have shown the usefulness of this gene for solving intergeneric and interspecific relationships among flowering plants example, Cucurbitaceae [7], Orchidaceae [8],

Rhamnaceae [9], Araucariaceae [10]. The present study was undertaken to evaluate the relationships at the intra and inter familial, intergeneric and interspecific levels, and also to examine the rates, patterns and types of nucleotide substitutions within the *rbcl* gene in all the selected taxa of each genera, through a computational experiment using 92 chloroplast *rbcl* gene sequences from 90 taxa, 12 genera, 10 families, and discussed the utilization of plastid-*rbcl* gene for molecular phylogeny.

### MATERIALS AND METHODS

Cladistic parsimony analyses of a total 92 *rbcl* nucleotide sequence data representing 90 species (Spp) and 2 duplicates from *Austrobryonia micrantha* and *Mentha longifolia* of 12 genera, 10 angiospermic plant families (both dicots and monocots) are presented here in the table-1, were withdrawn from GenBank database). The multiple sequence alignment was performed using GeneBee-ClustalW service. All gap characters were scored as missing data rather than a fifth character. Phylogenetic analyses of the sequence data was analyzed with UPGMA using MEGA software. The consistency of these generic wise groupings was further confirmed by the MP, NJ and ME methods as implemented in MEGA 4.1 software [11]. Tree statistics included the consistency index (CI) [12], retention index (RC) [13] and rescaled

consistency index (RCI) [13]. Branch lengths (ACCTRAN optimization with equal weights) and the level of support for branches of the phylogenetic trees was evaluated with the bootstrap analysis [14] to verify the strength of the branches based on 100 replicates, using branch and bound search. Bootstrap percentages are described as high (85-100%), moderate (75 – 84%) or low (50 – 74%) [15]. The number of nucleotide substitutions per site was estimated by Kimura's two parameter method [16] with the DNADIST program.

## RESULTS

The 12 genera of angiosperms namely, *Trichosanthes* (4 Spp), *Solanum* (2 Spp), *Austrobryonia* (4 Spp and 5 sequences), *Piper* (11 Spp), *Phyllanthus* (9 Spp.), *Saxifraga* (2 Spp.), *Drypetes* (10 Spp), *Asparagus* (3 Spp), *Salvia* (35 Spp.), *Cassia* (4 Spp), *Canna* (3 Spp), *Mentha* (3 Spp and 4 sequences) were selected for the study are highly divergent in several respects from one another. But the properties, such as, biochemical, morphological and cytological characteristics of species within each genus are exhibiting greater level of similarity. This in turn inferred that the molecular makeup of these organisms at the generic and family levels is also highly conserved. The analysis of the above studies strongly indicates that, out of the 12 selected genera, the members of *Trichosanthes*, *Austrobryonia*, *Phyllanthus*, *Solanum*, *Piper* and *Saxifraga* are nested into a separate clusters as a monophyletic group, within the major clades and sub-clades in the tree topology, this is strongly supported by high bootstrap replications (100%). Where as, *Drypetes*, *Asparagus*, *Cassia*, *Canna*, *Mentha* are paraphyletic and the members of the *Salvia* are distributed throughout these hiraoid clades, confirming the polyphyletic condition of this large genus. These observations were noticed in all UPGMA, MP, ME and NJ methods, but only the positions of the generic-wise clusters were altered into the tree topology in all these methods. The members of the *Asparagus* (Asparagales) and *Canna* (Cannaceae) were nested within the angiospermic dicot plant families also exhibiting the paraphyletic conditions of these genera with selected dicot plant families. The total length of the tree is 14029. Of the 92 sequences ranging between 968 – 1471 in the data matrix, of which only 877 nucleotide residues were used to infer the phylogenetic relationships among the selected taxa and were shown to be invariant and 305 were variable. These trees were characterized by a consistency index (CI) of 0.187540, retention index (RI) of 0.733592 and the rescaled consistency index i.e., RCI = 0.137578 (for all sites). The strict consensus of these weighted trees is shown in the Fig 1-4. Branch lengths

(ACCTRAN optimization with equal weights) and bootstrap are shown. The composition of adenine (A)-0.27, thymine (T)-0.29, guanine (G)-0.25 and cytosine (C)-0.19 nucleotides were observed as constant in all the test genera. But slight changes were noticed in the ratio of transition and transversion substitutions. In case of *Saxifraga* the ratio of transition and transversion is 1.83, *Asparagus* is 0.7, *Cassia* is 1.33, *Drypetes* is 1.63, *Salvia* is 2.7, *Mentha* is 1.8, *Trichosanthes* is 0.5, *Piper* is 1.0, *Phyllanthus* is 0.7. Perhaps the members within genera of *Austrobryonia*, *Solanum* and *Canna* are exhibiting highest level of similarity, the rate of transition and transversion ratios are almost negligible. In these selected taxa, there is no significant cluster at the level of subclass was found, particularly, there is no clear-cut segregation between monocots and dicotyledonous plants was observed in the tree topology. The members of the genera *Canna* and *Asparagus* belongs to monocotyledons are nested within the clades of the dicot plant families. The length of the *rbcl* gene sequence in the members of few genera included in the present study possess fixed number of nucleotide residues, which is evident from the genus *Trichosanthes* is 1356, *Phyllanthus* -1408, *Saxifraga* – 1346 and 1377, *Solanum* – 1370, *Austrobryonia* – 1355, *Piper* – 1428. Where as, considerable variation in substitution rates and length of the sequences were observed in some other selected genera namely, *Saxifraga stellaris* and *S. integrifolia*– 1346 and 1377, respectively. Similar observations were found with *Asparagus officinalis*, *A. cochinchinensis* and *A. capensis* – 1206, 1369 and 1340, respectively, *Drypetes* – 1398, except *D. littoralis* and *D. roxburghii* -1331 and 1420, respectively. *Canna indica* and *C. glauca* – 1327 but *C. tuerckheimii* – 1334; *Mentha rotundifolia*, *longifolia* (2) – 1420 and *M. suaveolens*, *M. longifolia* – 1402; *Cassia senna*, *C. didymobotrya* – 1368, *C. grandis* – 1421, *C. fistula*- 1465. However, the sequence length of *rbcl* in 35 members of the genus *Salvia* is ranging between 968 -1474. But the members of this genus located in the subclades-I of Clade-V are strictly containing 1323 and subclades-I of Clade-I containing 1371 nucleotide residues only, and these exhibited highest level of sequence similarity and originated from one common ancestral forms. Where as, the members of this genus located in the sub-clade-IV of clade-I, clade-II, and clade – III are found to be highly variable. The UPGMA method of phylogenetic analysis strongly suggests that, the members of the genus *Trichosanthes* are evolved first and forms a primitive, and then *Phyllanthus*, *Drypetes*, *Saxifraga*, *Solanum*, *Austrobryonia* are evolved in a sequential order. Where as, the members of the genus *Piper* are found to be late evolved and inferred them as highly advanced among the selected genera. Where as, the

members of the genus *Mentha* are evolved twice with the members of *Salvia*. *Mentha suaveolens* and *M. longifolia* are early evolved, they are found in the cluster-I and forms a basal group. But the *M. rotundifolia* and *M. longifolia* (2) are late evolved and nested in a cluster-V indicating that they are advanced. Among the four species of the *Cassia*, only the *C. senna* and *C. didymobotrya* exhibited high level of similarity, this is supported by high bootstrap values of 100% and occupying in the basal group. Where as, *Cassia grandis* nested with in the members of *Salvia* in clade-II and *Cassia fistula* originated sisterly with the members of the genus *Saxifraga*. Similarities between these individuals are strongly supported with bootstrap values as 96%. In case of the genus *Canna*, only the *C. indica* and *C. glauca* are highly similar, supporting with bootstrap values of 100% and located in the major clade-I. Another member of the same genus *C. tuerckheimii* is located in the clade-IV. Due to high sequence diversity among the three selected species of *Asparagus* are distributed distantly into the tree topology and indicated that, this is evolved several times during evolution. *A. capensis* is found in clade-I and referred as less evolved or primitive, where as, *A. cochinchinensis* in the cluster III and *A. officinalis* is in Clade-V and this *A. officinalis* is inferred as highly evolved among other selected species. Among the 35 species, the members located in the subclade-I of clade-I and members in the subclade -I of clade-V are exhibiting greater level of sequence similarity, members of the same genus located in the other clades and subclades are displaying much variable. It is evident that the members of this large genus evolved several times and are distributed into several patches all along the tree topology (FIG -1). The similar monophyletic, paraphyletic and polyphyletic conditions of these selected genera were also confirmed through other NJ, MP, and ME methods, indicating that these conditions are strictly conserved. Only the relative position of these generic level groupings was altered (FIG -2-4).

## DISCUSSION

The study presented here is an attempt to employ a set of 92 plastid *rbcl* gene sequences of 90 taxa belonging to 12 genera of 10 angiospermic plant families to address intra familial relationships. The analyses of *rbcl* nucleotide sequences presented here provide a great deal of support for previous hypotheses of relationships within the family and generic levels of some angiospermic plants. The members of the genera belongs to *Asparagus* (Asparagales) and *Canna* (Cannaceae) belongs to the sub-class monocotyledonous were nested within the angiospermic dicot plant families also exhibiting the paraphyletic conditions of these genera with

selected dicot plant families. The present data also reject the views of Burger, 1997; Taylor and Hickey, 1992 [17–18], that Piperaceae are basal among most of the primitive angiosperms and that, the monocots included in the present study were derived from them. In contrast to the Shu-Miaw *et al.*, 1997 [19], that the monocots included in the present studies are not clustered into a separate monophyletic group. The members of the genera *Canna* and *Asparagus* belongs to monocotyledons are nested within the major clades of the dicot plant families. Therefore, the plastid-*rbcl* gene sequence data lend support to Hutchinson's view that "the single cotyledon, parallel-veined leaves, absence of cambium, dissected stele and the adventitious root system of monocots are all regarded as apomorphies within the angiosperms. Moreover monocots are regarded as derived from dicots, the point of origin being Ranales [20]. These results show that analyses of the chloroplast DNA *rbcl* is a useful approach for inferring phylogenetic relationships especially at the supra-generic level.

## CONCLUSION

However, the present study, the tree relations among the genera are well resolved and providing support for the monophyly of large clades and subclades for many of the selected plant genera such as *Trichosanthes*, *Austrobryonia*, *Saxifraga*, *Solanum*, *Phyllanthus* and *Piper*. The polyphyletic condition of the genus *Salvia* is supported by the higher rate of the transition and transversion ratio is 2.7, 554 gaps as well as significant variation in the total number nucleotide residues in the different members of the genus *Salvia* among 35 species (i.e., 968 in *Salvia nubicola* to 1474 in *Salvia divinorum*). The present investigation also concludes that, the gene plastid-*rbcl* alone is not likely to provide robust estimates of phylogeny. Data from additional molecular (complete nuclear and chloroplast genetic systems) and morphological sources and their combined analyses are necessary to establish a stable and firm base for a plant systematics (Family classification) that better reflects phylogenetic relationships.

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**Table: List of the *rbcl* sequences using figure-1**

Sl. No	Accession Number (GenBank)	No of base pairs	TAXA USED	FAMILY	REFERENCES
01	AY663611	1408	<i>Phyllanthus polyphyllus</i>	Phyllanthaceae	[21]
02	AY663610	1408	<i>Phyllanthus nutans</i>	Phyllanthaceae	[21]
03	AY663609	1408	<i>Phyllanthus nummariifolius</i>	Phyllanthaceae	[21]
04	AY663608	1408	<i>Phyllanthus lokohensis</i>	Phyllanthaceae	[21]
05	AY663607	1408	<i>Phyllanthus juglandifolius</i>	Phyllanthaceae	[21]
06	AY663606	1408	<i>Phyllanthus fluitans</i>	Phyllanthaceae	[21]
07	AY663605	1408	<i>Phyllanthus flagelliformis</i>	Phyllanthaceae	[21]
08	AY663603	1408	<i>Phyllanthus calycinus</i>	Phyllanthaceae	[21]
09	AB233921	1331	<i>Phyllanthus flexuosus</i>	Phyllanthaceae	[22]
10	EF450315	1428	<i>Piper nigrum</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
11	EF450314	1428	<i>Piper chinense</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
12	EF450313	1428	<i>Piper laetispicum</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
13	EF450312	1428	<i>Piper hainanense</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
14	EF450310	1428	<i>Piper hancei</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
15	EF450309	1428	<i>Piper sarmentosum</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
16	EF450311	1428	<i>Piper betle</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
17	EF450308	1428	<i>Piper kadsura</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
18	EF450307	1428	<i>Piper wallichii</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
19	EF450305	1428	<i>Piper austrosinense</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
20	EF450304	1428	<i>Piper longum</i>	Piperaceae	Yang <i>et al.</i> , (Unpublished)
21	EU155605	1356	<i>Trichosanthes schlechteri</i>	Cucurbitaceae	[23]
22	EU155604	1356	<i>Trichosanthes pentaphylla</i>	Cucurbitaceae	[23]
23	EU155603	1356	<i>Trichosanthes cucumerina</i>	Cucurbitaceae	[23]
24	EU155602	1356	<i>Trichosanthes bracteata</i>	Cucurbitaceae	[23]
25	EF487553	1355	<i>Austrobryonia pilbarensis</i>	Cucurbitaceae	[23]
26	EF487552	1355	<i>Austrobryonia micrantha</i> HS4V	Cucurbitaceae	[23]
27	EF487551	1355	<i>Austrobryonia micrantha</i> HS411	Cucurbitaceae	[23]
28	EF487550	1355	<i>Austrobryonia centralis</i>	Cucurbitaceae	[23]
29	EF487549	1355	<i>Austrobryonia argillicola</i>	Cucurbitaceae	[23]
30	AF378764	1334	<i>Canna tuerckheimii</i>	Cannaceae	[24]
31	AF378763	1327	<i>Canna indica</i>	Cannaceae	[24]
32	AF378774	1327	<i>Canna glauca</i>	Cannaceae	Prince (Unpublished)
33	AY570390	1371	<i>Mentha longifolia</i>	Lamiaceae	[25]
34	Z37417	1420	<i>Mentha rotundifolia</i>	Lamiaceae	[26]
35	Z37415	1420	<i>Mentha longifolia</i> (2)	Lamiaceae	[26]
36	U28876	1402	<i>Mentha suaveolens</i>	Lamiaceae	[27]
37	L14407	1474	<i>Salvia divinorum</i>	Lamiaceae	[28]
38	AB295077	1323	<i>Salvia plebeia</i>	Lamiaceae	Sudarmono and Okada (Unpublished)
39	AB295076	1323	<i>Salvia glabrescens</i>	Lamiaceae	Sudarmono and Okada (Unpublished)
40	AB295073	1323	<i>Salvia nipponica</i>	Lamiaceae	Sudarmono and Okada (Unpublished)
41	AB295070	1323	<i>Salvia arisanensis</i>	Lamiaceae	Sudarmono and Okada (Unpublished)
42	AB295069	1323	<i>Salvia hayatana</i>	Lamiaceae	Sudarmono and Okada (Unpublished)
43	AB295068	1323	<i>Salvia pygmaea</i>	Lamiaceae	Sudarmono and Okada (Unpublished)
44	AB295067	1323	<i>Salvia lutescens</i>	Lamiaceae	Sudarmono and Okada (Unpublished)
45	AY570451	1162	<i>Salvia viscosa</i>	Lamiaceae	[25]
46	AY570450	1368	<i>Salvia viridis</i>	Lamiaceae	[25]
47	AY570449	1353	<i>Salvia staminea</i>	Lamiaceae	[25]

48	AY570448	1187	<i>Salvia tiliifolia</i>	Lamiaceae	[25]
49	AY570447	1349	<i>Salvia texana</i>	Lamiaceae	[25]
50	AY570446	1365	<i>Salvia sylvestris</i>	Lamiaceae	[25]
51	AY570445	1356	<i>Salvia taraxacifolia</i>	Lamiaceae	[25]
52	AY570444	1371	<i>Salvia summa</i>	Lamiaceae	[25]
53	AY570443	1350	<i>Salvia subincisa</i>	Lamiaceae	[25]
54	AY570442	1371	<i>Salvia spathaceae</i>	Lamiaceae	[25]
55	AY570439	1349	<i>Salvia sclarea</i>	Lamiaceae	[25]
56	AY570438	1371	<i>Salvia roemeriana</i>	Lamiaceae	[25]
57	AY570437	1177	<i>Salvia ringens</i>	Lamiaceae	[25]
58	AY570436	1364	<i>Salvia pratensis</i>	Lamiaceae	[25]
59	AY570435	1126	<i>Salvia polystachya</i>	Lamiaceae	[25]
60	AY570434	1371	<i>Salvia pentstemonoides</i>	Lamiaceae	[25]
61	AY570433	1186	<i>Salvia palaestina</i>	Lamiaceae	[25]
62	AY570432	1371	<i>Salvia pachyphylla</i>	Lamiaceae	[25]
63	AY570431	1355	<i>Salvia officinalis</i>	Lamiaceae	[25]
64	AY570430	968	<i>Salvia nubicola</i>	Lamiaceae	[25]
65	AY570427	1182	<i>Salvia nilotica</i>	Lamiaceae	[25]
66	AY570428	1113	<i>Salvia misella</i>	Lamiaceae	[25]
67	AY570426	1371	<i>Salvia lyrata</i>	Lamiaceae	[25]
68	AY570425	1357	<i>Salvia lycoides</i>	Lamiaceae	[25]
69	AY570424	1296	<i>Salvia involucrate</i>	Lamiaceae	[25]
70	AY570423	1115	<i>Salvia verbenaca</i>	Lamiaceae	[25]
71	AY570422	1135	<i>Salvia hirsuta</i>	Lamiaceae	[21]
72	AY663639	1398	<i>Drypetes staudtii</i>	Putranjivaceae	[21]
73	AY663638	1398	<i>Drypetes lateriflora</i>	Putranjivaceae	[21]
74	AY663637	1398	<i>Drypetes gilgiana</i>	Putranjivaceae	[21]
75	AY663636	1398	<i>Drypetes floribunda</i>	Putranjivaceae	[21]
76	AY663635	1398	<i>Drypetes fallax</i>	Putranjivaceae	[21]
77	AY663634	1398	<i>Drypetes diversifolia</i>	Putranjivaceae	[21]
78	AY663633	1398	<i>Drypetes deplanchei</i>	Putranjivaceae	[21]
79	AY663632	1398	<i>Drypetes brownie</i>	Putranjivaceae	[21]
80	M95757	1420	<i>Drypetes roxburghii</i>	Putranjivaceae	[29]
81	AB233926	1331	<i>Drypetes littoralis</i>	Putranjivaceae	[22]
82	AM234244	1421	<i>Cassia grandis</i>	Caesalpinioideae	[30]
83	U74195	1465	<i>Cassia fistula</i>	Caesalpinioideae	[31]
84	Z70155	1368	<i>Cassia senna</i>	Caesalpinioideae	[31]
85	Z70154	1368	<i>Cassia didymobotrya</i>	Caesalpinioideae	Kaess, (Unpublished).
86	AY149374	1206	<i>Asparagus officinalis</i>	Asparagaceae	McPherson <i>et al.</i> , (Unpublished)
87	AB029849	1369	<i>Asparagus cochinchinensis</i>	Asparagaceae	[32]
88	AM234843	1340	<i>Asparagus capsensis</i>	Asparagaceae	[30]
89	AF374732	1346	<i>Saxifraga stellaris</i>	Saxifragaceae	[33]
90	L01953	1377	<i>Saxifraga integrifolia</i>	Saxifragaceae	[33]
91	FJ914175	1370	<i>Solanum chilense</i>	Solanceae	Dillon <i>et al.</i> , (Unpublished)
92	FJ914174	1370	<i>Solanum pennellii</i>	Solanceae	Dillon <i>et al.</i> , (Unpublished)

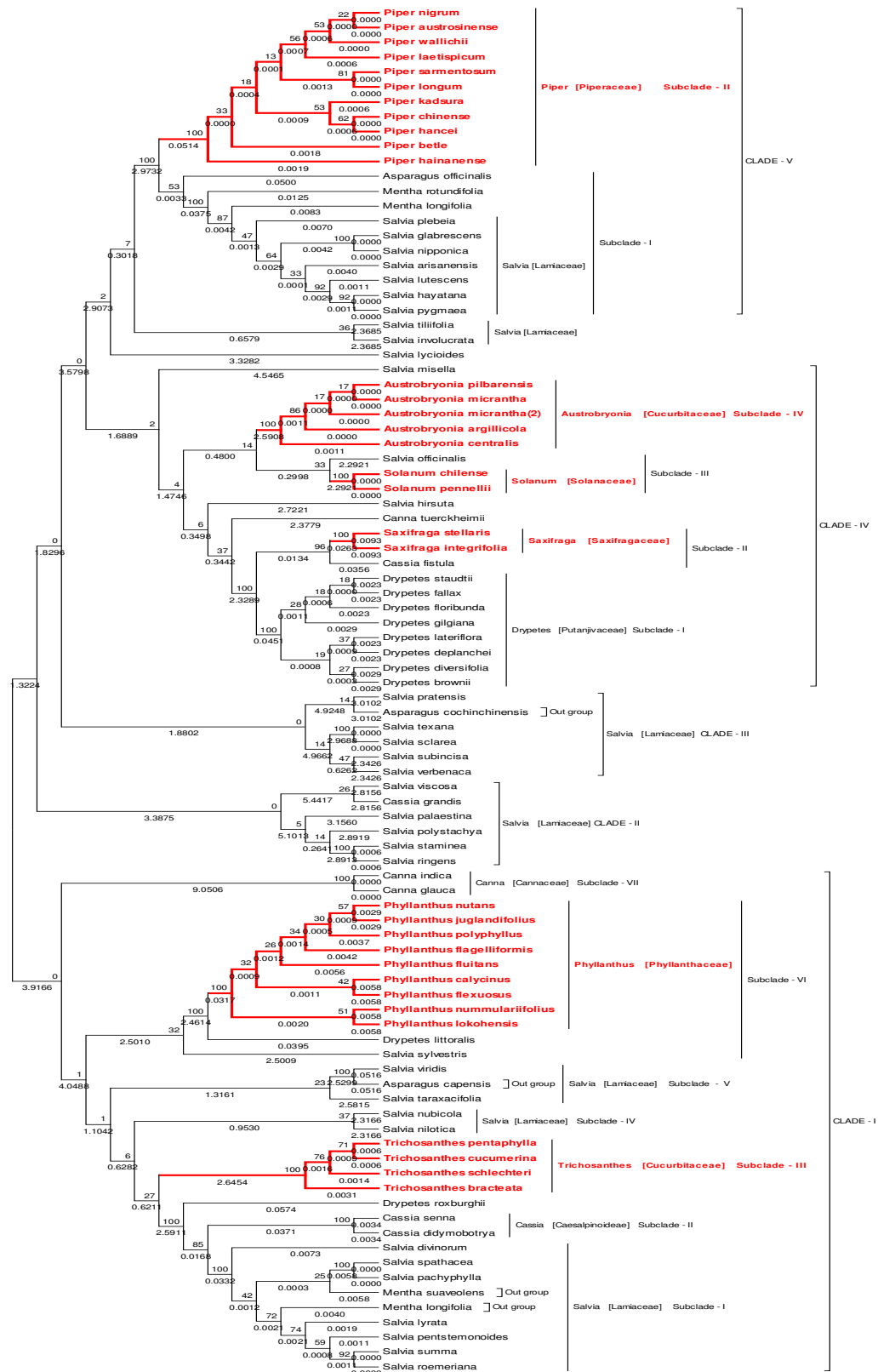


Fig -1 Phylogenetic tree from 92 *rbcL* sequences representing 90 taxa of family Cucurbitaceae, were inferred using UPGMA method. Bootstrap percentages and branch lengths are indicated above and below the branches, respectively.

Molecular phylogeny of angiospermic plant families using RBCL gene sequences

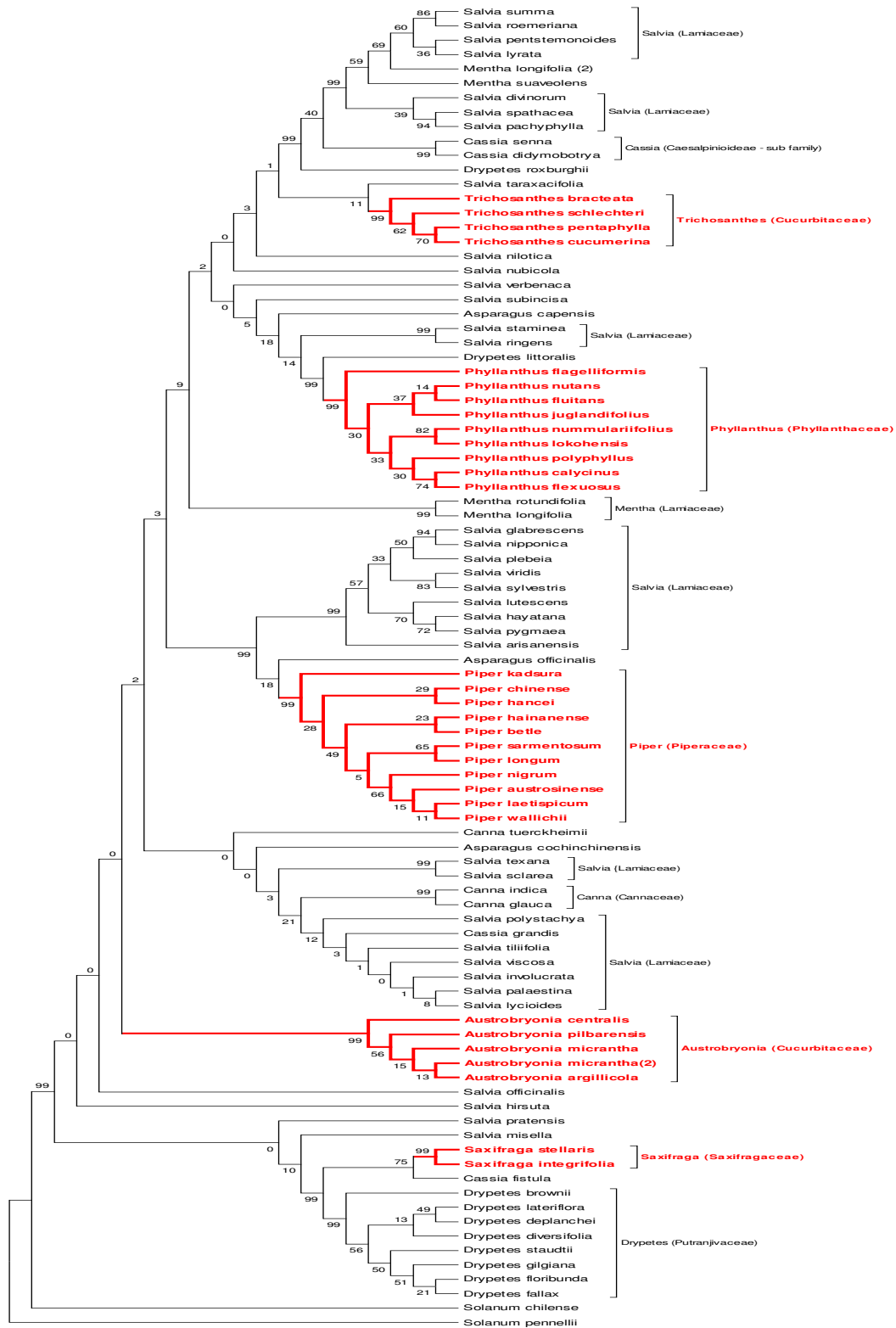


Fig-2 Phylogenetic tree from 92 *rbcL* sequences representing 90 taxa of family Cucurbitaceae, were inferred using the Maximum Parsimony method (length = 14029, CI = CI = 0.187540; RI = 0.733592; RCI = 0.137578 (for all sites).





Fig -3 Phylogenetic tree from 92 *rbcL* sequences belongs to 90 taxa of 12 genera and 10 families by the Neighbor-Joining method. Numbers above branches indicate percentage of 500 bootstrap replications. Horizontal bar indicates the nucleotide substitution scale

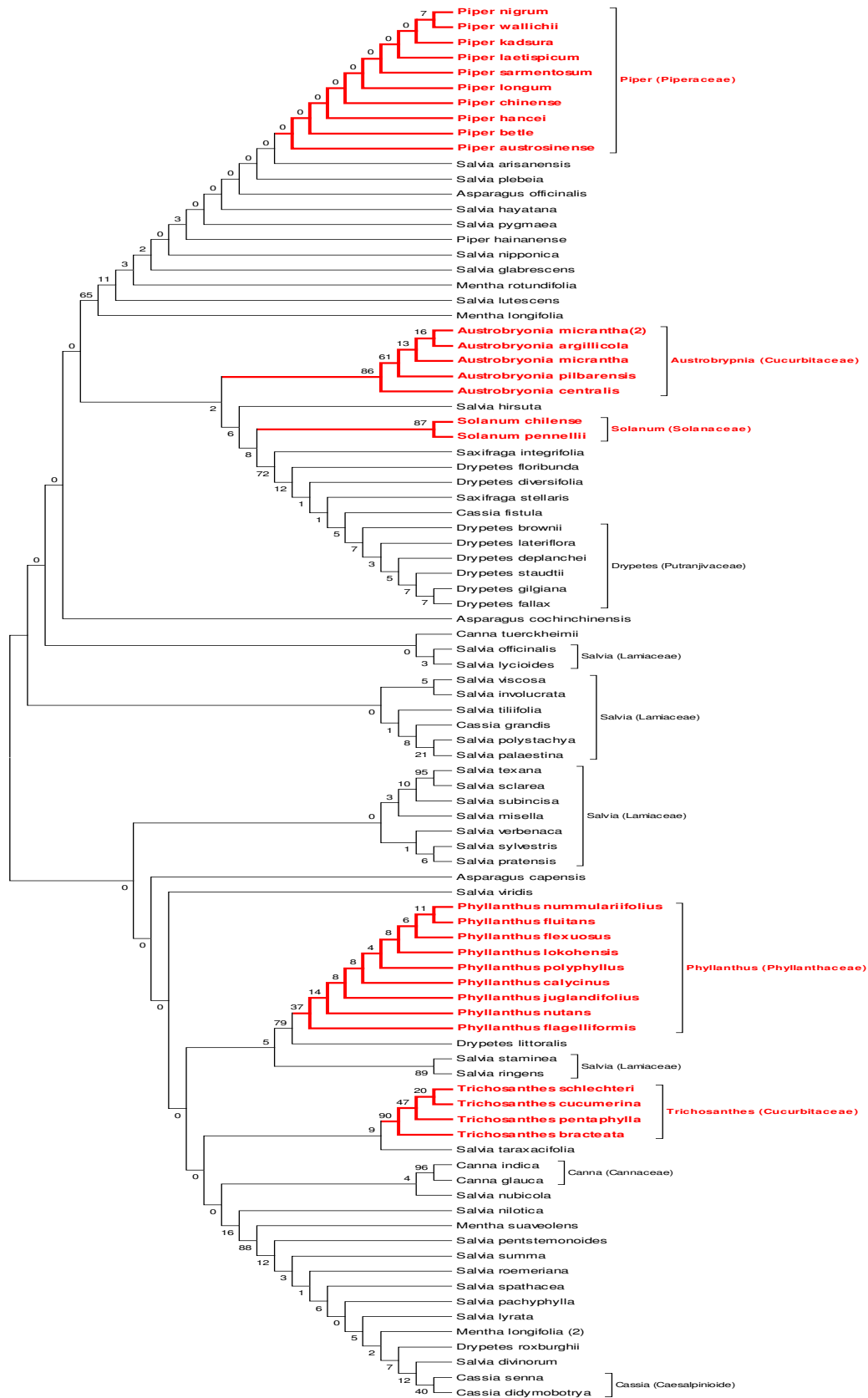


Fig -4 Phylogenetic tree from 92 *rbcl* sequences belongs to 90 taxa of 12 genera and 10 families using Minimum Evolution method. Numbers above branches indicate percentage of 500 bootstrap replications.