# A study on systematics and phylogenetic relationships of Asian *Leucas* (Lamiaceae: Lamioideae) based on molecular methods

Submitted to the
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the requirement for the degree of

**DOCTOR OF PHILOSOPHY IN BOTANY**

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**DEPARTMENT OF BOTANY**
**UNIVERSITY OF CALICUT**
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**2017**

# CERTIFICATE

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Certified that the thesis entitled **“A study on systematics and phylogenetic relationships of Asian *Leucas* (Lamiaceae: Lamioideae) based on molecular methods"** submitted by Mr. Vimal K.P., for the degree of **Doctor of Philosophy in Botany** of the University of Calicut is a bonafide record of research work done by him in this department under my supervision. This has not previously been formed the basis for the award of any degree/diploma.

Calicut University **Dr. Sunojkumar P.**

13 September 2017

# DECLARATION

I, Vimal K.P., hereby declare that the thesis entitled **“A study on systematics and phylogenetic relationships of Asian *Leucas* (Lamiaceae: Lamioideae) based on molecular methods”** submitted to the **University of Calicut**, for the award of the degree of **Doctor of Philosophy** in **Botany** is a bona fide record of the original research work carried out by me under the supervision and guidance of **Dr. P. Sunojkumar**, Assistant Professor, Department of Botany, University of Calicut and that it has not been submitted earlier either in part or full for the award of any degree/diploma to any candidate of any University.

C. U. Campus **Vimal K.P.**

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# CHAPTER 1

# INTRODUCTION

# INTRODUCTION

Systematics can be described as classifying organisms based on their evolutionary relationships. Phylogenetic analysis produces an evolutionary tree that shows how species are related to each other. Phylogenetics is the branch of science which deals with analysis of morphological or molecular sequencing data to understand the evolutionary relationships among groups of organisms. In the beginning, the phylogenetic tree construction was based on the classical morphological characters of plants. These studies involve phenetics, which use binary characters or multiple characters with many character states in the analysis. Numerical taxonomy or distance based approach has given its way to the molecular taxonomy, which uses many characters. Morphological data has been replaced by DNA or protein data. There are two different types of approaches for phylogenetic tree construction: distance approach and character state approach. Character state approach is an advanced method over distance approach.This approach will give us a good phylogeny with good and robust tree topology.

## Genus *Leucas*

Family Lamiaceae comprises of 236 genera and about 7173 species and are almost cosmopolitan in distribution. The genus *Leucas* was initially included in *Phlomis* by Linnaeus (1753), Forskal (1775), Retzii (1779), Jacquin (1781), Swartz (1788), Vahl (1794), Willdenovio (1800) and Roth (1821). Brown (1810) erected *Leucas* as a separate genus and transferred species to *Leucas*. The most comprehensive monograph of Labiatae with infra familial classification and descriptions of every then known species is that of Bentham (1832-36) which he later modified in De Candolle's *Prodromous* (1848) and the *Genera Plantarurm* (1876). He placed the genus *Leucas* in the tribe Stachydeae and subtribe Lamieae near to *Otostegia* and *Lasiocarys* together with other 21 genera. The genus *Leucas* R.Br. *s. l.* is one of the largest genera in subfamily Lamioideae of the angiosperm mint family Lamiaceae with about 100 species occurring on dry or disturbed ground in tropical to southern Africa and tropical and subtropical parts of Asia, South China and upto Australia (Harley *et al.,* 2004). The highest species richness and endemism are found in the northern part of East Africa and a second area of species richness is found in the Indian subcontinent, more specifically in southern India (Ryding, 1998; Singh, 2001) and especially in Western Ghats. Based on morphological studies it was suggested that the Asian and African *Leucas* species are not closely related despite being congeneric (Singh, 2001), and a cladistic analysis of a large morphological data set showed *Acrotome* and *Leonotis* to be nested within a group of African *Leucas* and the monotypic Arabian genus *Isoleucas* and a group of African *Otostegia* to be closely related to the *Leucas* group.

Calyx characters are used as key character for identification of species in genus *Leucas*. They also possess white flower with lower lip of corolla longer than the beared upper lip.

## Medicinal importance of the genus *Leucas*

The genus *Leucas* is known for its medicinal value, and used widely to cure many diseases in Ayurveda. In Sanskrit literature *Leucas* is known as ‘Dronapushpi’, and ‘Vaikundam’. In Hindi it is known as ‘Chota halkusa’. In Tamil ‘Tumbai’ and in Malayalam it is known as ‘Thumba’. Grind few leaves of *Leucas aspera* and mix it with butter milk, it is a good medicine against *Enterobius vermicularis.* Grind the whole plant and consume it for curing stomach pain and gas trouble. Grind whole plant and mix it with boiled butter milk and drink regularly to cure piles. Those who are suffering from jaundice can use the juice made from leaves as eye drops to reduce the yellow colour of the eyes. Grind few flowers of *Leucas aspera* with cumin seeds and civet cat semen and make it as a small tablet and consume it for curing hiccups. Dropping of the juice of the leaves inside nose will helps to cure severe coughs. Juice of the leaves is very good to prevent dandruff.

## Rationale for undertaking this work

The classical taxonomists Bentham (1830, 1834 and 1848) in his comprehensive works on the genus *Leucas* treated species found in Asia and those in Africa under separate infrageneric sections. This way of treatment was based on the character differences, he observed on the genus found in these two different geographical areas. Later Hooker (1885) and Gamble (1925) followed Bentham's concept of treating Indian species under four sections and this was supported by Mukerjee (1940) in his revision. Later, the taxonomic revision of *Leucas* undertaken by Sebald (1977a, 1977b and 1978) supported Bentham's conclusion and treated African species under 13 different sections and opined that Asian species share certain unique characters different from African species. He treated two Asian species found in Africa in different section from that of African species. Ryding (1995) clarified this fact clearly based on the pericarp anatomical data of species found in Asia and Africa. Later Ryding (1998) completed morphology based cladistics and the result clearly showed a monophyletic nature to Asian species. Based on his work he concluded an independent status to Asian species. Very recently Scheen & Albert (2009) completed molecular phylogeny of *Leucas* and results obtained clearly showed a monophyletic nature of Asian species and Paraphyletic nature to species found in Africa and Arabia. Based on this work they opined that the genus name *Leucas* is referable only to Asian species (Scheen & Albert, 2007).

In their molecular phylogenetic studies Scheen & Albert (2009) considered only 7 Asian taxa in analysis. Since there are more than 40 species of *Leucas* in Asia, a fair representation was not given in their work. Hence a continuation work by including maximum species from Asia is required to identify the phylogeny the genus as a whole. It is based on this problem the present work was undertaken with a view to give maximum representation of Asian taxa in the phylogenetic analysis with the following objectives.

**Objectives of the proposed study:**

1. Trace the molecular phylogeny of *Leucas* R.Br. with far better sampling from Asia.
2. Further investigation of the monophyly of Asian *Leucas* (*Leucas s.str.*)
3. Identify the molecular groups present in the Asian *Leucas* (*Leucas s.str.*) for systematic treatment.
4. Investigate the infrageneric relationship within *Leucas s.str*.

## CHAPTER 2

# REVIEW OF LITERATURE

# REVIEW OF LITERATURE

## Lamiaceae

Lamiaceae has long been established as one of the most distinctive angiosperm families, which can easily recognizable by its most unique distinguishing morphological characters like supra-reticulate pollen and fruit composed of nutlets (Bentham, 1876; Briquet, 1895–1897).

Information about the origin and evolution of Lamiaceae is controversial. Cantino (1992) pointed out that majority of Lamiaceae with gynobasic-style is supposed to be evolved in south China or Indomalesia. In 17th century, Bentham (1830, 1832-1836, 1848, 1876) divided the family Lamiaceae into eight tribes and two of the tribes Satureineae and Stachydeae was again sub divided into four sub tribes each. The genus *Leucas* was placed under the subtribe Lamieae of the tribe Stachydeae. Sectional classification of Bentham was purely based on floral characters.

Following Bentham, Biquet (1895-1897) used his own concepts to make alteration in the classification of Lamiaceae, which was widely accepted in recent years. He divided the family into seven subfamilies such as Ajugoideae, Prostantheroideae, Prasioideae, Scutellarioideae, Lavandulideae, Stachyoideae and Ocimoideae. The subfamilies Ajugoideae, Stachyoideae, and Ocimoideae were divided into several tribes and subtribes. He placed genus *Leucas* under the subtribe Lamiinae of the tribe Stachydeae, under the subfamily Stachyoideae. Out of the eight tribal classification of Bentham, four tribes (Nepeteae, Prasieae, Prostanthereae and Ajugoideae) and five subtribes (Ocimoideae = Lavanduleae, Satureieae = Pogostemoneae, Stachydeae = Melitteae, Stachydeae = Marrubieae, Stachydeae = Lamieae) are still recognizable at various hierarchical levels in Briquet's scheme. Briquest identified a large subfamily Stachyoideae (Lamioideae as per ICBN, Sanders and Cantino, 1984). Based on further evidences from palynological data (Abu-Asab & Cantino, 1992) and cladistics analysis of morphological and anatomical data, Cantino (1992) considered the family is polyphyletic. Conventionally his family has been classified according to their unique characters like gynobasic-style, supra-reticulate pollen and fruits composed of nutlets. The closest relative of Lamiaceae is found to be Verbenaceae. The position of style is an important synapomorphic character for separating Lamiaceae from Verbenaceae. There is no true disjunction between groups of Lamiaceae, like the Ajugoideae and Prostantheroideae, as they resemble Verbenaceae in having terminal style (Briquet, 1895-1897). These are considered as verbenoid Lamiaceae. This deadlock has been resolved by Junnel (1934) and he suggested that Lamiaceae bears only dorsal placentation and in Verbenaceae it is dorsal and ventral. He also suggested that only the Verbenoideae with ventral placentation should be retained in the Verbenaceae and rest of family with dorsal placentation should be transferred to Lamiaceae (Cantino, 1982, and 1992) and (Abu-Asab & Cantino, 1992, 1993a and 1993b). Cladistic and palynological studies had proved the close affinity of these two families.

Erdtman (1945) classified Lamiaceae on the basis of palynological features. He divided the family into two subfamilies viz. Lamioideae with tricolpate or tetracolpate pollens shed in two celled stage and Nepetoideae with hexacolpate or octa to dodecca colpate pollens shed in three celled stage. Leitner (1942) noticed the correlation between the number of colpi in the exines and the number of nuclei in the pollen grains. He also noticed a binucleate tricolpate grains and trinucleate hexacolpate grains. Erdtman's classification showed remarkable similarity with Bentham's (1830, 1830-1836, 1848, 1876) classification. Bentham's four tribes considered under subfamily Lamioideae and other three tribes under sub family Nepetoideae.

Bentham placed the genus *Leucas* under the subfamily Lamioideae. Classification based on essential oils had been completed by El-Gazzar & Watson (1970b). They noticed two phytochemically important groups in Lamiaceae; oil rich and oil poor. They belong to Nepetoideae and Lamioideae respectively. Phenetic studies conducted by El-Gazzar & Watson (1970a) shown to be congruent with the classification system of Erdtman (1945). Lamioideae is characterized with spatulate embryo, albuminous mature seeds and cotyledon length equal or greater than width. But in the case of Nepetoideae the seeds are exalbuminous; cotyledon length is less than its width and possesses an investing type of embryo. Presence or absence of chemical metabolic products was also used by Cantino & Sanders (1986) as a synapomorphy up to a certain extent. Lamioideae contains iridoid glycosides, limited amount of unsaturated seed oil and low volatile terpenoid content. Rosmarinic acid is absent and myxospermy is infrequent in this subfamily. On the contrary, the subfamily Nepetoideae, contains high amount of terpenoid content, highly unsaturated seed oil and rosmarinic acid. Myxospermy is frequent and iridoid glycosides are absent in this group. These two characters might have been the important synopomorphy in distinguishing Nepetoideae from Lamioideae. Cantino & Sanders (1986) opined that eventhough Erdtman's classification had strong character support it had shown a contradiction with widely accepted Briquet's system of classification. So that system should not be used.

Later, Wunderlich (1967) proposed a different classification scheme of Lamiaceae. He classified Lamiaceae into six important subfamilies such as Ajugoideae, Scutellarioideae, Stachyoideae, Saturejoideae, Stachyoideae and Prostantheroideae.

In 19th century, Briquet (1895-1897) had placed the genus *Leucas* in the subtribe Lamiinae together with *Ballota* L., *Lagochilus* Bunge ex Benth., *Leonotis* (Per.) R.Br., *Moluccella* L., *Otostegia* Benth., *Phlomis* L. and *Roylea* Wall. ex Benth. Genus *Leucas* and its allied genera were placed under the subfamily Lamioideae Cantino *et al.* (1992).

**Genus *Leucas* R.Br:**

The genus *Leucas* is one of the largest genera of the subfamily Lamioideae and with 100 species found in dry or disturbed regions of Tropical to Southern Africa, India, South China, Iran South East Asia upto Australia (Harley *et al.,* 2004). Northern part of East Africa is recognized as the highest species richness area in the world and Indian sub-continent is found to be the second area of species richness, more specifically the Southern peninsular India (Sebald, 1980; Ryding, 1998; Singh, 2001). Majority of the species are restricted to one of the two areas of Africa and Asia.

By a series of works, Sebald (1977a, b; 1978, 1980) revised the genus *Leucas* found in Africa. Sebald (1980) introduced 13 sections and a new subdivision to the genus. He also concluded that North East tropical African-Arabian *Otostegia* sect. *Otostegia* as the closest relative of *Leucas* and opined that the condition of easily breaking of the fruiting calyces were suggested to be the common origin in *Leucas* sect. *Physoleucas* and *Otostegia* section *Otostegia.* Species of *Leucas* found in Socotran Islands especially Yemen, shows remarkable variation and speciation. The monotypic *Ortholeucas* O. Schwartz from South Yemen was considered as the relative of the genera *Leucas* and *Otostegia.*

Ryding (1993) compared the pericarp anatomy of many species of *Leucas* and related genera like *Acrotome, Leonotis, Otostegia*, *Isoleucas* and found that they consists of thick sclerenchyma cells. But most of the African *Leucas* species and genera like *Acrotome* and *Leonotis* have a thin to strongly reduced sclerenchyma region and were suggested to be closely related. Asian species *Leucas hyssopifolia* Benth. was closer to the section *Leucas* than to *Plagiostoma* on the basis of the isodiametric cells of exocarp.

### Phylogenetic Systematics of Lamiaceae

Phylogenetic approach to the classification of the family Lamiaceae was started by the effort of El-Gazzar & Watson (1970a). They introduced and popularised numerical phenetic system of classification to this family. Different types of data such as morphology, anatomy, cytology, palynology, phytochemistry, etc were taken into account. They also concluded that type of stomata was also a good reliable character to determine taxonomic relationship at infra-generic level than the classification at higher levels.

Later on Cantino & Sanders (1986) thoroughly analyzed different classification systems and they came to a conclusion that in the case of tribal system of classification one should follow Erdtman's system of classification, and for infra-tribal classification should be based on Bentham's (1830, 1832-1836, 1848, 1876) classification. At present natural classification based on the then phylogenetic knowledge also had given the same result of Erdman's and Bentham's system of classification.

Olmstead (1989), using morphological and molecular data independent analyses were conducted in order to get phylogenetic relationships in the *Sutellaria angustifolia* complex. Phylogeny based on morphology was less resolved and was found to be less congruent with the molecular based phylogeny and made a conclusion that one portion of the phylogeny was well resolved using morphological data and one part was resolved using molecular data. The best estimate of phylogeny was also used to interpret the evolution of reproductive and vegetative characters, biogeography and habitat preference.

Nunez *et al.* (1990) proposed a new taxonomic division of the section *Sideritis* on the basis of chemical, morphological and cytological characters using cladistic analysis. *Angustifolia, Arborescens, Camarae, Chamaedryfolia, Flavovirens, Grandiflora, Gymnocarpae, Hirsuta, Lacaitae, Leucantha, Linearifolia, Ovata, Serrata, Scordioides* and *Stachydioides* are the new sections proposed by him. He also discussed the possible evolutionary pathways in his work.

Ryding (1992) examined the structure of the pericarp in order to reconstruct the phylogeny within the family Lamiaceae subfamily Nepetoideae of the tribe Ocimea. Pericarp structure of the 205 species, representing 43 out of the about 50 genera has been keenly inspected in and took into account for the analysis. The very basic structure of pericarp itself showed the similarity of the pericarp of Ocimeae and Nepetoideae. In the nutlet pericarp anatomy, the exocarp showed both mucilaginous and non-mucilaginous cells. Subdivision of Ocimeae showed correlation with the differences observed in pericarp anatomy by Briquet (1895). In this study he showed that few exceptional crystals were present in the bone cells of the subtribes Hyptidinae, Ociminae and the genus *Aeollanthus* in subtribe Plectranthinae. Plectranthinae except *Alvesia, Isodon* and *Siphocranion* has a plate like content in the mucilaginous cells but it is absent in other Labiatae. Systematic positions of the above mentioned genera were also analysed. On the basis of their pericarp anatomy; *Hoslundia, Fuerstia, Catoferia, Nosema, Benguellia, Octomeron* and *Ceratanthus,* Ryding tried an attempt to classify the genera, and he found that all of them were belonged to Ociminae, and *Neohyptis* and some of them to Plectranthinae. He had also suggested an informal method of classification of Ociminae. With an attempt to classify by considering the pericarp characters and the distribution of stamens a few genera *Capitanya, Pycnostachys* and *Solenostemon* were thought to be originated from *Plectranthus* of Ocimeae and they also have a larger plate like material present in the mucilaginous cells.

Ryding (1994) has intensively examined pericarp structure and Phylogeny of certain genera representing the genera of the subfamily Pogostemonoideae. The structure and shape of the pericarp has been closely examined in *Anisomeles, Colebrookea, Comanthosphace, Leucosceptrum, Pogostemon* and *Rostrinucula.* The structure of pericarp of *Anisomeles, Colebrookea* and *Pogostemon* were showed remarkable similarity with that of the subfamilies Lamioideae or Nepetoideae. Closely related three genera *Comanthosphace, Leucosceptrum* and *Rostrinucula* have a divergent type of slightly and indefinable pericarp, and scalariform celled secondary thickenings in the mesocarp. He concluded that the three closely related genera are suggested to be irrelevant in Pogostemonoideae and their systematic position is debatable.

Ryding (1995) noticed peculiarity of pericarp structure of different Lamiacean- Verbenacean complex and noticed that it could also be included in the phylogenetic analysis of Lamiaceae. Structure of pericarp was investigated in 158 species of the families Lamiaceae and Verbenaceae and 221 out of 262 genera of Lamiacae were selected for cladistic analyses on the basis of pericarp structure. Different groups formed by similarities in pericarp characters were listed out: 1. the gynobasic-styled labiates (subfamilies Pogostemonoideae, Lamioideae, Nepetoideae); 2. a Lamioideae-Pogostemonoideae-group; 3. Nepetoideae; 4.a *Westringia-Hemigenia-Hemiandra-Microcorys* group (in subfam .Chloranthoideae); 5. a *Lepechinia-Chaunostoma*-group (in Nepetoideae); and 6. a *Prunella-Cleonia*-group (in Nepetoideae).

Wagstaff *et al.* (1997) had thoroughly examined the phylogeny of Labiatae and Verbenaceae using *rbcL* molecular marker. The results supported monophyly of Labiatae *s.l.,* which includes Labiatae, Symphoremataceae and also includes subfamilies Caryopteroidiae, Chloranthoideae and Viticoideae of the Verbenaceae. Subfamily Verbenoideae did not form a monophyletic clade with Labiatae *s.l.* This also helped them to interpret character evolution.

Ryding (1998) did a cladistic analysis of gross morphological, cytological and anatomical data within the genus *Leucas*. He had chosen 47 different characters for the analysis.

Ryding (1998) had thoroughly investigated the phylogeny of the *Leucas* Group (Lamiaceae). Cladistic analyses were employed to investigate the inter-relationships within *Leucas* group using gross anatomical, cytological and morphological data. Sampling pattern includes fifty species including related genera like *Acrotome, Ballota, Ortholeucas, Leonotis, Leucas, Moluccella* and *Otostegia.* Separate analyses were conducted. The result suggested that characters with overlapping states and continuous characters also contain ample amount of phylogenetic information. The result revealed *Leucas* as paraphyletic and the endemic *Leucas* species from Asia excluding Arabia and Socotra as monophyletic. The monophyly of the endemic *Leucas* species from Asia excluding Arabia and Socotra is also proved. He suggested a new status to the African *Leucas milanjiana* Gürke and excluded this from the Asian section *Plagiostoma.* According to these studies of Ryding (1998) *Leucas* is suggested to have originated in North Eastern tropical Africa and migrated all the way from this area over Arabia to the Indian subcontinent.

Lindqvist *et al.* (2002) thoroughly analysed the different features of the Hawaiian Island endemic mints and found that they are the major Island population; showing remarkable diversity in floral, fruit and other vegetative features. They evaluated the origin of the Hawaiian mints using phylogenetic analysis of DNA data from the nuclear ribosomal *5S* non-transcribed spacer (Cron *et al.,* 1996 and plastid *rbcL 1F* (Fay *et al.,* 1997)., *1368R* (Fritsch *et al.,* 2001) and *trnL* (Taberlet *et al.,* (1991) intron loci and using the software NONA (Goloboff, 1998).

Barber *et al.* (2002) identified the Mediterranean region as the haven of Lamiacean plants. It consists of 10 species of annuals and perennials distributed mainly in Mediterranean region. Majority of the Mediterranean Lamieacean plants are coming under the continental subgenus *Sideritis* which consists of two perennial (*Sideritis* and *Empedoclea*) and two annual (*Burgsdorfia* and *Hesiodia*) sections. Out of these perennials, 23 species are woody perennials and are endemic to the Macronesian Islands like the Canary Islands and Madeira. Taxon sampling include 25 continental taxa representing all four sections of subgenus *Sideritis* and 7 island taxa drawn from the Macaronesian subgenus *Marrubiastrum*. Both chloroplast and nuclear markers are used to determine the continental origin of the insular group. The intron *trnL* were amplified using primer pairs C–D and *trnT–trnL* intergenic spacer using A–B of the universal primer designed by Taberlet *et al.* (1991). The *ITS* region were also amplified for the purpose. The complete *ITS* region, including the *5.8S* gene, was also amplified in many cases using primers *ITS-4* (White *et al.,* 1990) and *ITS-1A* (a modification of *ITS5*; Downie & Katz-Downie, 1996). Poor forward sequences of the *ITS-1* region for 11 taxa also were again subjected to an additional sequencing reaction using the reverse primer *ITS-2* of White *et al.* (1990). Phylogenetic analyses were conducted using PAUP version 4.0b4a (Swofford, 1999). In all the analyses it is found that *Sideritis cossoniana*, an annual species hails from Morocco, shows a closest relativeness to the Macaronesian group. The phylogenies also had given an implication that a distinct increase in woodiness among the Macaronesian species relative to their continental congeners, giving further support for the secondary nature of woodiness character in island plants.

Prather *et al.* (2002) investigated the phylogenetic relationships of 16 species of *Monarda* (Lamiaceae) using sequences of the internal transcribed spacer regions of nuclear ribosomal DNA. In the analysis *Mentha* and *Thymus* were used as outgroups and *Blephilia, Clinopodium, Conradina, Hesperozygis, Monardella, Pycnanthemum, and Ziziphora* were also included in the ingroup taxa to test the monophyly of the genus *Monard.* Parsimony analyses were conducted using PAUP version 4.0b4a (Swofford, 1999). After thorough analysis it was found that molecular phylogeny was showing a complete congruence with the earlier infrageneric classification of the genus. Their studies proved once again that the hypotheses of hybridization between *Monardella fistulosa* and *Monardella lindheimeri* in Texas. Despite considerable morphological variations among infra-generic species, especially in floral characters, little molecular diversification was found between the species groups. They could also observe the intra-specific polymorphism in *ITS* sequence in over half of the species examined during the analysis, and may be due to ancestral hybridization, polymorphism, or detection of paralogous loci.

Lindqvist *et al.* (2003) published a paper which showed Cladogenesis and reticulation in the Hawaiian endemic mints (Lamiaceae). In her work she analysed Hawaiian endemic mints which consists of 58 species of dry-fruited *Haplostachys* and fleshy-fruited *Phyllostegia* and *Stenogyne*, and which all constitutes a major island radiation that were likely to be originated from polyploid hybrid ancestors in the temperate North American *Stachys* lineage. Morphological and ecological diversity among taxa were distinguishable, but sequence variation in the nrDNA 5S non-transcribed spacer was found to be remarkably low. Later on, the analyses using standard parsimony result showed phylogenetic resolution among accessions of insect-pollinated *Phyllostegia* and bird-pollinated *Stenogyne*. Multiple inter-island dispersal events might have created a diversified environment. In order to consider them in the data set these ambiguities were recoded as new character states and further analyses were done. The *5S-NTS* primer was used for the amplification purpose. Cladistic analysis was done using non additive parsimony analysis using NONA (Goloboff, 1998), and geographical and morphological character state optimizations were performed using WinClada (Nixon, 2002). Finally he came into a conclusion that the relatively greater extent of lineage sorted polymorphisms in *Stenogyne* may indicate selective differentiation from other important fleshy-fruited taxa, perhaps through the agency that highly specific avian pollinators, might have restricted the gene flow with other Hawaiian mint morphotypes.

Paton *et al.* (2004) studied phylogeny and evolution of basils and allies (Ocimeae, Labiatae) based on three plastid DNA regions such as *trnL* intron, *trnL–trnF* (Taberlet *et al.*, 1991) intergenic spacer and *rps16* (Oxelman *et al.,* 1997) intron. They used Bayesian Inference method to construct the phylogenies. They analysed the distribution of morphological, pericarp anatomy, chemical, and pollen characters and the geographical distribution of the clades. Tribe Ocimeae is monophyletic and could easily be diagnosable with its key morphological synapomorphies. There are monophyletic clades within Ocimeae that are subtribes: Lavandulinae, Hyptidinae, Ociminae, and Plectranthinae. Among these only Lavandulinae has clear non-molecular synapomorphies others do not have. Most of the currently recognised genera in Lamiaceae are not monophyletic. Specialised floral structural morphology consistent with sternotribic pollination is most common feature in Ocimeae, but independent departures from this particular model are also noticed. Buzz pollination is also dominant in some species, and were observed within Lamiaceae. Phytochemical compounds like Quinone diterpenoids and flavones in the leaf exudates and other plant parts differ in their distributions across each lineage of phylogeny. Mapping geographic distribution on to an ultrametric phylogenetic tree reconstructed using more specific non-parametric rate smoothing supports an Asiatic origin for Ocimeae.

Trusty *et al.* (2004) used molecular data to test the biogeographic connection of the well-known Macaronesian genus *Bystropogon* (Lamiaceae) to the New World Lamiaceae. Previous taxonomic studies based on morphology concluded that this genus is closely related to the western South American *Minthostachys.* Phylogenetic analyses were conducted using internal transcribed spacers (*ITS*) and *5.8S* subunit of nuclear ribosomal DNA, *trnL* intron and *trnL-trnF* intergenic spacer of the chloroplast genome. Phylogenetic analyses were conducted using 33 out of 72 genera in the Mentheae tribe. Combined data set were subjected to Maximum Parsimony analysis resulted in 63 most parsimonious trees. The strict consensus tree of this analysis showed that *Bystropogon* is sister to the Old World taxa *Acinos, Ziziphora,* and *Clinopodium vulgare* with moderate bootstrap support of 74%. Separate analyses were conducted in order to reconstruct the phylogeny using each gene. It was observed that the *ITS* and *trnL-F* data sets do not agree with the sister group to *Bystropogon,* and could see that none of the phylogeny supports a sister relationship with *Minthostachys*. The cpDNA phylogeny strongly supported a relationship between *Bystropogon* with a clade of New World mint taxa with 90% bootstrap support. Apparent conflicts between the chloroplast and nuclear characters observed in the phylogenies. Combined analysis resulted that all of the *Mentheae* genera sampled in this study form a monophyletic group with 100% bootstrap support and a derived clade of ten New World genera were also found.

According to the study conducted by Bräuchler *et al.* (2005) on the polyphyly of the genus *Micromeria* using cpDNA data, they used *trnK* (Johnson & Soltis, 1994) and *trnL-F* molecular markers for the amplification purpose. Mediterranean region are well known for the genus *Micromeria*

(Lamiaceae, Nepetoideae). To test the monophyly of the genus and to expound it's phylogenetic relationship with sub-tribe Mentheae Bräuchler *et al.* (2005) performed parsimony analyses of *trnK* intron (Johnson & Soltis, 1994) sequence on 51 taxa belongs to 15 genera of Nepetoideae and two genera of subfamily Ajugoideae. Topology of the trees reconstructed conceded a well-supported "core group" with four distinct lineages with convincing Bootstrap support. The first lineage comprises three species of *Saturejas;* the second one included the sampled taxa of the genus Old and the New World *Clinopodium.* The genus *Clinopodium* was found to be paraphyletic with respect to *Monarda* and two species of *Micromeria* of the section *Pseudomelissa*. The third clade which constitutes a group contains all samples of the remaining *Micromeria* species. Result of the analyses were end up with some sort of ambiguity like a Western lineage taxa from North West Africa, the Balearic, and the Canary Islands, were grouped together and found to be sister to an eastern lineage with species distributed from South East Asia to the Western Mediterranean region. The genera *Thymbra, Thymus*, and *Origanum* were grouped together and formed a separate clade. Combined analysis using a reduced dataset of *trnK* (Johnson & Soltis, 1994) and *trnL-F* sequences increased support for the infrageneric resolution within *Micromeria* and helped to reduce the ambiguity to a minimum. Phylogenetic reconstructions concluded that the genus currently circumscribed is polyphyletic in nature. Results were discussed in various contexts of biogeography, morphology, karyology and ended up with a unanimous decision of the necessity of removing the section *Pseudomelissa* from *Micromeria*.

Trusty *et al.* (2005) conducted a molecular phylogenetic study of the Macaronesian-endemic genus *Bystropogon* (Lamiaceae) of paleo islands. Main focus areas were ecological shifts and the inter-island colonizations of the genus. One nuclear molecular marker *ITS* and two chloroplast introns *trnL* gene and *trnL-trnF* were used for the analyses. Phylogenetic data proved that *Bystropogon* has a recent origin. This phylogenetic pattern was followed by most of the species endemic to the palaeo-islands of Tenerife. Over time, the two sections currently sticked together in *Bystropogon* clade formed two monophyletic groups. Taxa belong to the section *Bystropogon* clade showed inter-island colonization restricted to the Canary Islands with ecological shifts only among three ecological zones. Canariense clade showed significant variation when compared to the other clades and showed interisland colonization both within the Canary Islands and in between the Canary Islands and other nearby Island Madeira. Speciations events are more likely to occur within this clade are confined to the dense forests. The genus has followed a colonization route all the way from the Canary Islands towards Madeira. Some of the Macronesian endemics are also following the same colonization route. Current infrasectional classification is incongruent with the molecular phylogeny. Major incongruence was the varieties of *Bystropogon canariensis* and *Bystropogon origanifoliu*s do not form two monophyletic groups. According to our knowledge it could have been formed two different monophyletic clades. The other defect was the widespread *Bystropogon origanifolius* appeared to be the progenitor of the other species in section *Bystropogon* with highly restricted distribution.

Rossello *et al.* (2006) conducted a study on the topic of phylogenetic systematics and intragenomic diversity of wild Rosemaries (*Rosmarinus officinalis,* Lamiaceae). In their study nuclear ribosomal sequences (*ITS*) were used to infer species boundaries and to analyse the phylogenetic patterns in wild Rosemaries (*Rosmarinus officinalis, Rosmarinus eriocalyx* and *Rosmarinus tomentosus*). Phylogenetic reconstructions of cloned sequences and test for monophyly suggested that both *Rosmarinus officinalis* and *Rosmarinus tomentosus* were monophyletic. But in the analysis another species *Rosmarinus tomentosus* clones were nested within a paraphyletic *Rosmarinus eriocalyx.*

Edwards *et al.* (2006) conducted a recent study on molecular phylogeny of Conradina and Other Scrub Mints (Lamiaceae) from the Southeastern USA and Evidence for hybridization in pleistocene refugia. South Eastern United States showed high endemism and comprised of six allopatric species. It is considered as the cradle of evolution of *Conradina* (Lamiaceae). Out of the six species four of them were critically endangered or threatened. The objectives of this study were to test the monophyly of *Conradina* and its relationship to other endemic mints of the South Eastern United States, to understand the structure and patterns of multifarious switch over diversification in *Conradina*, and to clarify the credibility of species relationships. DNA amplifications were done using actions to relax secondary structure. The *ITS-1*, *5.8S,* and *ITS-2* regions of the nuclear ribosomal DNA were amplified using the primers *N-ncl8S10* and *C26A* (Wen & Zimmer., 1996) *trnT-trnL* intergenic spacer region and the *trnL* intron were amplified using the A-B primer pairs and C-D Taberlet *et al.* (1991) respectively. Sang *et al.* (1997) developed primers for the *psbA-trnH* spacer region. The entire *trnK* intron region including the matK gene was amplified using the primers *trnK1F* Manos & Steele (1997) and *trnK2R* Johnson and Soltis (1994). The phylogenetic analyses using nuclear *ITS*, revealed the outcome strongly supported the monophyly of *Conradina*, in accordance with evidence from morphology based classical taxonomy. But the plastid sequence data do not support a monophyletic *Conradina* instead of that it placed the genus as paraphyletic to *Clinopodium, Stachydeoma,* and *Piloblephis.* Perhaps shared ancestral polymorphism might have caused the similar plastid haplotypes that were shared by different genera. In the analyses, a contradiction and incongruence between nuclear and plastid molecular data were noticed. The authors opined that it might be due to the varability of rapidly evolving sequence data from nuclear markers. Within *Conradina*, *ITS* sequence failed to resolve species relationships, and plastid sequence data do not support the monophyly among the most traditionally defined species of *Conradina* that are distinguishable by morphology as in classical taxonomy. More rapidly evolving primers are needed to clarify relationships in *Conradina* mint genus from the Southeastern United States.

Wilson *et al.* (2012) conducted a study and published an article on Molecular phylogeny and systematics of the Australian endemic *Prostanthera* (Lamiaceae) commonly known as mint bush. *Prostanthera* constitutes the largest genus of Lamiaceae in Australia. Many species are endemic to Australia. In order to examine the anomaly in the current classification of the genus based on the homology of the morphological characters, molecular analyses were carried out using chloroplast *trnT–F, ndhF–rpl32* and nuclear *ETS* sequence data for 71 species. Phylogenetic reconstruction was carried out using separate and combined datasets by using maximum parsimony and Bayesian inference methods. The result indicated that *Prostanthera* showed paraphyly with respect to *Wrixonia*, and the latter should be synonymised with the *Prostanthera*. Eventhough combinations of different datasets provided some sort of infrageneric resolution within *Prostanthera s.l.*, still ambiguity persists in its sections. The two sections *Prostanthera* section *Prostanthera* (Entomophilous) and *Prostanthera* section *Klanderia* (Ornithophilous) did not form a sister clade and both of them were found to be paraphyletic. All species of *Prostanthera* section *Klanderia* nested within *Prostanthera*. Section *Prostanthera* point out the credibility of the formation of a paraphyletic group. The phylogeny of *Prostanthera* based on molecular data was found to be incompatible with the morphology based classical Taxonomy.

Ryding (2007) found a remarkable correlation between calyx structure and amount of calyx fibers. He conscientiously examined calyces of 306 species comprising of 181 out of the 236 genera of Lamiaceae pinpointed particularly the characters like amount of fibres and xylem cells. Presence of much larger amounts of fibres and similar xylem cells in the calyx tube is the peculiarity of most of the species in the subfamilies Lamioideae and Scutellarioideae and it also helps to differ from most other labiates, particularly from Nepetoideae. This result revealed a credential support for the cladistic hypotheses based on plastid DNA showing that Lamioideae and Scutellarioideae are closely related than is related to Nepetoideae. The phylogeny within Lamioideae has been changed shortly after the introduction of this new character. It is found that high amount of correlation is also seen between the amount of fibres, calyx size, calyx width, aridity of the habitat and a ballistic dispersal mechanism in *Scutellaria*.

Walker & Kenneth (2007) described staminal evolution in the genus *Salvia* (Lamiaceae) and molecular phylogenetic evidence for multiple origins of the staminal lever. They concluded that in *Salvia,* independent origin of the staminal lever mechanism is established. It was observed that *Salvia* formed a clear polyphyletic clade, with five other related genera nested within it, and staminal evolution has proceeded in many other different ways in each of the three different lineages of *Salvia.* But in all the cases it resulted in peculiarly similar staminal morphologies.

Oliveira *et al.* (2007) carried out an interesting study in the genus *Dicerandra*, which is an endemic mint of the south eastern United States. Of the nine species, all are endangered or threatened. Mainly they are concentrated to sand-hill vegetation region and to the mosaic like scrub habitats. Molecular Phylogenetic analyses of 13 taxa of the genus *Dicerandra* were carried out on using nuclear and plastid molecular markers data. This analysis identified two strongly supporting clades, with four annuals and five perennials in separate lineage. During the analyses, the nuclear and plastid trees were found to be incongruent in their placement of two perennial taxa, *Dicerandra cornutissima* and *Dicerandra immaculata* var. *savannarum.* The authors opined that these kinds of disparity might have due to the lineage sorting and ancient hybridization. Based on these analyses, the widespread *Dicerandra linearifolia* was found to be polyphyletic with populations of *Dicerandra linearifolia* var. *linearifolia* and grouped together either in Western or Eastern clades. The western clade, with good populations of *Dicerandra linearifolia* var. *linearifolia* and *Dicerandra linearifolia* var. *robustior* occurs in the river drained areas toward the Gulf of Mexico. On the other side it was observed that eastern clade, comprising populations of *Dicerandra linearifolia* var. *linearifolia*, *Dicerandra densiflora*, *Dicerandra odoratissima*, and *Dicerandra radfordiana* consisting of all the perennial species, seen on the banks of rivers that are flowing to the Atlantic Ocean. This type of genetic population differentiation was reported for the first time in the case of present day land plants. A revised subgeneric classification was also done to get a clear picture of annual and perennial clades.

Barber *et al.* (2007) conducted a study on the hybridization in Macaronesian *Sideritis* (Lamiaceae). Incongruence of nuclear and chloroplast sequence datasets were noticed. The genus *Sideritis* consists of suffrutescent to woody perennials capable of establishing in various diverse ecological zones are the major plant radiations in the Macronesian archipelagos. Here in this study they used nuclear ribosomal DNA internal transcribed spacers (*ITS*) and *trnL* intron, *trnT-trnL* intergenic spacer plastid regions. Here the phylogenies built using independent data sets showed incongruence; i.e., nuclear marker phylogeny showed remarkable difference with that of the plastid phylogeny. It was opined that the root cause of incongruence is due to potential cytoplasmic introgression, and the authors suggested that hybridization might have played are important unavoidable incident in the evolution of *Sideritis* in the Macaronesian archepelago (Rieseberg & Soltis, 1991; Doyle, 1992).

Scheen & Albert (2007) conducted molecular studies of the subfamily Lamioideae. They found that most of the genera coming under Lamioideae do not form a monophyletic clade as currently circumscribed. To tackle this issue of monophyly in *Leucas,* taxonomically, a new genus *Rydingia* A.C.Scheen & V.A.Albert was described over here with four species transferred from *Otostegia.* The number of species of *Moluccella* L. is increased to three with the recognition of *Moluccella aucheri* (Boiss.) A.C.Scheen transferred from *Otostegia.* The number of species in *Isoleucas* O.Schwartz was increased from one to two with the recognition of *Isoleucas somala* (Patzak) A.-C.Scheen, transferred from *Otostegia*. Two important groups of *Leucas* were established as per the nomenclatural priority, they are *Leucas s.str.* and the African *Leucas* clade consisting of African *Leucas, Otogegia, Leonotis,* and *Ortholeucas* respectively.

Guerin (2008) studied the phylogeny of tribe Westringieae (Lamiaceae: subfamily Prostantheroideae) using a concatenated morphological and molecular data. The analyses were conducted to test monophyly of the tribe and found that the tribe is monophyletic and *Prostanthera labill* is found to be the sister to the remaining genera. Both *Hemigenia* and *Microcorys* are found to be polyphyletic. The typical section with a large group of Western Australia species of *Hemigenia* is also polyphyletic and reported to be placed in a separate clade. The author also suggested the formation of a new section of *Hemigenia* to include the type species *Hemigenia purpurea.*

A preliminary cladistic analysis was conducted by An-Ming (1990) to figure out the relationships between important families of the superorder

Lamiiflorae *sensu* Dahlgren. Several character complexes were inspected, and 29 very important informative characters were used for the study. Clethraceae, Oleaceae and Solanaceae were included as outgroup for further comparison and to determine the exact topology of the clade. Concatenated data of 23 ingroup families were used to reconstruct the data matrix. The cladistic parsimony program Hennig 86 by Farris (1989) was used to analyse the data. Three equally parsimonious trees were observed. Inter family relationship could not be resolved. The Retziaceae nested as the sister group to the remaining families. Half of the important genera formed and nested to form polytomy in the consensus tree. The monophyletic complex of families involving the Lamiales with the other families Callitrichaceae, Lamiaceae, Phrymaceae and Verbenaceae, and three isolated families Trapellaceae, Hippuridaceae, and Hydrostachyaceae. Within this complex, Lamiaceae and Verbenaceae came out as sister groups, like in Callitrichaceae and Hydrostachyaceae, with Hippuridaceae. Here Hippuridaceae considered as sister to Callitrichaceae and Hydrostachyaceae. Outcome of the analysis ended up with controversy.

Gobert *et al.* (2006) did reconstruction of the phylogeny of mints related to Mentha × piperita. In their study they used molecular markers such as nuclear DNA (*ITS*), chloroplast DNA (non-coding regions *trnL* intron, intergenic spacers *trnL-trnF,* and *psbA-trnH* and *AFLP* and *ISSR*, markers. Taxonomic sampling consisted of hybrids, diploid and polyploid genomes. Trees reconstructed were shown incongruent between *ITS*, chloroplast DNA, and *AFLP-ISSR*. One important conclusion is that DNA fingerprinting data were shown to be much more congruent with morphological classification.

Maki *et al.* (2010) studied phylogenetic relationships between the 14 taxa of *Isodon* collected from Japan and South Korea using 11 chloroplast DNA regions. Concatenated data consisted of 6478 base pairs. The resulted phylogenetic tree showed three main clades comprised of different sections. Complexity of the phylogeny might be due to lineage sorting of various chloroplast DNA regions due to the rapid divergence of the species within the genus.

Bräuchler *et al.* (2010) conducted a study on the molecular phylogeny of subtribe Menthinae. Due to higher amount of homoplasies in morphological characters, relationships between tribes, subtribes and genera within the subfamily Nepetoideae were poorly studied. The workers used DNA sequence data of the three chloroplast regions *trnK*, *trnL-F* intergenic spacer; nuclear *ITS* of 278 taxa, representing 38 out of 40 genera of the subtribe Menthinae and 11 outgroup genera. Character state approach like Maximum Parsimony and Bayesian analyses were performed to reconstruct the phylogeny based on nuclear and chloroplast sequence. Data obtained were incongruent in all the cases. In the independent analyses they used both the nuclear and chloroplast datasets, which supported three major lineages. They could see that the branching pattern itself was very incongruent among the data sets. This incongruence might have been due to chloroplast capture, incomplete lineage sorting, and ancient hybridization. The *ITS* phylogeny indicated that *Cyclotrichium* and *Mentha,* were closely related. It was also found that *Micromeria cymuligera* is close to *Mentha* and might have been a representative of this genus. *Acinos* species which was part of *Clinopodium* were found to be conjoined with species of *Ziziphora*, which created chaos. All the data set result proved monophyly of the New World taxa. It might have been due to long distance dispersal from the Old World to the New World rather than an explanation of the vicariance. *Bystropogon* regarded as the connecting link between two lineages. They thoroughly analysed the result and came into the conclusion that low level sequence divergence is the root cause of low phylogenetic resolution.

Ryding (2010) analysed the structure of pericarp and it influence in phylogeny of tribe Mentheae (Lamiaceae). Structure of the pericarp was investigated in 143 specimens belonged to 59 genera and 134 species about 2,000 species of Lamiaceae subfamily Nepetoideae of the tribe Mentheae. It was found that there was remarkable variation in pericarp structure and there was correlation with the variations in DNA and with gross morphological characters. The results showed considerable difference in the structure of the pericarp in the two subtribes, Menthinae and Nepetinae, but it was not easy to distinguish them as due to their overlapping characters. On the basis of differences in the pericarp structure he suggested that Menthinae could be divided into three important monophyletic groups such as (1) the main bulk of the tribe, (2) the characteristic genus *Lycopu*s. (3) a group of *Prunella* and *Cleonia* and perhaps also *Horminum*. Most of the sections were found to be convincing, but it was found that pericarp data disagreed with the genus *Melissa.* It was enlisted with the wrong sub tribe Salviinae. Modified version of the subtribal classification of Mentheae was also discussed.

Ryding (2010) studied the crystals in calyces of Lamiaceaen plants and their implications in phylogeny. Diversity of crystal types in fruiting calyces were examined for 435 out of the 7,200 species, and 184 out of the 236 genera of Lamiaceae. Members of the Nepetoideae and Lamioideaen sub families were given importance. The results showed that Calcium Oxalate crystal types can be used as a tool for classification. Crystals were grouped on the basis of their structure such as prismatics in the inner epidermis, prismatics in fibres, prismatics in the mesophyll, prismatics in sclereids, and druses in the mesophyll. Synapomorphic character is the shared derived character. Presence of epidermal prismatics was a synapomorphy of the subfamily Nepetoideae or it could also be regarded as a subclade within this subfamily comprises of the two largest tribes Mentheae and Ocimeae. The presence of narrow mesophyll prismatics is the main characteristics of the clade of Lamioideae genera. Recent phylogenetical classification also agreed with this result. Closed calyces and calyces with a narrow mouth and internally hairy calyces were consisted of epidermal prismatics. Overall study and its findings and the position of the crystals in the calyces were good enough to support the hypothesis that calcium oxalate crystals protect the fruit from the insect predators.

Scheen *et al.* (2010) conducted a phylogenetic analysis of Lamiaceae subfamily Lamioideae based on the plastid genome sequences of the *trnL* intron, *trnL-trnF* intergenic spacer, and *rps16* intron. Monophyly of Lamioideae *s.l.* was proved once again. The neighbor hood clade including Pogostemonoideae is well supported, with *Cymaria* Benth and which is subsumed in Lamioideae. Based on the phylogenetic analysis, the subfamily Lamioideae is divided into nine tribes. Based on this analysis three new tribes were also established: Gomphostemmateae Scheen & Lindqvist, Phlomideae Mathiesen, and Leucadeae Scheen & Ryding. The other six tribes comprised of Pogostemoneae Briq., Synandreae Raf., Stachydeae Dumort., Leonureae Dumort., Lamieae Coss. & Germ and Marrubieae Vis. Reestablsihment of the genus *Betonica L*. was also observed. The results also strongly suggest that the genera *Stachys* L., *Sideritis* L.*, Ballota* L., and *Leucas* R. Br. were shown to be polyphyletic or paraphyletic.

Bendiksby *et al.* (2011) analysed and reconstructed a phylogeny of the subfamily Lamioideae, the second largest and poorly investigated subfamily in Lamiaceae. An environment updation of the subfamily was done with the earlier published data as well as 71 new DNA sequences from outgroup taxa, based on DNA sequence data from four chloroplast regions such as *trnL* intron, *trnL-F* spacer, *rps16* and *matK*. On the basis of this study ten out of 13 previously ambiguous small or monotypic Asian lamioid genera got a taxonomic position and additionally, 37 lamioid species have been identified. Updation of the classification was done accordingly. Results obtained from maximum parsimony and Bayesian phylogenetic methods, were found to be congruent with the earlier results. Overall branch support and phylogenetic resolution were better in these analyses. New genera were added and assigned to earlier established tribes or the new tribe Paraphlomideae*,* which includes *Ajugoides, Matsumurella* and *Paraphlomis*. Later on *Acanthoprasium* has given a genus status. *Lamium* and *Otostegia* were found to be monophyletic. On the contrary ten important genera remain non-monophyletic like *Ballota s.str.*, *Lagopsis, Leonotis, Leonurus, Leucas, Microtoena, Phlomoides, Sideritis, Stachys, Thuspeinanta*, *Eriophyton* and *Stachyopsis.* They belonged to Lamieae, *Hypogomphia* in Stachydeae, and *Loxocalyx* in Leonureae. *Betonica, Colquhounia, Galeopsis,* and *Roylea* were still in uncertainity to be classified at the tribal level. Three East Asian *Galeobdolon* species and *Lamium chinense* were transferred to *Matsumurella*. Four *Otostegia* species and *Sulaimania* were transferred to *Moluccella*. *Alajja* along with three *Lamium* species were transferred to *Eriophyton*. In total, 14 new amalgamations were made, one at the rank of subgenus and 13 at the rank of species.

Mathiesen (2011) carried out a study on the genus *Phlomis,* which is regarded as one of the largest genera of subfamily Lamioideae (Lamiaceae) with more than 100 recognised species distributed in Asia, Northern Africa and Southern Europe. Classical Morphological studies splitted and classified the genus into two morphologically distinct groups. This was considered to be as the first molecular phylogenetic study of the genus *Phlomis.* Sequence variation in the *trnL* intron, the *trnL-F* intergenic spacer, and the *rps16* intron was very low, and polymorphism was also occurred that lead to a sparse, poor phylogenetic resolution, particularly among the Mediterranean and South West Asian taxa. So ecological, classical and morphoilogical data also included in the analysis in order to get more convincing result. TNT

(Goloboff *et al.,* 2003a) was used for the phylogenetic reconstruction. The results splitted up the genus into two separate groups such as genera *Phlomoides* and *Phlomis* by reducing taxonomic compexity. The study also showed that a small Asian genus *Notochaete*, showing more close relatedness to the genus *Phlomis* clade. Outcome of the study indicated that *Notochaete hamosa* and the two monotypic genera *Lamiophlomis* and *Pseuderemostachys* were nested within Phlomoides and three new combinations were also made. Character optimization analyses of all taxa based on geographic distributions were performed using Winclada (Nixon 2002) and concluded that the groups obtained by molecular phylogenetic reconstruction analysis were congruent well with biogeography. The results suggested a Central Asian origin of the genus *Phlomis* and have a major diaspora in South West China.

Agostini *et al.* (2012) have been conducted a preliminary phylogenetic analysis of the genus *Cunila* (Lamiaceae) based on *ITS* and *trnL-F* regions. These species have aromatic and medicinal property and were commonly used in folk medicine. Distribution of the genus was all the way from North America to Southern South America. According to the classical taxonomic classifications, the species of Southern South America were classified into three botanical sections i.e., *Incanae*, *Incisae* and *Spicatae.* The present study applied a new molecular phylogenetic approach to infer the genetic relationships among the South and North American *Cunila* species. One of the primary objectives of the study was to test the monophyly of the genus. The results based on the concatenation of the sequences obtained using nuclear (*ITS* rDNA) and chloroplast (*trnL-F*) primers showed significant irony with the classical taxonomic classification. Phylogenetic tree reconstructions based on maximum likelihood, maximum parsimony, and Bayesian analyses, pointed out the non-monophyletic status of *Cunila.* Phylogeny showed that the genus was separated into three segregated clades. A South American subshrub clade with the species of the section *Spicatae.*

The second clade with species of the sections *Incanaea* and *Incisae,* also known as South American shrub clade and the third clade is known as the North American *Cunila* clade. Complexity at genus circumscription was resolved to a great extent after this study.

Curto *et al.* (2012) critically analysed importance and the the use of single copy number plant nuclear markers in plant phylogenetics which were showing higher level of variability in general when compared to the chloroplast DNA molecular markers. Due to its ability to determine reticulation patterns these markers were used to infer species level phylogeny. This study found that high frequency of significant polyploidization lead to the prevalence of gene duplication events during the evolution of higher plants. In particular cases, strategical method of marker development should be needed to get a consistent phylogeny. Markers were developed for the phylogenetic analysis in *Micromeria*, using publicly available DNA sequences and *EST*s from related genera from Lamiaceae, subfamily Nepetoideae. They omitted the markers showing gene duplication patterns during the fourth step of marker development. Finally they were able to select 19 primer pairs that resulted in orthologous products for all the species that had already been included in this study. Most of the primers were used to test the evolution of the genus *Micromeria* in the Canary Islands. Some hypotheses which were formulated before were supported by all markers. Phylogenetic reconstruction was conducted using PAUP v4.0. An East West split, of Tenerife and Madeira and the one from the eastern islands and Gran Canaria clades were well supported by 12 markers. Out of the 19 molecular markers, 7 of them showed contradictory results. After the through analyses of the results they found that inter-island gene flow and reticulation might have played a significant role in the evolution of the genus *Micromeria.*

Krawczyk *et al.* (2013) did a review of *Galeobdolon luteum* from the historical and taxonomic perspectives. In this recent study it was showed that *Galeobdolon luteum* must be included in the genus *Lamium.* Testing of the hypothesis was done by a comparative molecular phylogenetic analysis between the representatives of the genera *Galeobdolon* and *Lamium* using four DNA regions of *ITS*, *accD*, *rpoC1* and *trnH-psbA*. The analyses supported phylogenetic relationships among the studied taxa *Galeobdolon luteum* and also proved that there was no clear genetic difference between sequences of *Galeobdolon luteum* and *Lamium* to consider both as distinct genera.

Salmaki *et al.* (2013) conducted a recent study of molecular phylogeny of the tribe Stachydeae of the family Lamiaceae of the subfamily Lamioideae. Tribe Stachydeae of the family Lamiaceae is considered to be monophyletic. Relationships within the tribe were still poorly resolved. Paraphyly, considerable morphological plasticity, wide range of ploidy levels, and frequent natural hybridization increased the complexity of Stachydeae. Parsimony and Bayesian analyses were conducted on nuclear *ITS* and plastid *trnL* intron, *trnL–trnF* intergenic spacer, and *rps16* intron. Bulk amount of the DNA sequence data from a taxonomically and geographically broad sampling of the tribe were performed to identify the major evolutionary lineages within it and to test and prove the taxonomic hypotheses within the largest lamioid tribe Stachydea. We included 121 species with 143 accessions, representing both Old and New World species of the all 12 recognized genera of the tribe *Stachydeae.* Phylogenetic reconstructions showed that both nuclear and plastid data proved monophyly of the tribe, with *Melittis* as sister to all the other Stachydeae. The authors suggested a phylogenetic name *Eurystachys* to the other well supported clade. Monophyly is supported by both nuclear and plastid data for several named and unnamed groups. Majority of the early recognized taxa appeared to be para or polyphyletic. The authors opined that the impact of hybridization in the evolution of Stachydeae is the root cause of the plastid–nuclear incongruence.

Roy *et al.* (2013) carried out a study on the Phylogeny and biogeography of New World Stachydeae (Lamiaceae) with emphasis on the origin and diversification of Hawaiian and South American taxa. Hawaiian Archipelago regarded as a unique ideal place to study the biogeography, population biology and phylogeny of different taxa. Isolation of this island itself had restricted the gene flow between other places of earth. The Hawaiian mints consisted of three endemic genera were appeared to be the largest radiations in the island. Previous studies had enlisted Hawaiian mints to the dry-fruited *Stachys* clade; might have due to hybridization events. Stachydeae, the largest tribe in the subfamily Lamioideae (Lamiaceae), is showing a taxonomic complexity, and exhibiting incredible chromosomal diversity. In this paper they attempted to unravel the mysterious relationships between the Hawaiian mints and the New World mint taxa and also attempted to discover the origin and diversification of the mints in the New World. During the Middle to Late Miocene, the genus *Stachys* had witnessed at least two independent migrational events to the New World and towards the beginning of the Pliocene, respectively. The outcome indicated the incongruence between the rDNA and cpDNA phylogenies and also had given some suggestions like a reticulated, New World origin for the Hawaiian mints. Dispersal event to Hawaii Island was supposed to be happened only once during the Pliocene. Low copy number nuclear loci had given further insights into the phylogenetic relationships between the New World mints.

Meng & Zhang (2013) conducted a study on the diversification of plant species in the arid areas of Northwest China. For this purpose, species level phylogeographical history of *Lagochilus* (Lamiaceae) was analysed. Qinghai-Tibetan Plateau is considered as the cradle of evolution of many of the species found in China. Cooled climate with strong dessication in the Pleistocene, and the rapid upliftment of mountain ranges of Qinghai-Tibetan Plateau had major impacts on the genetic structure of the flora. In order to investigate the mystery behind the evolution of plant species, accessions of *Lagochilus* of different regions like temperate steppes and desert regions of Northwest China were studied. They sequenced the plastid regions, *psbA-trnH* (*Sang et al.,* 1997) and *trnS-trnG* (Hamilton *et al.,* 1999) of the known ten species of the genus. Phylogenetic trees were estimated using Neighbor-Joining and Bayesian inference methods. The divergence time estimation of major lineages were done with BEAST (Drummond *et al.,* 2007) and IMa2 (Hey, 2010) softwares. Genetic structure, variability and demographic history were inferred using the AMOVA, Bayesian skyline plot analyses, mismatch distribution, and neutrality tests. The overall outcome of the data indicated that most chloroplast haplotypes were species-specific and the phylogeny of *Lagochilus* is geographically structured. The estimated Bayesian chronology and IMa implied that the main divergence events of the species between major Eastern and Western portions of the then Chinese desert occurred at the Pliocene or Pleistocene boundary, ie, about 2.1–2.8 Mya (Million Years ago), and it had coincided with the formation of the deserts in Northwest China due to the changes in the pattern of the geography and climate. The regional demo graphic expansions, in the eastern at ca. 0.06 Ma in the western region at ca. 0.39 Ma, and, or across all regions at ca. 0.26 Ma, showed the response to aridification. Rapid cooling of the Pleistocene period increased aridity at alarming rate in the Chinese deserts. The significant changes reflect a major influence of the geologic and climatic pattern on the evolution of species of *Lagochilus.* Finally as per the result they concluded that diversification was the result of the past fragmentation due to the aridification events and Adaptation to the cold, dry desert environment.

Jenks *et al.* (2013) had thoroughly analyzed the phylogeny of New World *Salvia* subgenus *Calosphace* (Lamiaceae). It comprised of about 500 species and are economically and ethno medicinally important. In order to identify the phylogenetic relationships, and major lineages using chloroplast intergenic spacer *psbA-trnH* (Sang *et al.,* 1997) and nuclear *ITS*, 73 sections of *Calosphace* were sampled. Phylogenetic analysis of the concatenated data sets established monophyly of seven sections (*Blakea, Corrugatae, Erythrostachys, Hastatae, Incarnatae, Microsphace,* and *Sigmoideae*) and four major lineages (*Salvia axillaris*, *Hastatae* clade, *Uliginos*ae clade, and core *Calosphace* clade. Geographic origin of *Calosphace* was found to be Mexico. Current disjunct distribution could explain through seven dispersal events.

Silveira *et al.* (2013) conducted a study on phylogenetic systematics of the Lamiacean Mesa mint *Pogogyne*. It is a small genus consists of annuals, seen in almost entirely in fresh pool habitats. DNA sequence data from the *trnQ-rps16* (Shaw *et al.,* 2007) chloroplast spacer and both *ETS* and *ITS* nuclear regions were obtained using all the existing seven species to reckon the phylogeny of this group, and several outgroups. Bayesian inference and Maximum parsimony reconstruction methods were used for the tree construction. Morphological characters were subjected to ancestral state reconstructions and the outcome showed separate groups within *Pogogyne*. Monophyly of the subgenus *Hedeomoides* has been proved with good clade credibility support. Two apomorphies were also seen, a reduced corolla size and a reduction in stamen number. Subgenus *Pogogyne* was found to be paraphyletic, with poor clade credibility support. The crown clade of the genus *Pogogyne* has short branches when compared to the relatively long stem lineage of *Pogogyne.* The crown clade had the mean divergence time of 0.9 to 1.9 million years ago and the stem node is 5.1 to 7.7 million years ago.

These results supported well with the hypothesis that a rapid diversification in response to specialization has been occurred in the members of *Pogogyne.*

Xiang *et al.* (2013) studied the newly established tribe Gomphostemmateae (Lamiaceae), which consistsrted of *Bostrychanthera Chelonopsis,* and *Gomphostemma*. In this present study, they used DNA sequences of two nuclear regions of *ITS* and *ETS* and five chloroplast loci such as *psbA-trnH*, *rps16*, *trnL intron, trnL-trnF spacer,* and *trnS-trnG* to reconstruct the estimated phylogeny of the East Asian genus *Chelonopsis.* By combining the data set of nuclear and chloroplast they got well supporting trees with good topology. The result proved monophyly of the tribe Gomphostemmateae and Bostrychanthera was embedded within *Chelonopsis.* Genus *Chelonopsis* comprised of two master clades, one *Chelonopsis* subgenus *Aequidens*, *Chelonopsis* subgenus *Chelonopsis* and the genus *Bostrychanthera*. The resulting lineage split is due to the variation in morphological characters. Split has been resolved by adding more morphological characters and using cytological data. After conducting a thorough analysis it could be seen that the position of the genus *Bostrychanthera* has been changed and it could be nested within *Chelonopsis.* Apart from these, the result also indicated that *Chelonopsis* subgenus *Aequidens,* section *Aequidens* and section *Microphyllum* were also found to be monophyletic.

Dndar *et al.* (2013) conducted morphological revision and phylogenetic analysis of *Stachys* L. sect. *Eriostomum* (Hoffmanns & Link) Dumort. (Lamiaceae) using nuclear ribosomal internal transcribed spacer (nr*ITS*) sequences. The phylogenetic analysis was shown to be incongruent with the classical morphological classification. Instead of three sub sections phylogeny showed two subsections, such as *Creticae* and *Germanicae spectabiles.* A potentially novel species of *Stachys* was also observed in the tree. Monophyly of *Eriostomum* is proved and new status of the subgenus Betonica (L.) R.Bhattacharjee to genus is proved.

Puppo *et al.* (2014) studied the influence of geological history on diversification in isolated species of *Micromeria* (Lamiaceae) and the genetic and morphological patterns of *Micromeria* in Tenerife, one among the Canary Islands. Prime objective of the study was to understand the variegation of the two groups of the *Micromeria* species in Tenerife. Using eight nuclear loci from 37 samples they calculated and calibrated phylogeny. Data from 54 specimens were subjected to principal components analysis (PCA) for 27 morphological characters, sampled from Tenerife. The result of the phylogenetic reconstruction showed that the species from Tenerife could be subdivided into three main clades: one composed of the species occupying the palaeo Island of Anaga (*Micromeria glomerata , Micromeria rivas-martinezii* and *Micromeria teneriffae*) another composed of the species present in the palaeo Island of Teno (*Micromeria densiflora*); and a third group that includes all the central species (*Micromeria hyssopifolia, Micromeria lachnophylla, Micromeria lasiophylla* and *Micromeria varia*. Morphometric analyses designated two main groups interrelated to the palaeo Island species and the central ones. Their study pointed out that a relationship could be seen between the diversification within *Micromeria* and the geological history of Tenerife. They also concluded that journey of the first appearance of *Micromeria* was in Anaga. Diversification has been started from there and it has been subsequently colonized in Teno and from there engrossed in the central part indubitably after the formation of the Teide volcano. The species of *Micromeria* in Tenerife Island is regarded as a model plant species to study how species diversification could happen on oceanic islands like Tenerife. Volcanic eruption might have caused that much of endemism in the Canary Islands especially in Tenerife.

Gao *et al.* (2014) studied diversification and vicariance of desert plants using chloroplast DNA sequence variation of the selected genus *Lagochilus ilicifolius* (Lamiaceae). In order to understand mechanism of diversification of the desert taxa and to record arid Northern China as a diversification center for desert plants, they used five cpDNA spacers *trnL-trnF*, *rps16*, *psbA-trnH*, *psbA-trnH-f* (Sang *et al.,* 1997), *psbA-trnH-r* (Tate and Simpson, 2003), *psbK-psbI* (Lahaye *et al.,* 2008) and *trnS-trnG* (Shaw *et al.,* 2007). Phylogenetic reconstruction analyses showed that *Lagochilus ilicifolius* comprised of two well defined distinct lineages, one distributed in China, and the other one in Mongolia–Russia. Biogeographic inferences, on the basis of the Bayesian uncorrelated lognormal model conjoin with molecular dating, propounded that the main diversification events happened within the species occurred in the Pleistocene ca. 1.38–0.30 Mya (Million Years Ago). It is due to the fact that the transition of the atmospheric climate of Eurasia to a dry-cold pattern and the desertification events caused by the vicariance of desert plants.

The molecular phylogeny and biogeographic antiquity of *Isodon* were studied by You *et al.* (2014) using sequences obtained by the amplification of three plastid markers, the nuclear ribosomal internal transcribed spacer (nr*ITS*), and a low-copy nuclear gene *LEAFY* intron II, which was amplified using *LFsxl-2* and *LFtxr* (Frohlich & Meyerowitz, 1997). Different probabilistic models were used to investigate the evolutionary changes in the chromosomes. The results supported monophyly of *Isodon,* which includes the two disjunct African species, both of them were supposed to be formed through allopolyploidy. Authors opined that the disjunct distribution of *Isodon* might have due to an overland migration from Asia to Africa through Arabia during the early Miocene period. Floristic exchange in between Asia and Africa might have distrupted due to the formation of the Red Sea in the middle Miocene. In addition to this a rapid radiation of *Isodon* possibly should have occurred in the late Miocene. The major upliftment of the QTP had wreaked havoc to the then climate in the area and lead to the subsequent aridification events. They concluded that climatic and geographic variations could have a major effect in the species diversification in the QTP region.

With a focus on the section Hymenosphace, Dizkirici *et al.* (2015) investigated the phylogenetic relationships of the genus *Salvia euphraticas. s.l.* and its closely allied species. They used *trnL* intron, *trnL-trnF* intergenic spacer, *trnT-trnL* intergenic spacer, and *trnV* intron in order to screen the specimens. They used 19 sequences from seven *Salvia* taxa along with sequences from Gene Bank. Phylogeny reconstruction revealed well supported clades which were congruent with the latest taxonomic treatments on *Salvia pseudeuphratica* and *Salvia cerino-pruninosa.* The results concluded that section *Hymenosphace* is a non-monophyletic clade. The results revealed that *ITS* had given trees with highest resolution for discriminating studied taxa. Chloroplast regions showed poor resolution. Chloroplast regions were good enough to discriminate species from the different clades.

Barrabe *et al.* (2015) conducted molecular studies to recircumscribe *Oxera* by reconstructing a phylogenetic tree based on the *trnD-T* region, including the *trnE-trnY, trnY-trnD,* and *trnT-trnE* intergenic spacers, and the *trnE* and *trnY* (Demesure *et al*., Peti (1995) the intergenic spacers, *trnL* intron, *trnL-trnF* and *trnT-L* intergenic spacer, the *trnS-fM* region consists of *trnG-trnfM, psbZ-trnG, and trnS-psbZ,* intergenic spacers, and the *psbZ* and *trnG* genes (Demesure *et al.,* 1995) and the *trnS-G* region, using *trnS-G* intergenic spacer and the *trnG* intron (Hamilton,1999). Nuclear ribosomal loci like *ITS* and *ETS*. Altogether 30 ingroup taxa were included. *Clerodendrum* and its closely related genera were found to be sister to *Faradaya* and its allies, *Hosea* and *Oxera.* It could be seen that *Hosea* formed a sister clade to all the species of *Oxera* and *Faradaya.* Polyphyly of the *Faradaya* was proved and could be seen that it was partly nested within *Oxera*. However, *Oxera* share strong similar characters with the three species of *Faradaya*. In order to overcome the dilema in phylogeny; polyphyletic *Faradaya* was placed in synonymy with *Oxera* and they propounded three combinations *Oxera amicorum, Oxera lehuntei* and *Oxera splendida.*

Seyedi & Salmaki (2015) examined the morphology of trichome and its significance in the systematics of the genus *Phlomoides* (Lamiaceae; Lamioideae; Phlomideae). Using modern techniques like Scanning Electron Microscopy (SEM) and light microscopy structure of the trichomes of 32 species of the genus *Phlomoides* were examined. Variation in trichome characters played a significant role not only in classification at sectional rank, but also in separating different species of *Phlomoides* from their relatives. Outcome of the analysis indicated that the presence of branched trichomes with papillate surface as characteristic features of indumentum in the type species of this section. (1) multi nodal branched trichomes were found to be primitive against the branched ones (2) extremely long trichomes were found to be primitive than the long or short simple trichomes, and (3) loss of glandular trichomes considered as the most advanced characteristic feature.

Mirzaei, *et al.* (2015) conducted a phytochemical study of selected Menthol producing Lamiacean plants. Prime objective of the study was to determine phylogenetic relationships of different Lamiacean members within the species with menthol dehydrogenase gene. Based on the menthol content, three genera *Mentha, Micromeria* and *Salvia,* of Lamiaceae, were selected for phylogenetic and phytochemical analyses. Phylogenetic tree construction was estimated using MEGA4 software for various species. Purposefully done phytochemical analysis divulged that *Mentha spicata* and *Micromeria hyssopifolia* respectively contained the highest and lowest amounts of both menthol and essential oil respectively. The species *Mentha piperita* and *Mentha spicata,* which were nested within the same clade, contained the highest amounts of menthol and essential oil. *Micromeria* species, were found to be too distant from the crown clade, contained the lowest amount of menthol and essential oil. They concluded that, varying essential oil and menthol content had also placed different genus in different clades and it was also in accordance to the earlier morphological and molecular studies.

Salmaki *et al.* (2015) conducted a recent Molecular phylogeny of the enigmatic *Stachys persepolitana* and placed the same in *Lamium* (Lamiaceae: Lamioideae). The main purpose of this study was to test the hypothesis that *Stachys persepolitana* is unnecessarily placed in *Stachys* tribe Stachydeae, subfamily Lamioideae. Based on the result they changed its status and included in the genus *Lamium* (Lamieae, Lamioideae). Phylogenetic position of *Stachys persepolitana* was studied using plastid chloroplast regions like *rps16 i*ntron, *trnL-F* and *matK* regions and nuclear *nrITS* DNA sequence data. Phylogenetic trees were estimated using Maximum parsimony and Bayesian inference approaches. Plastid and nuclear data strongly supported and concluded that *Stachys persepolitana* should be included into the genus *Lamium*. Classical taxonomy on the basis of morphological characters also corroborated its placement in *Lamium.* Synapomorphic characters like peculiarity of anterior pair of stamens, which bends outward after pollination, triquetrous mericarps and hairy anthers, were the important characters that lead to the placement of *Stachys persepolitana* to *Lamium.*

Manchester *et al.* (2015) assessed the fossil record of Asterids in the context of the present phylogenetic evidence. Lamiaceae is coming under the subclass Asteridae. The fossil record imparted good evidence for the minimum ages of important episodes in the geographic radiation and diversification of Asteridae, with earliest examples. It could be concluded that the genesis of Asteraceae and Lamiaceae might have been from the middle to late Miocene in North Eastern China. They reiterated that dislodged pollen, taken forth with megafossil and mesofossil data, to fill the void in our meager knowledge of the paleobotanical archives.

Roy & Lindqvist (2015) investigated the evolutionary relationship within the Lamioid tribe Synnandreae using a low-copy number nuclear Pentatricopeptide Repeat Region or PPR and cross checked the outcome of the phylogeny with previously published sequence data using a combined data set consisted of four chloroplast DNA loci. They incorporated representatives of all 10 lamioid tribes analyzed the data using Bayesian inference. They also estimated the ancestral areas and divergence times for major nodes.

Yao *et al.* (2016) had recently studied the genus *Pogostemon* (Lamiaceae; Lamioideae) *s.l.* It is a large genus comprised of ca. 80 species with a distinct Asian and African distribution. Due to the morphological capriciousness and wildly accepted convergent evolution within the genus the infrageneric taxonomical classification of the genus was found to be problematic. Conspicuously, some species of *Pogostemon* are strictly following aquatic, life. *Pogostemon* might be the only Lamiaceaen taxa which exhibits this adaptivity. Phylogenetic trees were estimated using the nuclear *ITS* and chloroplast regions such as *trnL-F, rps16, rbcL, matK* and *trnH-psbA* and firmly established the monophyly of *Pogostemon* and its sister similarity and relationship with the genus *Anisomeles.* Genus *Pogostemon* was resolved into two major clades, and it could also be seen that none of the three morphologically defined subgenera of *Pogostemon* were found to be monophyletic. In accordance to the result a new infrageneric classification of *Pogostemon* with two subgenera was also proposed. Biogeographic diversification analyses conjointly with molecular dating suggested that in the early Miocene period itself the genus *Pogostemon* splitted from its sister clade genus in Southern and South East Asia. The upliftment of the Qinghai–Tibetan Plateau and early hype of the Asia monsoon system might have played an important role in the subsequent diversification of the genus.

Welch *et al.* (2016) studied plastid phylogenomics of endangered and extinct Hawaiian endemic mints (Lamiaceae). Contemporary radiation of the Hawaiian endemic mints had shown minimal sequence divergence and poor phylogenetic resolution. In order to get clear cut evidence and to trace the reticulate evolutionary history of the lineage, they used 12 complete or nearly complete plastid genomes from different Hawaiian endemic mint species and its allies. Rare or extinct taxa also were included in the analysis. They also used next generation amplicon sequencing approach. This procedure helped to increase the total number of variable sites over the previous analyses. The result obtained using next generation sequencing method also failed to give a good and robust tree with good topology.

Eiji & Salmaki (2016) had investigated the variation in the trichome characters of the genus *Salvia* and *Pleudia* in order to estimate this onset of morphological evidence to tackle problems related to the genus level delimitation and sub-generic arrangement and classification. Using light microscopy and scanning electron microscopy (SEM), trichomes of 46 *Salvia* spp., of the three subgenera from Iran, were scrutinized. Most remarkable feature of them was, they were showing constant characters among different populations of a certain species, but to the surprise, they were also showed a degree of variability, which was very useful in the delimitation of taxa. More specifically; at lower taxonomic levels, trichome characters include number of composing cells uni, bi or multicellular etc, types of glandular hair, size and thickness; branching pattern; and its unique superficial papillae. Non-glandular trichomes could be simple and branched like glandular trichomes can be subsessile or sessile or stalked. Such characters which were discussed above were potential enough for the determination of evolutionary trends in *Salvia* and its allies.

With reference to the molecular phylogeny of the tribe Elsholtzieae Ya-Ping *et al.* (2016) had recently studied the phylogenetic position of *Ombrocharis* (Lamiaceae). They used two-step approach to resolve the phylogenetic placement of the genus. At the very beginning, the subfamilial affiliation of *Ombrocharis* was determined based on a combined *ndhF* and *rbcL* datasetof all seven subfamilies of Lamiaceae. Outcome showed that *Ombrocharis* is included in the subfamily Nepetoideae, main synapomorphy is found in the hexacolpate pollen grains. In the latter set of analyses, two nuclear DNA like *ITS* and *ETS* and four chloroplast DNA *ycf1*, *rps15-ycf1*, *trnL-F* and *rpl32-trnL* markers were used to unravel the mystery behind the position of *Ombrocharis* within Nepetoideae. Outcome of the result had placed *Ombrocharis* and another monotypic genus, *Perillula*, to a unique single clade that was sister to the remaining genera of the tribe Elsholtzieae. This study had given a light into the fact that the tribe Elsholtzieae is monophyletic. In these analyses they tried to merge *Keiskea* with *Collinsonia,* and *Elsholtzia*. But the result showed that the decision to merge them was in vain due to lack of support value.

Roy *et al.* (2016) carried out a study on the subfamily Lamioideae (Lamiaceae), which consists of ten tribes. Two out of the ten belongs to Old World members and only Stachydeae and Synandreae are of New World members. Lots of previous studies have been conducted to infer the phylogenetic relationships among the members of Synandreae, which could purely base on nuclear and plastid molecular markers. Here a different low-copy number nuclear loci such as *COR* Curto *et al.* (2012), *PHOT1* and *PHOT2* (Yuan & Olmstead, 2008 and PPR Roy & Lindqvist, 2015) molecular markers were used in order to re-examine the phylogenetic relationships within Synandreae. The result proved both the monophyly of the tribe Synandreae, and the sister relationships between *Brazoria* and *Warnockia*. It also proved the monophyly of *Physostegia*. They could also observe the incongruence in the interrelationships of *Macbridea* and *Synandra*. They failed to include Synandreae within Lamioideae. Migration pattern and the colonization events of the New World Synandreae have been occurred in the Mid-Miocene. Different climatic events, edaphic conditions and intolerance of soil acidity might have been played an important role in the restricted distribution of most genera of Synandreae to South Eastern and South-Central North America. They concluded that polyploidy might have influnzed colonization capability of *Physostegia*.

# CHAPTER 3

# AREA OF PRESENT STUDY

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The present study focused on the phylogeny of Asian *Leucas* taxa found in India. Plants were collected from all geographical regions of India and special attention were given to diversity rich regions. The diversity and distribution of *Leucas* varies in different geographical regions based on various agroclimatic factors. Diversity was found more towards southern part, particularly in Southern Western Ghats. Endemic diversity also showed similar patterns.

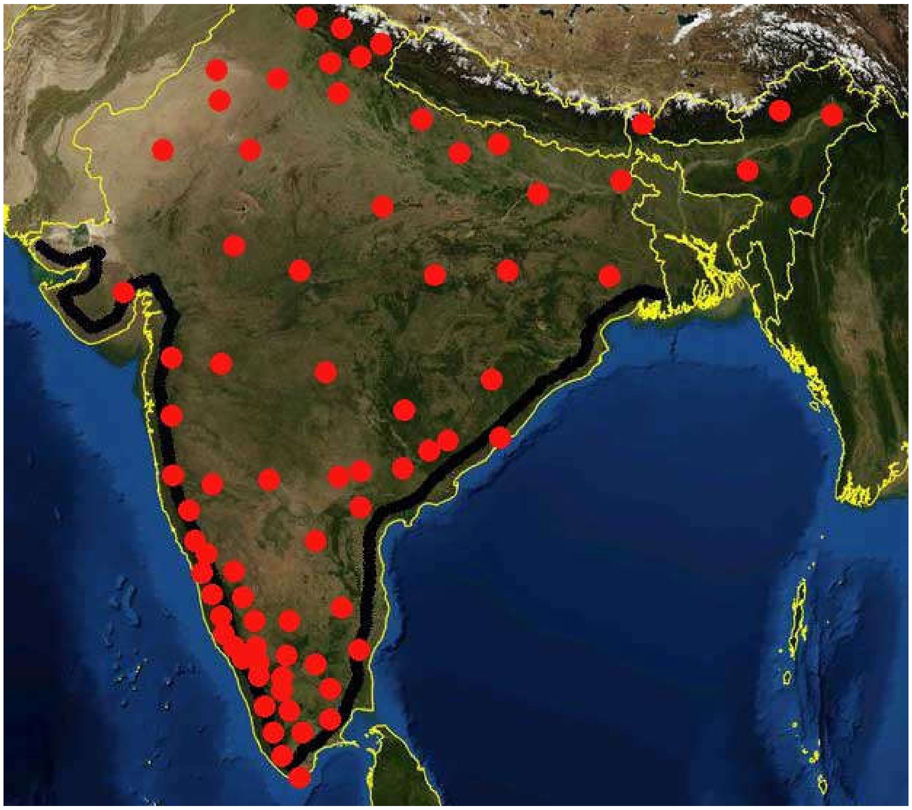

**Figure 3.1:- Map of India showingspecimen collection spots**

## India

Geographical position of India lies between latitudes 8° 4' and 37° 6' north and between longitudes 68° 7' and 97° 25' east. It relies entirely to the north of the equator with a large area in the subtropical zone which mainly lies on the Tropic of Cancer. India represents the largest peninsula of continent of Asia. Northern stretch of India is blessed with the Great wall of Himalayas. North West boundary constitutes the Thar Desert of Rajasthan. In the southern most part Capecomorin by Indian Ocean and the Eastern peninsula by the Bay of Bengal. In the western side of the Arabian Sea a group of Islands Laccadive and Minicoy Islands are also a part of Indian sub-continent. The countries which border India are Pakistan, Afghanistan, Nepal, Bhutan, China, Bangladesh and Myanmar (Singh, 1971).

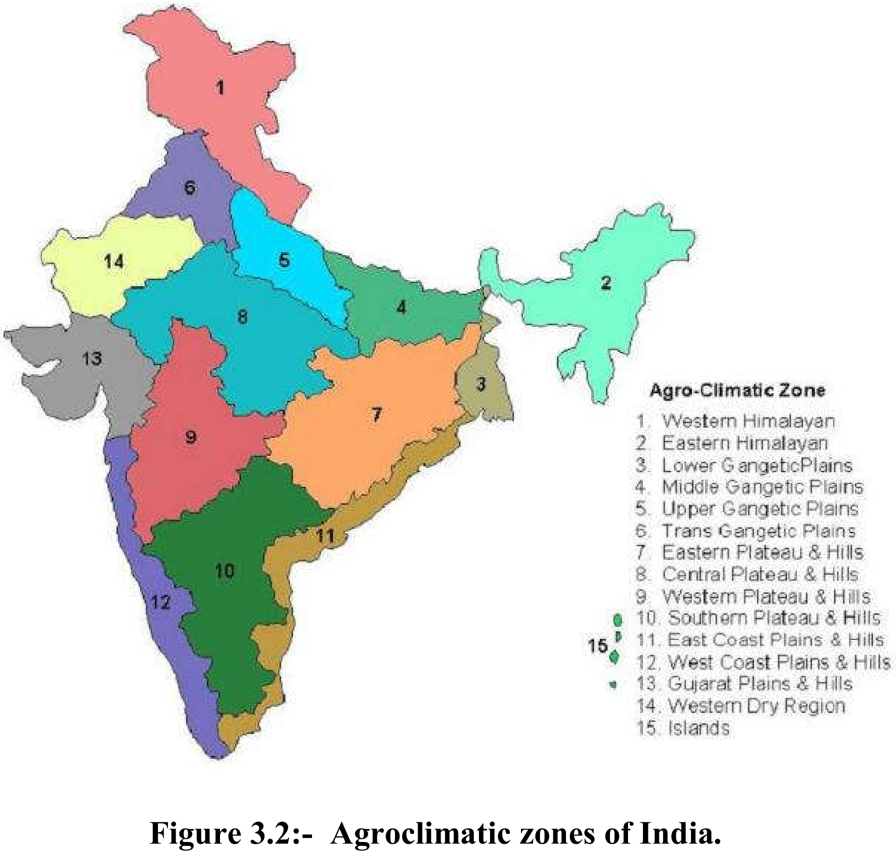

**Figure 3.2:- Agroclimatic zones of India.**

There are 15 Agro-Climatic regions in India. It includes Western Himalaya, Eastern Himalaya, Lower Gangetic planes, Middle Gangetic planes, Upper Gangetic planes, Upper Gangetic planes, Trans Gangetic planes, Eatern Plateau and Hills, Central Plateau and Hills, Western Plateau and Hills, Southern plateau and hills, East coast plains and hills, West coast plains and hills, Gujarat plains and Hills, Western Dry Regions, and Islands.

With varying number of species, *Leucas* is represented in all these agroclimatic zones in India. Highest diversity is seen in the west cost plains and hills, particularly in Western Ghats.

**Soil**: - Soil with particular vegetation are quiet often be considered as the product of rocks of a particular area. The soil which is made up of five components include, dead decaying organic matter, mineral particles, soil atmosphere, soil water and biological system which include important soil microorganisms. According to the present classification system there are 8 different types of soils in India. Laterite soils, Black cotton soils, Red soils, Alluvial soils, Alkali soils and saline soils, Peaty and other organic soils, Desert soils, and scanty soils of mountains. Laterite soils, Black cotton soils, Red soils and Alluvial soils are considered to be the important soils types in India. Others are found to be less important in the agro-climatic point of view.

**Laterite soil: -** This kind of soil is found to be generally reddish or yellow-red and turn black on sun light exposure and generally occurs in parts of Rajasthan, Madhya Pradesh, Bihar, West Bengal, Assam, Orissa, Tamil Nadu, and Eastern and Western Ghats. These distributed to an area of 248000 sq km. *Leucas* diversity is found more in these type of soil.

**Black cotton soil: -** This kind of black soil is the best for cotton cultivation. This is also known as 'regur'. Distribution covers some areas of Tamil Nadu, especially in Ramnad and Tirunelveli districts, some parts of Karnataka, Andhra Pradesh, Western Madhya Pradesh, some districts of Rajasthan and Gujarat. Total distribution area covers 546000 sq km.

**Red soil**:- Red soil, which occurs mainly in the peninsular regionsof Karnataka, Andhra Pradesh, Maharashtra, Madhya Pradesh, and Odisha. Total area covers an area of 35000 sq.km.Which is not useful for cultivation.

**Alluvial soil:-** This soil type is considered to be the largest and the most important soil groups of India. Largest share of the agricultural wealth is fulfilled with this soil type. This soil covers 1500000 sq km area and holds the most populous portions of India. Distribution area includes parts of Rajasthan, Punjab, Uttar Pradesh, West Bengal, Orissa and Assam. Ganges and Brahmaputra riverine system has been playing an important role in the distribution of this soil across India. Narmada and Tapti rivers in Madhya Pradesh, Godavari, Krishna, and Kaveri in the peninsular states, and Yamuna, Ganga and their tributaries are also considered to be the carriers of the bulk amount of alluvial soil.

### Geographical representation of downloaded sequences

In order to accommodate all the possible taxa and to get a good sampling pattern, tree resolution and a robust phylogeny, I used most of the *Leucas* sequences from gene bank to represent diversity from all around the world. Downloaded sequences represent diversity from Africa, Arabia, Yemen, Socotran Islands and other geographical areas in the Afro Arabian belt and Asia. According to Hedge (1992) Africa is considered to be the origin of *Leucas s.l.* but the dispersal and vicariance are still unknown to the world. In order to unravel the pattern of diversity and evolution we need sequences representing different parts of the world. Moreover avoiding diverse geographical and agro climatical zones where *Leucas* are distributed may end up with the partial phylogeny and that may lead to the wrong assumption and pattern of shifting of many of the taxa and poor node support values.

## World Map

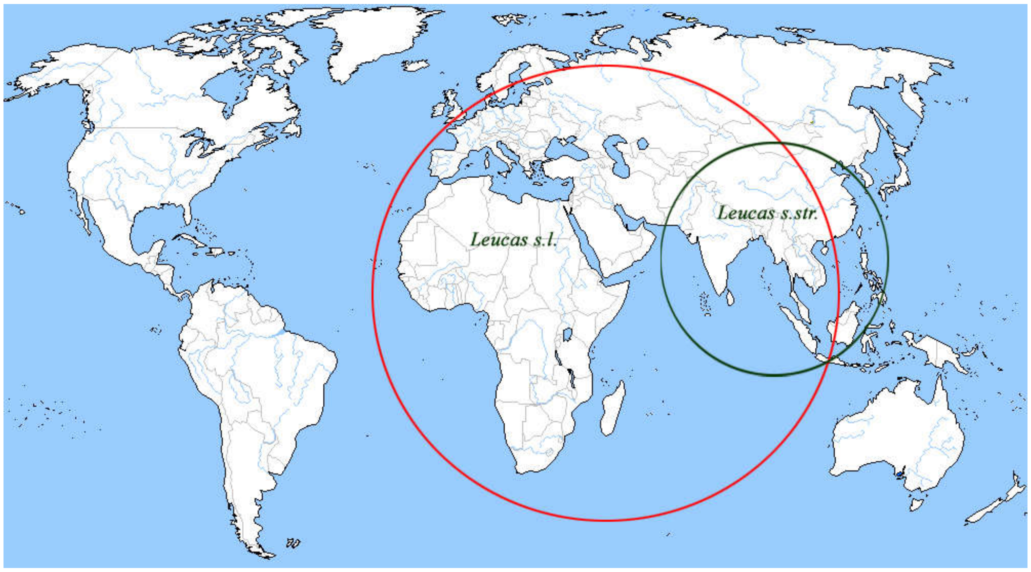

**Figure 3.3:- Area of natural distribution of *Leucas s.l.* and Asian *Leucas***

Asia can be divided into different biogeographic regions.

There is no significant geographic separation between Asia and Europe. The globally accepted boundaries are: the Northern side consists of Arctic Ocean, East by Pacific Ocean, South by  Caucasus Mountains and the Caspian and Black Seas, east of the Suez Canal, the Ural River, and the Ural Mountains.

Asia is one of the most diverse biogeographic regions of the world. The mainland of Asia lies between 34.0479° N, and 100.6197° E. It has a land boundary of about 44.58 million km². India is located in southern part of Asian continent. Except one, all *Leucas* species found in Asia are also seen in India.

# CHAPTER 4

# MATERIALS AND METHODS

# MATERIALS AND METHODS

The present work included maximum number of taxa from both the ingroup genus *Leucas* and selected outgroups. This study used more samples from Asia than the earlier works. Thorough field studies were conducted and a sum total of 39 *Leucas* taxa were collected from different parts of India. Except a few, great majority of Asian *Leucas* species and infrageneric variants are seen in India. Occurrence of species was identified primarily from the earlier works of Singh (2001) and Sunojkumar (2005). Information was also obtained from the herbarium specimens already preserved in CALI herbarium. Outgroup taxa from 'African *Leucas*' and genera like *Acrotome, Ballota, Gomphostemma, Ortholeucas, Lamium, Leonotis, Microtoena, Otostegia, Phlomis, Roylea, Scutellaria,* and *Rydingia* were downloaded from gene bank. Details regarding materials and methods adopted are given below.

## MATERIALS

### 4.1. Selection of taxa for molecular sequencing

The success of any phylogenetic study depends on the inclusion of maximum number of ingroup taxa for sequence generation. Two factors become very important:

**a.** Representation of all morphological groups and

**b.** Inclusion of samples from all geographical area

This study focused on collection of maximum number of *Leucas* specimens from different parts of India. A prior knowledge regarding the availability of specimens was obtained from earlier works by Singh (2001) and Sunojkumar (2005). These works were adopted in identifying the collected specimens. Attention was given to get representation from all infra-generic sections identified by Bentham (1830; 1848), Singh (2001) and Sunojkumar (2005). Similarly all the phytogeographical regions of India were visited for specimen collection. Altogether 39 taxa were included in the study and details regarding specimen collection, including taxa name, locality of collection, collection number, GPS reading are given in table 4.1 given in next page. As it was impossible to collect some species like *L. mathewiana*, *L. longifolia* etc samples were obtained from herbarium specimens.

Field collection trips were conducted and identified using data from previous works (Singh, 2001., Sunojkumar, 2005). Flowering twigs (4-5 nos) were collected from the field, wrapped in news paper and kept in plastic bags with moisture. These twigs were dried in herbarium press and mounted on sheets with proper labels. These voucher specimens are preserved in CALI for further reference. Young plants were uprooted from the field, transported to Calicut University Botanical Garden and planted there for further studies. Tender leaves were hand picked and kept in plastic zip bags with silica gel for DNA extraction and further molecular studies. Proper labelling was done on each zip bag and it included the collection locality, collection number, date of collection and species name.

**Table 4.1: List of plant collected, voucher specimen information, locality, and Genbank accession numbers of *trnL* intron, *trnL-F* intergenic spacer and *rps16* intron.**

| Sl No | Name of the Taxa | Locality | Collection No | GPS Reading | Gene Bank Accession No *trnL* intron | Gene Bank Accession No *trn L-F* interg nic spacer | Gene Bank Accession No *rps 16* intron |
|---|---|---|---|---|---|---|---|
| 1 | *Leucas angularis* Benth. | Lakkidi, Wayanad | 139521 | N.11.52539,<br>E.76.023009 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 2 | *Leucas biflora* (Vahl) Sm. | Maharashtra | 135590 | N15.765234<br>E74.002885 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 3 | *Leucas chinensis* (Retz.) Sm. | Nelliampathy, Palakkad | 135999 | N.10.555601<br>E.76.714598 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 4 | *Leucas deodikarii* Billmore & Hemadri | Ganesh Kindy, Junnar, Maharashtra. | 139519 | N.19.186220<br>E.73.861710 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 5 | *Leucas dhonimalayensis* sp. nov | Palamala, Dhoni Forest Palakkad | 133357 | N10°54’37.”<br>E76°37’41.” | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 6 | *Leucas eriostoma* Hook.f. | Periya, Wayanad | 088169 | N11.832644<br>E75.853354 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 7 | *Leucas helianthemifolia* Desf. | Rajamala, Idukki | 290159 | N10.169866<br>E77.063721 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 8 | *Leucas hirta* (B.Heyne ex Roth) Spreng | Mattuppetty, Idukki | 135992 | N10.107156<br>E77.123020 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 9 | *Leucas lamiifolia* Desf. | Anamudi Shola National Park, Idukki | 290142 | N10.187393<br>E77.185866 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10 | *Leucas lanata* var. *candida* Haines | Marappalam, Berliar Road, The Nilgiris | 135909 | N11.339878<br>E76.843624 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 11 | *Leucas lanceifolia* Desf. | Koodam, Silent Valley, Kerala | 138369 | N11.169097<br>E76.468334 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 12 | *Leucas lavandulifolia* Sm. | Calicut University Campus, Kerala | CU49602 | N11.132908<br>E75.888877 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 13 | *Leucas lavandulifolia* var. *decipiens* Hook.f. | Ootty, The Nilgiris | 135926 | N11.405194<br>E76.725195 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 14 | *Leucas longifolia* Benth. | Maharashtra | 139603 | N15.965234]<br>E74.002685 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 15 | *Leucas marrubioides* var. *pulneyensis* Hook.f. | Kodanad View Point, The Nilgiris | 135925 | N11.524688<br>E76.916029 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 16 | *Leucas martinicensis* (Jacq.) R.Br. | Maharashtra | 122267 | N18.746209<br>E73.403468 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 17 | *Leucas pubescens* Benth. | Deviculam, Idukki | 49695 | N10.056252<br>E77.119641 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 18 | *Leucas sebaldiana* Sunojk. | Chembra Peak, Wayanad | 88129 | N11.546625<br>E76.083825 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 19 | *Leucas suffruticosa* Benth. | Royal Valley Estate, The Nilgiris | 88147 | N11.473869<br>E76.542543 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 20 | *Leucas urticifolia* (Vahl) Sm. | TNAU Campus, Coimbatore | 49685 | N11.015173<br>E76.932693 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 21 | *Leucas wightiana* Wall. ex Benth. | Vijayanarayanam, Tamil Nadu | 49685 | N8.398895<br>E77.763224 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 22 | *Leucas zeylanica* (L.) | Calicut University Campus | 139563 | N11.133120<br>E75.888711 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |

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|---|---|---|---|---|---|---|---|
| 23 | *Leucas ciliata* Benth. | Idukki, Kerala | 135965 | N10.178185<br>E77.108228 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 24 | *Leucas rosmarinifolia* Benth. | Attappadi, Palakkad, Kerala | 4177 | N11.070186<br>E76.568168 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 25 | *Leucas mathewiana* Sunojk. | Kalasamudram, Andhra Pradesh | 135906 | N14.204798<br>E78.110742 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 26 | *Leucas diffusa* Benth. | Tirunelveli, Tamil Nadu | 139564 | N8.718408<br>E77.695840 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 27 | *Leucas manipurensis* N.P. Singh | Mawphlang Sacred groove, Meghalaya | 139625 | N25.446726<br>E91.751628 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 28 | *Leucas helicterifolia* Haines | Valmiki Tiger Reserve, West Champaran District, Bihar | 139652 | N27.339489<br>E84.306238 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 29 | *Leucas lanata* Benth. | Mussurie, Dehradun, Utharakhand | 139661 | N30.436405<br>E78.079157 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 30 | *Leucas macrantha* Blatt. & Hallb. | Mount Abu, Rajasthan | 139653 | N24.573859<br>E72.732494 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 31 | *Leucas deodikarii* Billmore & Hemadri | Ganesh Kindy Junnar, Maharashtra | 138315 | N19.200456<br>E73.857448 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 32 | *Leucas beddomei* (Hook.f.) Sunojk. & P. Mathew | Chembra peak, Wayanad | CU49631 | N. 1 1' 32.14,<br>E.0.76' 04.976 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 33 | *Leucas prostrata* (Hook.f.) Gamble | Kodanad estate, The Nilgiris | 4510 | N11.524785<br>E76.915674 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 34 | *Leucas aspera* Willd. Link | Kanyakumari, Tamil Nadu | 139510 | N8.084615<br>E77.538718 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 35 | *Leucas martinicensis* (Jacq.) R.Br. | Arakku Valley, Andhra Pradesh | 139693 | N18.327122<br>E82.877657 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 36 | *Leucas mukerjiana* Subba Rao & Kumari | Araku Valley, Andhrapradesh | 139694 | N18.327632 E82.878012 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 37 | *Leucas decemdentata* var. *decemdentata* | Araku Valley, Andhra Pradesh | 139692 | N18.3270613 E82.874621 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 38 | *Leucas montana* (Roth) Spreng. | Talakkona water Falls, Andhra Pradesh | 139682 | N13.634849 E79.425956 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 39 | *Leucas nepetifolia* Benth. | Maruthwamala, Kanyakumari District,Tamil Nadu | 151007 | N08.131137 E77.504935 | Not Yet Deposited | Not Yet Deposited | Not Yet Deposited |
| 40 | *Gomphostemma javanicum* (Blume) Benth. | Africa | Nil | Nil | AF502028 | HQ911735 | FJ854038 |
| 41 | *Sideritis macrostachyos* Poir. | Africa | JB 254 | Nil | AF335648 | FJ854194 | FJ854079 |
| 42 | *Sideritis romana* L. | Africa | JB 209 | Nil | AF335653 | FJ854196 | FJ854081 |
| 43 | *Acrotome angustifolia* G.Taylor. | Africa | C. Skarpe 423 (UPS | Nil | EU138374 | EU138297 | EU138222 |
| 44 | *Acrotome fleckii* (Gürke) Launert | Africa | M. Strohbach & K.Bremer s.n., 05.03.1992 (UPS) | Nil | EU138375 | EU138298 | EU138223 |
| 45 | *Acrotome hispida* Benth. | Africa | P. Herman 1990 (C) | Nil | EU138376 | EU138299 | EU138224 |
| 46 | *Acrotome inflata* Benth. | Africa | R. Seydel 3454 (US) | Nil | EU138378 | EU138301 | EU138225 |
| 47 | *Acrotome pallescens* Benth. | Africa | I. Ortendahl 105 (UPS) | Nil | EU138379 | EU138302 | EU138226 |
| 48 | *Ballota acetabulosa* (L.) Benth. | Africa | M.Bendiksby & A.-C. Scheen 04-12 (O) | Nil | EU138442 | EU138365 | EU138296 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 49 | *Ballota pseudodictamnus* (L.) Benth. | Africa | M.Bendiksby & A.C. Scheen 04-20 (O) | Nil | EF546857 | EF546857 | EU138295 |
| 50 | *Isoleucas arabica* O.Schwartz | Africa | M. Thulin *et al.* 8402 (UPS) | Nil | EU138380 | EU138303 | EU138227 |
| 51 | *Lamium tomentosum* Willd. | Africa | J. Klackenberg 820620-27 (S) | Nil | EF546855 | EF546855 | EU138293 |
| 52 | *Leonotis grandis* Iwarsson & Y.B.Harv. | Africa | M. Iwarsson & O. Ryding 972 (UPS) | Nil | EU138381 | EU138304 | EU138228 |
| 53 | *Leonotis leonurus* (L.) R.Br. | Africa | F. Venter & P. Vorster 171 (US) | Nil | EU138382 | EU138305 | EU138229 |
| 54 | *Leonotis myricifolia* Iwarsson & Y.B.Harv. | Africa | M. Iwarsson & O. Ryding 986 (UPS) | Nil | EU138383 | EU138306 | EU138230 |
| 55 | *Leonotis myrothamnifolia* Iwarsson & Y.B.Harv. | Africa | M. Iwarsson & O. Ryding 959 (UPS) | Nil | EU138384 | EU138307 | EU138231 |
| 56 | *Leonotis nepetifolia* var. *africana* (P.Beauv.) J.K.Morton. | Africa | O. Ryding *et al.* 1564 (UPS) | Nil | EU138385 | EU138308 | EU138232 |
| 57 | *Leonotis nepetifolia* var. *nepetifolia* | Africa | R. Abdallah *et al.* 493 (UPS) | Nil | EU138386 | EU138309 | EU138233 |
| 58 | *Leonotis ocymifolia* var. *raineriana* (Vis.) Iwarsson | Africa | F. G. Meyer 8140 (US) | Nil | EU138388 | EU138311 | EU138235 |
| 59 | *Leonotis ocymifolia* var. *schinzii* (Gurk e) Iwarsson. | Africa | R. Blomberg *et al.* 713 (UPS) | Nil | EU138389 | EU138312 | EU138236 |
| 60 | *Leucas abyssinica* var. *sidamoensis* Sebald | Africa | O. Ryding *et al.* 2194 (UPS) | Nil | EU138392 | EU138315 | EU138239 |
| 61 | *Leucas abyssinica* var. *brachycalyx* (Chiov.) | Africa | O. Ryding *et al.* 2189 (UPS) | Nil | EU138391 | EU138314 | EU138238 |
| 62 | *Leucas aequistylosa* Sebald | Africa | I. Friis *et al.* 3572 (UPS) | Nil | EU138393 | EU138316 | EU138240 |
| 63 | *Leucas argentea* Gürke | Africa | I. Friis *et al.* 3766 (UPS) | Nil | EU138394 | EU138317 | EU138241 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 64 | *Leucas bracteosa* Gürke | Africa | A. Hemp 4440 (O) | Nil | EU138397 | EU138320 | EU138244 |
| 65 | *Leucas calostachys* Oliv. | Africa | M. Iwarsson 280 (UPS) | Nil | EU138398 | EU138321 | EU138245 |
| 66 | *Leucas capensis* (Benth.) Engl. | Africa | C. Skarpe 374 (UPS) | Nil | EU138399 | EU138322 | EU138246 |
| 67 | *Leucas cuneifolia* Baker**.** | Africa | M. Thulin *et al.* 10643 (UPS) | Nil | EU138401 | EU138324 | EU138248 |
| 68 | *Leucas deflexa* var. *deflexa* | Africa | A. Hemp 4285 (O) | Nil | EU138403 | EU138326 | EU138250 |
| 69 | *Leucas densiflora* Vatke | Africa | S.T. Iversen *et al.* 86805 (UPS) | Nil | EU138404 | EU138327 | EU138251 |
| 70 | *Leucas discolor* Sebald | Africa | O. Ryding *et al.* 2261 (UPS) | Nil | EU138405 | EU138328 | EU138252 |
| 71 | *Leucas flagellifera* (Balf.f.) Gürke | Africa | M. Thulin & A.N. Gifri 8750 (UPS) | Nil | EU138406 | EU138329 | EU138253 |
| 72 | *Leucas glabrata* (Vahl) Sm. | Africa | O. Ryding 2351 (UPS) | Nil | EU138407 | EU138330 | EU138254 |
| 73 | *Leucas glabrata* var. *glabrata* | Africa | O. Hedberg 6816 (UPS) | Nil | EU138408 | EU138331 | EU138255 |
| 74 | *Leucas grandis* Vatke | Africa | A. Hemp 4386 (O) | Nil | EU138409 | EU138332 | EU138256 |
| 75 | *Leucas inflata* Benth. | Africa | M. Thulin *et al.* 3869 (UPS) | Nil | EU138410 | EU138333 | EU138257 |
| 76 | *Leucas jamesii* Baker. | Africa | O. Hedberg *et al.* Somalia Medicinal Plant Project 85 (UPS) | Nil | EU138411 | EU138334 | EU138258 |
| 77 | *Leucas kishenensis* (Radcl.-Sm.) Sebald | Africa | M. Thulin & A. N. Gifri 8531 (UPS) | Nil | EU138412 | EU138335 | EU138259 |
| 78 | *Leucas martinicensis* (Jacq.) R.Br. | Africa | O. Ryding *et al.* 2190 (UPS) | Nil | EU138416 | EU138339 | EU138263 |
| 79 | *Leucas masaiensis* Oliv. | Africa | A. Hemp 4419 (O) | Nil | EU138417 | EU138340 | EU138264 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 80 | *Leucas milanjiana* Gürke | Africa | O. Ryding 2329 (UPS) | Nil | EU138418 | EU138341 | EU138265 |
| 81 | *Leucas minimifolia* Chiov | Africa | M. Thulin & A. M. Dahir 6706 (UPS) | Nil | EU138419 | EU138342 | EU138266 |
| 82 | *Leucas neuflizeana* Courbon. | Africa | O. Ryding *et al.* 1082 (UPS) | Nil | EU138421 | EU138344 | EU138268 |
| 83 | *Leucas nubica* Benth. | Africa | M. Thulin *et al.* 3793 (UPS) | Nil | EU138422 | EU138345 | EU138269 |
| 84 | *Leucas oligocephala* Hook.f. | Africa | A. Hemp 4438 (O) | Nil | EU138423 | EU138346 | EU138270 |
| 85 | *Leucas sexdentata* Skan | Arica | O. Ryding 2310 (UPS) | Nil | EU138424 | EU138347 | EU138271 |
| 86 | *Leucas spiculifolia*(Balf.f.) Gürke | Africa | M. Thulin & A.N. Gifri 8688 (UPS) | Nil | EU138425 | EU138348 | EU138272 |
| 87 | *Leucas stachydiformis* (Benth.) Hochst. ex Briq | Africa | K. Hylander *et al.* 97 (UPS) | Nil | EU138426 | EU138349 | EU138273 |
| 88 | *Leucas urticifolia* var. *urticifolia* | Africa | O. Ryding & N. Sileshi 1763 (UPS) | Nil | EU138427 | EU138350 | EU138274 |
| 89 | *Leucas usagarensis* Gürke | Africa | S. Bidgood *et al.* 3873 (UPS) | Nil | EU138428 | EU138351 | EU138275 |
| 90 | *Leucas virgata* Balf.f. | Africa | A. G. Miller *et al.* 10142 (UPS) | Nil | EU138429 | EU138352 | EU138276 |
| 91 | *Leucas volkensii* var. *parviflora* Sebald. | Africa | E. Rob & Th.C.E. Fries s.n., 16.01.1922 (UPS) | Nil | EU138430 | EU138353 | EU138277 |
| 92 | *Marrubium vulgare* L. | Africa | E. Rob & Th.C.E. Fries s.n., 16.01.1922 (UPS) | Nil | EU138443 | EU138366 | EU138294 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 93 | *Otostegia erlangeri* Gürke | Africa | M.G. Gilbert *et al.* 7965 (UPS) | Nil | EU138432 | EU138355 | EU138279 |
| 94 | *Otostegia fruticosa* (Forssk.) Schweinf. ex Penzig | Africa | I. & O. Hedberg 9218A (UPS) | Nil | EU138433 | EU138356 | EU138280 |
| 95 | *Otostegia hildebrandtii* (Vatke & Kurtz) Sebald | Africa | M. Thulin *et al.* 8143 (UPS) | Nil | EU138434 | EU138357 | EU138281 |
| 96 | *Rydingia integrifolia* (Benth.) | Africa | M. Thulin *et al.* 8161 (UPS) | Nil | EU138435 | EU138358 | EU138282 |
| 97 | *Otostegia modesta* S. Moore. | Africa | M.G. Gilbert & D. Sebsebe 8631 (UPS) | Nil | EU138437 | EF546856 | EU138283 |
| 98 | *Rydingia persica* (Burm.f.) | Africa | K. Nikookar s.n., 09.11.1963 (S) | Nil | EU138438 | EU138361 | EU138284 |
| 99 | *Isoleucas somala* (Patzak) | Africa | M. Thulin & A.M. Warfa 5395 (UPS) | Nil | EU138439 | EU138362 | EU138285 |
| 100 | *Otostegia tomentosa* subsp *ambigens* (Chiov.) Sebald | Africa | O. Ryding *et al.* 1654 (UPS) | Nil | EU1384411 | EU138364 | EU138287 |
| 101 | *Phlomoides milingensis* C.Y.Wu & H.W.Li | Africa | C.Mathiesen & J. M. Taylor 17 (O) | Nil | EU138448 | EU138371 | EU138292 |
| 102 | *Phlomis purpurea L.* | Africa | M. Thulin *et al.* 4782 (UPS) | Nil | EU138449 | EU138372 | EU138291 |
| 103 | *Roylea cinerea* (D.Don) Baill | Africa | O. Polunin *et al.* 837 (UPS) | Nil | EU138450 | EU138373 | EU138290 |
| 104 | *Scutellaria hirta* Sm. | Africa | M.Bendiksby & A.C. Scheen 04-11 (O) | Nil | EF546927 | EF546847 | EU138289 |
| 105 | *Scutellaria sieberi* Benth. | Africa | M.Bendiksby & A.C. Scheen 04-18 (O) | Nil | EF546928 | EF546848 | EU138288 |
| 106 | *Achyrospermum wallichianum* (Benth.) Benth. ex Hook.f. | Africa | K. Larsen 9364 (C) | Nil | HQ911666 | HQ911734 | HQ911594 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 107 | *Eurysolen gracilis* Prain | Africa | Soerensen & al. 3046 (C) | Nil | HQ911665 | HQ911732 | HQ911593 |
| 108 | *Pogostemon benghalensis* (Burm.f.) Kuntze | Africa | Rebecca G. Troth 677 (US) | Nil | HQ911663 | HQ911731 | HQ911731 |
| 109 | *Pogostemon quadrifolius* (Benth.) F.Muell. | Africa | K. Larsen & B. Hansen 6313 (C) | Nil | HQ911661 | HQ911729 | KR608643 |
| 110 | *Pogostemon stellatus* (Lour.) Kuntze | Africa | R. Geesink & al. 5870 (C) | Nil | HQ911660 | HQ911728 | KR608651 |
| 111 | *Microtoena delavayi* Prain | Africa | T. Simeon 1291 (C) | Nil | HQ911658 | HQ911726 | HQ911588 |
| 112 | *Colebrookea oppositifolia* Sm. | Africa | Unknown 8354 (S) | Nil | HQ911657 | HQ911725 | HQ911587 |

## METHODS

## 4.2 WET LAB EXPERIMENTS

Tender leaves of each species were collected and hand cut pieces were properly preserved in plastic zip covers using silica gel. The plastic covers were labelled with informations including name, collection locality, GPS reading, collection date etc. After each field trip, young and uprooted plants were planted in Calicut University Botanical garden for further studies.

### 4.2. A. DNA extraction

Silica dried leaves collected during field explorations were used for DNA extraction using DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufactures instructions.

DNeasy Plant Mini kit provides quick and easy way to isolate and purify DNA from plant specimens. Both wet and lyophilized leaf materials were used in this study. A specified quantity up to 100 mg of wet tissue material or 25 mg lyophilized tissue was processed using the DNeasy Plant Mini Kit. DNeasy Plant Mini Kit provided pure total DNA (genomic, mitochondrial and chloroplast) for reliable PCR in less than 1 hour. Purification of the isolated DNA requires no phenol or chloroform extraction or alcohol precipitation, and involves minimal handling and less amount of time. This makes DNeasy Mini Kit, highly suitable for simultaneous processing of multiple samples. One of the peculiarities of the standard procedure is that, purified DNA is eluted in low-salt buffer or water, and ready for use in other various applications. Most of the DNA isolated and purified using DNeasy Plant Kit is up to 40 kb in size, with fragments of 20–25 kb predominating. Isolation and purification procedures were maintained in such a way that the DNA of this higher length should show complete denaturation in PCR and should show the highest amplification size. The

DNeasy membrane ensures complete removal of all inhibitors of PCR and other enzymatic reactions. DNA purified using DNeasy Plant Kits is highly suited for use in all downstream applications, including sequencing.

**Table 4.2:- The composition of buffers are shown below**

| 2X CTAB Buffer | (Per 200 ml) |
|---|---|
| 100 mM Tris-HCl (pH8.0) | 20 ml 1 M Tris-HCl pH 8.0 |
| 1.4 M NaCl | 56 ml 5 M NaCl (or 16.4 g) |
| 20 mM EDTA | 16 ml 0.5 M EDTA |
| 2% CTAB | 4.0 g powder |
| 2% PVP40 | 4.0 g |
| 0.2% β-mercaptoethanol | add right before use |

The pH was adjusted to 5 using HCl and made upto 200 ml using distilled water.

Just before starting the extraction, the mortars and pestles were chilled in -20°C freezer overnight. The water bath was set to a temperature of 65°C and the 2X CTAB solution was incubated prior to use. Right before use, the β-mercapto ethanol buffer was added to CTAB buffer. (10ml pre - 2X CTAB + 20 µl β-mercapto ethanol per sample).

### 4.2. A.1. Standard procedure of DNA isolation using DNeasy plant mini kit

1. Disrupted samples (≤100 mg wet weight or ≤20 mg lyophilized tissue) using a mortar and pestle.

2. Added 400 µl Buffer AP1 and 4 µl RNase A. Vortex and incubated for 10 min at 65°C. Inverted the tubes 2-3 times during incubation. Do not mix Buffer AP1 and RNase A before use.

3. Added 130 µl Buffer P3. Mixed and incubated for 5 min on ice.

4. Centrifuged the lysate for 5 min at 20,000 × g (14,000 rpm).

5. Pipeted the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuged for 2 min at 20,000 × g. (remaing cell debris and salt precipitates were removed by centrifugation through a QIAshredder spin column. The preparation of a cleared lysate was essential to prevent clogging of the DNeasy spin column in the following step).

6. Transferred the flow-through into a new tube without disturbing the pellets. Added 1.5 volumes of Buffer AW1, and mixed by pipetting.

7. Transfered 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuged for 1 min at ≥ 6000 × g (≥8000 rpm).

8. Placed the spin column into a new 2 ml collection tube. Added 500 µl Buffer AW2, and centrifuged for 1 min at ≥ 6000 × g. discarded the flow-through.

9. Added another 500 µl Buffer AW2. Centrifuged for 2 minutes at 20,000 × g. Removed the spin column from the collection tube carefully so that the column does not come into contact with the flow-through.

10. Transferred the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.

11. Added 100µl buffer AE for elution. Incubated for 5 minutes at room temperature (15-25°C). Centrifuged for 1 minute at ≥6000 × g.

12. Repeated the step 11.

The DNA was visualized and quantified on 1% agarose gel using standard commercial DNA ladders using transilluminator (Biotech R&D Laboratories, Yercaud, Salem District, Tamil Nadu State).

### 4.2. B. PCR amplification, agarose gel electrophoresis and sequencing

The *trnL* intron and *trnL-trnF* intergenic spacer were amplified using universal primers of Taberlet *et.al.* (1991), either as one fragment (hereafter referred to as the *trnL-F* region) using primers *c* and *f* or as two separate fragments using primers *c* and *d* and *e* and *f*, respectively. The *rps16* intron was amplified using the primers *rpsF* and *rpsF2R* (Oxelman *et al*., 1997).

### 4.2. B.1. Primer Dilution

The *trnL* forward lyophilized primer of 22.4nm concentration was diluted with 224 µl of Milli-Q water. In the same way 25.8nm of *trnL* reverse primer added with 258 µl of Milli-Q water, 29.30 nm of *trnL-F* forward added with 293 µl and 27nm of reverse primer with 270 µl Milli-Q water, *rps16* forward primer 31.6 nm of *rpsF* with 316 µl and *rps2R* of 29.80 nm with 298 µl of Milli Q water. Overnight incubation was done for the diluted primers. Water was added to each tube in order to convert the primer concentration to 100 µM.

After overnight incubation take 1µl from each primer tube and make up to 10 µl as working stock.(1:10, primer:milli-Q water). This is to be done in order to dilute the concentration to 10µm solution. This is the working stock.

**Table 4.3:-** Primers for amplification of cpDNA regions.

| | Region | Primers used | Reference |
|---|---|---|---|
| 1 | *trnL* introns | Forward (Name:-a) CGAAATCGGTAGACGCTACG | Taberlet *et al.* (1991) |
| | | Reverse (Name:-b) GGGGATAGAGGGACTTGAAC | |
| 2 | *trnL-trnF* intergenic spacers | Forward (Name:-e) GGTTCAAGTCCC TCTATCCC | |
| | | Reverse (Name:-f) ATTTGAACTGGTGACACGAG | |
| 3 | *rps16* intron | ForwardGTGGTAGAAAGCAACGTGCGACTT | Oxelman *et al.* (1997) |
| | | ReverseTCGGGAT GAACATCAATTGCAAC | |

Polymerase Chain Reactions (PCR) were done in 25µl using GT PCR Mater Mix (TAKARA BIO INC, Seta 3-4-1, Otsu, Shiga 520-2193, Japan), 1 µM of each primer, and 1 µl genomic DNA. The DNA amplifications were performed using Master Cycler (Nexus gradient, Eppendorf, Germany) using a programme consisting of an initial denaturation step of 94°C for 4 minutes followed by 30 cycles of 30 seconds at 94°C , 30 seconds annealing at 60°C, and 1 minute extension at 72°C, ending with final 4 minutes extension at 72°C.

### 4.2. B.2. Agarose Gel Electrophoresis of PCR products

The PCR products were checked in 1.2% of agarose gels prepared in 0.5X TBE buffer containing 0.5µg/ml Ethidium Bromide. 1 µl of 6X gel loading dye (Himedia) was mixed with each samples and ran the gel using Electrophoretic unit (Enduro, Labnet International inclusive) at 75V power supply with 0.5 X TBE as electrophoretic buffer for about 1-2 hours, until the Bromophenol Blue Dye front had migrated to such an extent ie. almost at the bottom of the gel. The 100 bp DNA ladder (Invitrogen 100 bp DNA ladder of

0.1µg/µl concentration was used to analyse the size of the amplified DNA. Gels were visualized in the UV transillumianator (Biotech R&D Laboratories, Yercaud, Salem, Tamil Nadu) and the particular images were documented using the Gel Documentation system (Enduro GDS, Labnet International Inclusive, aplegen).

### 4.2. B.3. DNA Sequencing

We followed Sanger's method (Sanger *et al.,* 1977) of sequencing using the Big Dye Terminator v3.1. These amplified products were subjected to the Sangers sequencing using the PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, USA) followed by manufacturer's instructions.

**Table 4.4:-** Composition of DNA sequencing solution

| **Particulars used** | **Amount** |
|---|---|
| PCR Product (ExoSAP treated) | 10-20 ng |
| Primer | 3.2 pM (either forward or Reverse) |
| Sequencing Mix | 0.28 µl |
| DMSO | 0.30 µl |
| 5X Reaction buffer | 1.86 µl |
| Sterile distilled water | make upto 10 µl |

Sequencing PCR was carried out. It consists of first and foremost denaturation step of 96°C for 2 minute, followed by 30 cycles of 96°C denaturation for 30 seconds, 50 °C annealing for 40 seconds and elongation at 60 °C for 4 minutes.

### 4.2. B.4. Procedure for Post Sequencing PCR clean up

The products after sequencing were cleaned up using Master Mix-I, Master Mix-II and 70% ethanol. 12 μl Master Mix-I and 52 μl Master Mix-II were needed for each sequenced reaction product.

**Composition of the Master Mix**

Master Mix-I - 2 μl of 125 mM EDTA in 10 μl Milli Q Water.
Master Mix-II - 2 μl of 3M Sodium Acetate at pH 4.6 in 50 μl of ethanol.

To the 10 μl of each and every reaction product 12 μl of Master mix -I was added. Thoroughly mixed and to this 52 μl of Master Mix-II was added. The contents were mixed by inverting the tubes many times during incubation at room temperature for 30 minutes. After the incubation period, proper centrifugation has been carried out at 14,000 rpm for 20 minutes. Later on the supernatant was discarded and the 70% ethanol wash was repeated. The supernatants were decanted and 70% of ethanol wash were repeated. After decanting the supernatant the resultant pellet was kept for air drying. The cleaned air dried products were sequenced in ABI 3730/3500 Genetic Analyser (Applied Biosystems) at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

### 4.2. B.5. Editing of sequence

Quality of the sequence data has checked using Quality Value (QV) i.e. a widely accepted and established metric for determining quality of the standard sequenced data. The QV>20, means the probability that the base was miscalled is not greater than 1%, is the acceptable standard for a good sequence reaction. For all the samples sequenced, showed a QV>20. Thus practically all samples sequenced could be used for further studies.

### 4.2. B.6. Multiple Sequence Alignment

Forward and Reverse sequences were combined using Bioedit (Hall, 1999) software. Sequences were aligned using Clustal W (Thompson *et al.,* 1994) incorporated within MEGA6 (Tamura *et al.,* 2013) software. Alignments were thoroughly checked and manually edited using MEGA6 (Tamura *et al.,* 2013) software.

## 4.3. PHYLOGENETIC ANALYSIS

Partition Homogeneity Test i.e. Incongruence Length Difference (ILD) (Lee *et al.,* 2001) test was done for each data set to know whether these genes can be concatenated or not. Depends upon the credibility of ILD values, all the three different genes were concatenated. Model of sequence evolution of three different genes were separately calculated using jModelTest 2.0 (Darriba *et al.,* 2012) and Partition Finder v1.1.0 (Lanfear, 2012). In the partitionfinder it could be seen that model of sequence evolution was TVM+G for *trnL* and *rps16* introns. But in the case of *trnL-F* intergenic spacer, model of sequence evolution was found to be TrN+G. Simultaneously two separate trees were run using RAxML version 8: (Stamatakis, 2014) and MrBayes version 3.2 (Ronquist & Huelsenbeck, 2003) for the two different model of sequence evolution obtained from partitionfinder and Jmodeltest 2.0 and best fit model were included in the data set during the analysis. Maximum Likelihood and Bayesian analyses were performed using the concatenated dataset using RAxML version 8: (Stamatakis, 2014) and MrBayes version 3.2 (Ronquist & Huelsenbeck, 2003) softwares respectively to get the standard phylogenetic reconstruction of DNA sequence data. In these analyses gaps were coded as missing.

## 4.3. A. PARTITION HOMOGENEITY TEST (ILD TEST)

One of the major issues in systematics is the incongruence between the data sets. Presently, one of the most widely accepted and used methods for evaluating incongruence within the platform of parsimony framework is the homogeneity test of Farris *et al.* (1995). It is also usually termed as the Incongruence Length Difference (ILD) test (Cunningham, 1997a; Johnson & Soltis 1998) or the Partition Homogeneity Test (Swofford 2000). The test is the widely accepted and has long been established test to produce more accurate results than the other tests (Cunningham, 1997a) and it is also easy to implement using PAUP* (Swofford, 2000).

Partition Homogeneity Test using heuristic search has been done using PAUP 4b10 version (Swofford 2003). Number of replicates of 100 was used and parsimony was used as the optimality criterion. Total of 2151 characters of 112 taxa were used. All the characters were set to un-ordered. All the characters had equal weights, 1538 characters were constant and 256 variable characters were found to be parsimony-uninformative. Number of parsimony informative character was 357. Gaps were treated as missing. Branch-swapping algorithm used was Tree Bisection Reconnection (TBR) and random sequence addition was used. Branches collapsed if the branch length was zero. P value obtained should be less than 0.5.

## 4.3. B. PartitionFinder v1.1.0

PartitionFinder v1.1.0 is the software which helps to find out the partitioning schemes and model of molecular evolution for nucleotides alignment. This program is mainly used for three purposes. It works within the platform of Python 2.7.

1. In order to find out the best-fit partitioning scheme for a given dataset.

2. To compare any number of user-defined partitioning schemes.

3. In order to find out the best-fit models of molecular evolution for each subset in any partitioned dataset.

After running the analysis and it could be seen that *trnL* and *rps16* followed the same evolutionary models and *trnL-F* followed a different substitution model. Results obtained using this software was found to be accurate and much more robust.

### 4.3. C. RAxMLGUI 1.3 - Randomized Axelerated Maximum Likelihood

Commonly RAxML (Randomized Axelerated Maximum Likelihood) is regarded as a sequential and parallel Maximum Likelihood based inference program to construct large phylogenetic trees. Analysis started with Importing 112 taxa contained 2151 characters into the RAxML GUI 1.3 software. Settings had been changed accordingly to the data set: ML + thorough bootstrap were used; number of replications was set to 1000, bootstrap per Branch Length was selected, edited the set/edit partitions and changed to DNA, p1 = 1-574, 1171-2151 and DNA, p2 = 575-1170, evolutionary model was set to GTRGAMMA and run the analysis. Tree with robust topology was obtained after a couple of hours with good bootstrap supporting values. Same procedure was used for *trnl, trnL-F, rps16* introns and with concatenated data set separately using different partition schemes such as the jModelTest 2.0 (Darriba *et al.*, 2012) and Partition Finder v1.1.0 (Lanfear, 2012) respectively.

### 4.3. D. Bayesian analysis using MrBayes Version 3.2

MrBayes version 3.2 was used to perform the most reliable method of tree construction i.e., Bayesian inference to get a good and robust tree topology. MrBayes is command based software. In order to get the best posterior probability support for the nodes in Bayesian analysis, convergence has been attained using Metropolis Coupled Monte Carlo Markov Chain (MCMCMC) or (MC) $^3$. The idea of the method was to introduce a series of Markov chains samples from the heated posterior probability distributions. The Potential Scale Reduction Factor (PSRF) (Gelman & Rubin, 1992) was analysed to get an appropriate convergence. PSRF compares variance among runs with the variance within runs. As the chain converges and a proper tree has been obtained at that time PSRF value should approach 1.0. Two simultaneous independent runs with 4 Markov chains were performed for 1000000 generations and tree sampling was done in every 100 generation resulting in 10000 trees. The first 25 percentage of the trees were considered to be in burnin phase and discarded after the analysis. A majority- rule consensus tree based on the remaining 7500 trees were computed and the posterior probability of the branches were shown at the each nodes. Trees were observed in FigTree v1.4.2 (Rambaut, 2014).

**Various steps used to construct a bayesian tree**

**Getting data into Mrbayes: -** MrBayes only accepts NEXUS file format of an aligned amino acid, nucleotide or morphological data. The concatenated DNA data set of three different genes *trnL* & *rps16* introns and *trnL-F* intergenic spacer of 2151bp length of 112 taxa were converted into NEXUS file formats. The file was imported into the software using the command **execute** <**filename**>. The commands were typed at the MrBayes command prompt.

**Specifying the model: -** The command **'Lset'** is used to define the structure of the model and '**prset'** is used to define the prior probability distributions on the parameters of the model. These two commands were commonly used to specify the evolutionary models. Usually specified model can be obtained using the command **'showmodel'**. The general structure of the substitution model is determined by settings of the 'Nst' (Number of substitution types) value. This value may change based on the evolutionary models. Here evolutionary model obtained was TVM+G for *trnL* and *rps16* introns data sets. TrN+G model was selected for *trnL-F* intergenic spacer. A value of Nst =6 has been set for the three different data sets. Rates at each site is found to be gamma distributed and the command used was rates=gamma.

**Setting up of the priors: -** Next step is to set up the priors for the specified model. There are six types of parameters in the model.

a. The topology

b. The branch lengths

c. The four stationary frequencies of the nucleotides.

d. The six different nucleotide substitution rates

e. The proportion of invariable sites.

f. The shape parameter of the gamma distribution of rate variation.

Commonly, by default; priors will work well for most of the analyses. By typing '**help prset'** command I got the default settings for the parameter in our model.

**lset applyto=(2) nst=6 rates=gamma; prset applyto=(2) revmatpr = dirichlet (1,1,1,1,1,1) statefreqpr=dirichlet(1,1,1,1) shapepr=uniform(0.1, 50);** This commands were used for the model=**TVM+G;**

**lset applyto=(4) nst=6 rates=gamma; prset applyto=(4) revmatpr=dirichlet(1,1,1,1,1,1) statefreqpr=fixed(equal) shapepr= uniform(0.1,50) pinvarpr=uniform(0,1);** These commands were used for the model=**TrN+G.**

JModelTest 2.0 (Darriba *et al.*, 2012) had resulted in GTR+G model for *trnL* and *rps16* introns. During the analysis for *trnL-F*, It could be seen that TIM+G model was the best. Two separate models were set up for the three different genes.

**Checking the model: -** The command '**showmodel'** was used to check the model before the analysis. Here I checked and reconfirmed the model and gave a nod to continue to the next step.

**Setting up the analysis: -** Usually analysis starts with **mcmc** command. Before starting the analysis, one has to look back to the run settings by typing the command **help mcmc**. Here number of generations by default was found to be 1000000. So, no need to change the settings and the analysis was run for 1000000 generations. Number of chains were set to be 2 (Nchains=2).

**Running the analysis: -** To start the analysis the command **mcmc** was executed. The analysis was run for few hours to 2 days depend upon the complexity of the dataset.

**When to stop the analysis: -** At the end of the run MrBayes asks "whether or not you want to continue with the analysis"? Before typing a reply command **NO** the average standard deviation of split frequencies have to be checked If it tends to .01, sampling was good enough to stop the analysis. Then type the command **NO** to stop the run.

**Summarizing samples of substitution model parameters: -** During each run, samples of the substitution model parameters were written to the .p files for the every sample frequency generation.

The command '**sump**' was used to summarize the sampled parameter values. Burn-in percentage was set to 25%. Here 10000 trees were generated of which 2500 has been discarded by the system.

**Summarizing samples of trees and branch lengths: -** Trees and branch lengths were printed to the '.t' files. The command **sumt burnin=2500** was used to summarize the tree and branch length information. Sumt command also provides output summary statistics of the data set bipartitions, trees with clade credibility values, and a phylogram. Execution of this commands also generate additional files. One among them is '.parts' file. It contains list of taxon bipartitions, their posterior probabilities, and branch lengths. The '.con' file includes two consensus trees, one with the posterior probability values and branch lengths. The second tree gives only branch lengths. These trees were viewed in FigTree (Rambaut, 2014) package.

MrBayes version 3.2 was used to perform the most reliable method of tree construction i.e., Bayesian inference to get a good and robust tree topology. MrBayes is command based software. In order to get the best posterior probability support for the nodes in Bayesian analysis, convergence has been attained using Metropolis Coupled Monte Carlo Markov Chain (MCMCMC) or $(MC)^3$. The idea of the method was to introduce a series of Markov chains samples from the heated posterior probability distributions. The Potential Scale Reduction Factor (PSRF) (Gelman & Rubin, 1992) was analysed to get an appropriate convergence. The PSRF value compares variance among runs with the variance within runs. When the chain converges and the tree is obtained, the PSRF value at that time should approach 1.0. Two simultaneous independent runs with 4 Markov chains were performed for 1000000 generations and tree sampling was done in every 100 generation resulting in 10000 trees. The first 25 percentage of the trees were considered to be in burnin phase and discarded after the analysis. A majority- rule consensus tree based on the remaining 7500 trees were computed and the posterior probability of the branches were shown at the each nodes. Trees were observed in FigTree v1.4.2 (Rambaut, 2014).

# CHAPTER 5

# RESULTS

# RESULTS

## 5. A. Agarose Gel Electrophoresis

Amplification of the gene regions *trnL* intron *trnL-F* intergenic spacer and *rps16* introns were checked using 1.2% agarose gel electrophoresis. From the amplified PCR product 3µl was taken to run Agarose Gel Electrophoresis. Size of the amplicons were viewed properly and photographs were also taken using the Gel Documentation System (Labnet, Enduro GDS, aplegen)

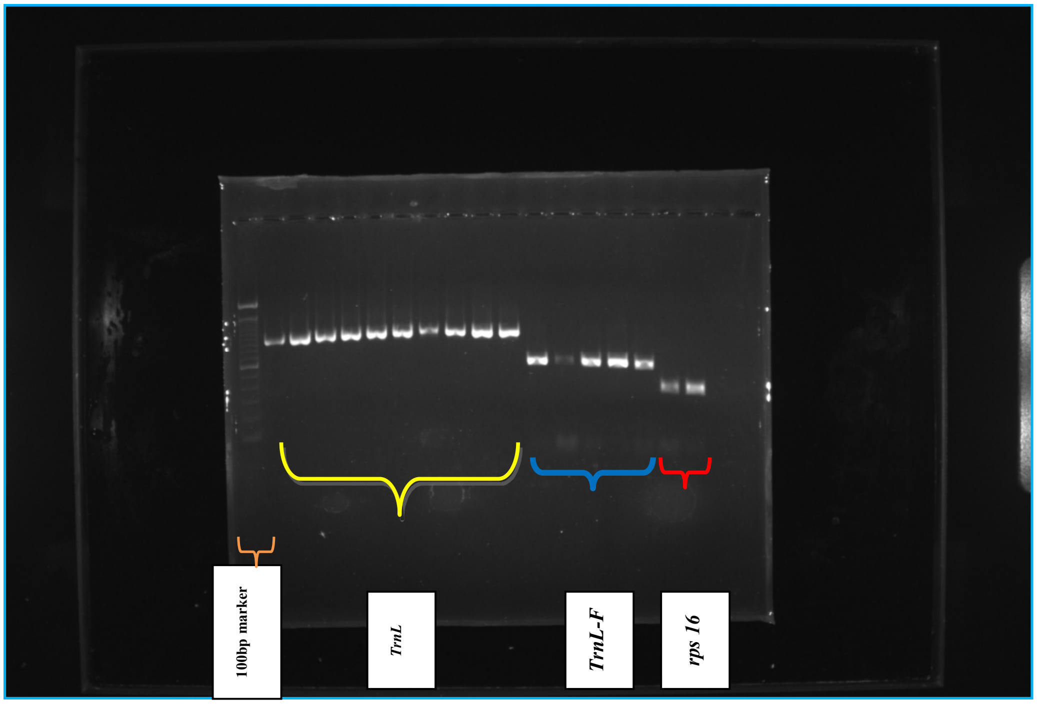


**Figure 5.1:- Agarose Gel Electrophoresis image of three genes**

For reference, DNA ladder of 100 bp (Invitrogen 100 bp DNA ladder of 0.1µg/µl concentration) was used. It is observed that size of the *trnL* intron was in between 500-600 basepairs and *TrnL-F* intergenic spacer in between 600-700. Finally the size of the *rps16* intron was also found be in between 900-1000 base pairs.

### 5. B. Partition homogeneity test

Incongruence Length Difference (ILD) Test was conducted using the software PAUP 4b10 version. All of the 112 taxa were considered for analysis and each of this carries a total of 2151 characters. Of this 1538 characters were constant, 256 variable characters were found to be parsimony-uninformative and 357 were parsimony informative. All characters have equal weights. Tree Bisection Reconnection (TBR) was used as branch swapping algorithm.

**Table 5.1:- Result of Incongruence Length Difference Test conducted with a total of 2151 characters for 112 taxa**

| Sum of tree lengths | Number of replicates |
|---|---|
| 1159* | 1 |
| 1169 | 1 |
| 1170 | 2 |
| 1171 | 1 |
| 1172 | 7 |
| 1173 | 9 |
| 1174 | 8 |
| 1175 | 11 |
| 1176 | 12 |
| 1177 | 4 |
| 1178 | 17 |
| 1179 | 9 |
| 1180 | 6 |
| 1181 | 3 |
| 1182 | 4 |
| 1183 | 5 |

* = sum of lengths for original partition. P value = 1 - (99/100) = 0.010000.

## 5. C. Finding out partitioning schemes and model of molecular evolution

**Table 5.2: - PartitionFinder v1.1.0 is used to find out the different model of sequence evolution for the *trnL*, *rps16 and trnL-F* intergenic spacer. Settings used**

```
alignment        : trnllfrps16.phy
branchlengths     : linked
models              : K81+I, GTR+I, F81+I+G, GTR+G, TrN+G, F81, K81+G,
F81+G, TIM, TIM+I+G, TVM+G, JC+I+G, TVMef+G, TVMef+I, TVM+I,
TrNef+I, F81+I, JC, TIMef+G, HKY, K81uf+I, TVMef, K81uf+G, K81+I+G,
TIMef+I, TVM, SYM+I, K81uf, JC+I, K80, K81, K80+I+G, K80+I,
SYM+G, K81uf+I+G, K80+G, TrN+I, GTR+I+G, GTR, TIM+G, HKY+I+G,
TIMef, TIM+I, TVMef+I+G, TVM+I+G, HKY+G, SYM+I+G, TrN, HKY+I,
SYM, TIMef+I+G, JC+G, TrNef, TrNef+G, TrNef+I+G, TrN+I+G
model_selection   : bic
search            : greedy
Best partitioning scheme
Scheme Name       : step_1
Scheme lnL        : -8744.81585
Scheme BIC        : 19300.6220985
Number of params  : 236
Number of sites   : 2151
Number of subsets : 2
Subset | Best Model | Subset Partitions               | Subset Sites                  |
Alignment
1      | TVM+G      | Gene1, Gene3                    | 1-574, 1171-2151              |
.\analysis\phylofiles\2be70985527782406b5ab4b5dd6b0325.phy
2      | TrN+G      | Gene2                           | 575-1170                      |
.\analysis\phylofiles\54fc9d7849dfa031b85750f9229d8ddc.phy
Scheme Description in PartitionFinder format
Scheme_step_1 = (Gene1, Gene3) (Gene2);
RaxML-style partition definitions
DNA, p1 = 1-574, 1171-2151, DNA, p2 = 575-1170
```

The best-fit model of sequence evolution according to the PartitionFinderV1.1.1_Windows analysis for *trnL* and *rps16* intron regions was found to be TVM+G and for the *trnL-F* region was TrN+G.

## 5. D. Sequence editing and multiple sequence alignment

Chromas lite 2.1.1 was used to edit chromatograms. BioEdit was used to construct consensus sequences. Multiple Sequence Alignment was performed using Clustal W incorporated in MEGA6.

### 5. D.1. *trnL* based DNA sequenced data

Total no of 112 taxa were included in the analysis. The length of the *trnL* varied from 466 base pairs, which was observed in *Lamium tomentosum* and the longest base pair length was upto 580 base pairs and this was observed in *Leucas sebaldiana.* Inorder to get accurate result, the aligned sequences were trimmed at the tail end. The final aligned and trimmed sequence length was 574 base pairs. Among this data set 422 were observed as conserved sites, 138 variable sites, 69 parsimony informative (Pi) sites and 68 were singletons.

**Table 5.3:- Nucleotide Frequencies of *trnL* intron in percentage**

| | **Name of the Taxa** | **Percentage of** | | | | **Total length of the base pair** |
|---|---|---|---|---|---|---|
| | | **T** | **C** | **A** | **G** | |
| 1. | *Leucas angularis* | 27.8 | 15.2 | 38.4 | 18.6 | 521 |
| 2. | *Leucas biflora* | 28.0 | 15.3 | 38.3 | 18.4 | 522 |
| 3. | *Leucas chinensis* | 27.8 | 15.4 | 38.4 | 18.4 | 521 |
| 4. | *Leucas deodikarii* | 27.9 | 15.4 | 38.7 | 18.1 | 520 |
| 5. | *Leucas dhonimalayensis* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 6. | *Leucas eriostoma* | 28.0 | 15.3 | 37.9 | 18.8 | 522 |
| 7. | *Leucas helianthemifolia* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 8. | *Leucas hirta* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 9. | *Leucas lamiifolia* | 27.9 | 15.6 | 37.9 | 18.7 | 520 |
| 10. | *Leucas candida* | 27.8 | 15.4 | 38.4 | 18.4 | 521 |
| 11. | *Leucas lanceifolia* | 27.7 | 15.6 | 38.1 | 18.6 | 527 |
| 12. | *Leucas lavandulifolia* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 13. | *Leucas lavandulifolia* var. *decipiens* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 14. | *Leucas longifolia* | 27.7 | 15.2 | 38.5 | 18.6 | 527 |
| 15. | *Leucas marrubioides* var. *pulneyensis* | 27.8 | 15.4 | 38.4 | 18.4 | 521 |
| 16. | *Leucas martinicensis* | 27.5 | 15.2 | 38.8 | 18.6 | 528 |
| 17. | *Leucas pubescens* | 27.5 | 15.6 | 38.3 | 18.6 | 527 |
| 18. | *Leucas sebaldiana* | 27.6 | 15.4 | 38.5 | 18.4 | 532 |
| 19. | *Leucas suffruticosa* | 27.7 | 15.6 | 38.1 | 18.6 | 527 |
| 20. | *Leucas urticifolia* | 27.3 | 15.3 | 38.8 | 18.6 | 528 |
| 21. | *Leucas wightiana* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 22. | *Leucas zeylanica* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 23. | *Leucas ciliata* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 24. | *Leucas rosmarinifolia* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 25. | *Leucas mathewiana* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 26. | *Leucas diffusa* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 27. | *Leucas manipurensis* | 27.7 | 15.4 | 38.3 | 18.6 | 527 |
| 28. | *Leucas helicterifolia* | 27.8 | 15.4 | 38.4 | 18.4 | 521 |
| 29. | *Leucas lanata* | 28.1 | 15.2 | 38.7 | 18.1 | 520 |
| 30. | *Leucas macrantha* | 27.7 | 15.0 | 38.3 | 19.0 | 527 |
| 31. | *Leucas deodikarii* | 27.9 | 15.4 | 38.7 | 18.1 | 520 |
| 32. | *Leucas beddomei* | 27.5 | 15.6 | 38.3 | 18.6 | 527 |
| 33. | *Leucas prostrata* | 27.7 | 15.4 | 38.1 | 18.8 | 527 |
| 34. | *Leucas aspera* | 27.8 | 15.4 | 38.4 | 18.4 | 526 |
| 35. | *Leucas nepetifolia* | 27.6 | 15.7 | 38.2 | 18.5 | 529 |
| 36. | *Leucas martinicensis* | 27.2 | 15.5 | 38.6 | 18.7 | 536 |
| 37. | *Leucas mukerjiana* | 27.7 | 15.7 | 38.1 | 18.6 | 528 |
| 38. | *Leucas decemdentata* var. *decemdentata* | 27.7 | 15.7 | 38.1 | 18.6 | 528 |
| 39. | *Leucas montana* | 27.5 | 15.7 | 38.1 | 18.7 | 530 |
| 40. | *Gomphostemma javanicum* | 27.1 | 15.7 | 37.6 | 19.6 | 542 |
| 41. | *Sideritis macrostachyos* | 26.8 | 16.1 | 38.2 | 19.0 | 542 |
| 42. | *Sideritis romana* | 26.8 | 15.8 | 38.3 | 19.1 | 549 |
| 43. | *Acrotome angustifolia* | 27.5 | 15.4 | 38.7 | 18.4 | 527 |
| 44. | *Acrotome fleckii* | 27.5 | 15.4 | 38.7 | 18.4 | 527 |
| 45. | *Acrotome hispida* | 27.5 | 15.0 | 38.9 | 18.6 | 527 |
| 46. | *Acrotome inflata* | 27.5 | 15.4 | 38.7 | 18.4 | 527 |
| 47. | *Acrotome pallescens* | 27.5 | 15.4 | 38.7 | 18.4 | 527 |
| 48. | *Ballota acetabulosa* | 27.3 | 15.1 | 38.4 | 19.1 | 523 |
| 49. | *Ballota pseudodictamnus* | 27.3 | 15.1 | 38.4 | 19.1 | 523 |
| 50. | *Isoleucas arabica* | 27.8 | 15.4 | 38.6 | 18.3 | 526 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 51. | *Lamium tomentosum* | 28.1 | 16.3 | 36.3 | 19.3 | 466 |
| 52. | *Leonotis grandis* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 53. | *Leonotis leonurus* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 54. | *Leonotis myricifolia* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 55. | *Leonotis myrothamnifolia* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 56. | *Leonotis nepetifolia* var. *africana* | 27.7 | 15.4 | 39.2 | 17.7 | 520 |
| 57. | *Leonotis nepetifolia* var. *nepetifolia* | 27.7 | 15.4 | 39.2 | 17.7 | 520 |
| 58. | *Leonotis ocymifolia* var. *raineriana* | 26.6 | 15.5 | 39.4 | 18.5 | 470 |
| 59. | *Leonotis ocymifolia* var. *schinzii* | 27.7 | 15.4 | 38.7 | 18.3 | 520 |
| 60. | *Leucas abyssinica* var. *sidamoensis* | 27.3 | 15.3 | 38.9 | 18.6 | 517 |
| 61. | *Leucas abyssinica* var. *brachycalyx* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 62. | *Leucas aequistylosa* | 27.6 | 15.2 | 38.8 | 18.4 | 526 |
| 63. | *Leucas argentea* | 27.5 | 15.4 | 38.7 | 18.4 | 527 |
| 64. | *Leucas bracteosa* | 27.3 | 15.4 | 38.9 | 18.4 | 527 |
| 65. | *Leucas calostachys* | 27.5 | 15.4 | 38.7 | 18.4 | 527 |
| 66. | *Leucas capensis* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 67. | *Leucas cuneifolia* | 27.1 | 15.4 | 38.9 | 18.6 | 527 |
| 68. | *Leucas deflexa* var. *deflexa* | 27.3 | 15.2 | 38.9 | 18.6 | 527 |
| 69. | *Leucas densiflora* | 27.2 | 14.9 | 38.9 | 18.4 | 527 |
| 70. | *Leucas discolor* | 27.4 | 14.7 | 38.9 | 18.4 | 527 |
| 71. | *Leucas flagellifera* | 28.1 | 15.2 | 38.5 | 18.1 | 519 |
| 72. | *Leucas glabrata* | 27.5 | 15.4 | 38.7 | 18.4 | 527 |
| 73. | *Leucas glabrata* var. *glabrata* | 27.5 | 15.4 | 38.7 | 18.4 | 527 |
| 74. | *Leucas grandis* | 27.7 | 15.0 | 39. 3 | 18.0 | 527 |
| 75. | *Leucas inflata* | 27.5 | 15.4 | 38.7 | 18.4 | 527 |
| 76. | *Leucas jamesii* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 77. | *Leucas kishenensis* | 28.1 | 15.2 | 38.3 | 18.3 | 519 |
| 78. | *Leucas martinicensis* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 79. | *Leucas masaiensis* | 27.3 | 15.4 | 38.9 | 18.4 | 527 |
| 80. | *Leucas milanjiana* | 27.5 | 15.4 | 38.9 | 18.2 | 527 |
| 81. | *Leucas minimifolia* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 82. | *Leucas neuflizeana* | 27.5 | 15.0 | 38.9 | 18.6 | 527 |
| 83. | *Leucas nubica* | 27.9 | 15.4 | 38.5 | 18.3 | 520 |
| 84. | *Leucas oligocephala* | 27.5 | 15.0 | 38.9 | 18.6 | 527 |
| 85. | *Leucas sexdentata* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 86. | *Leucas spiculifolia* | 28.1 | 15.2 | 38.5 | 18.1 | 519 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 87. | *Leucas stachydiformis* | 27.6 | 15.4 | 39.0 | 18.1 | 526 |
| 88. | *Leucas urticifolia* var. *urticifolia* | 27.3 | 15.4 | 38.9 | 18.4 | 527 |
| 89. | *Leucas usagarensis* | 27.7 | 15.2 | 38.9 | 18.2 | 527 |
| 90. | *Leucas virgata* | 27.9 | 15.2 | 38.7 | 18.1 | 519 |
| 91. | *Leucas volkensii* var. *parviflora* | 27.5 | 15.2 | 38.9 | 18.4 | 527 |
| 92. | *Marrubium vulgare* | 27.2 | 15.1 | 38.5 | 19.2 | 522 |
| 93. | *Otostegia erlangeri* | 27.8 | 15.2 | 38.8 | 18.3 | 526 |
| 94. | *Otostegia fruticosa* | 27.6 | 15.2 | 38.7 | 18.5 | 525 |
| 95. | *Otostegia hildebrandtii* | 27.8 | 15.2 | 38.8 | 18.3 | 526 |
| 96. | *Rydingia integrifolia* | 28.4 | 15.6 | 38.1 | 17.9 | 525 |
| 97. | *Otostegia modesta* | 27.6 | 15.2 | 38.8 | 18.4 | 526 |
| 98. | *Rydingia persica* | 28.2 | 15.4 | 38.3 | 18.1 | 525 |
| 99. | *Isoleucas somala* | 27.8 | 15.4 | 38.6 | 18.3 | 526 |
| 100. | *Otostegia tomentosa* subsp *ambigens* | 27.9 | 15.4 | 38.6 | 18.1 | 513 |
| 101. | *Phlomis milingensis* | 28.0 | 15.2 | 38.5 | 18.3 | 507 |
| 102. | *Phlomis purpurea* | 28.5 | 14.4 | 38.5 | 18.7 | 520 |
| 103. | *Roylea cinerea* | 27.6 | 15.2 | 38.7 | 18.5 | 514 |
| 104. | *Scutellaria hirta* | 25.7 | 16.7 | 37.5 | 20.1 | 502 |
| 105. | *Scutellaria sieberi* | 25.7 | 16.7 | 37.5 | 20.1 | 502 |
| 106. | *Achyrospermum wallichianum* | 27.4 | 15.3 | 38.9 | 18.3 | 470 |
| 107. | *Eurysolen gracilis* | 27.2 | 15.1 | 38.3 | 19.3 | 522 |
| 108. | *Pogostemon benghalensis* | 27.9 | 15.6 | 38.1 | 18.5 | 520 |
| 109. | *Pogostemon quadrifolius* | 26.9 | 16.2 | 38.7 | 18.3 | 476 |
| 110. | *Pogostemon stellatus* | 27.4 | 15.7 | 38.3 | 18.6 | 515 |
| 111. | *Microtoena delavayi* | 27.0 | 16.0 | 38.7 | 18.3 | 481 |
| 112. | *Colebrookea oppositifolia* | 25.1 | 17.2 | 39.3 | 18.4 | 407 |
| | Average | 27.6 | 15.4 | 38.5 | 18.5 | 521.6 |

### 5. D.2 *trnL-F* based DNA sequenced data

Length of the *trnL-F* intergenic spacer DNA region varied from short base pair length of 221 in *Sideritis macrostachyos* and 222 in *Sideritis romana* respectively. The length of *Leucas mukerjiana* extended up to 525 base pairs and it was considered to be the longest. After aligning the sequences using Clustal W, total base pair length was found to be 596. Among these 244 were observed as conserved sites, 190 variable sites, 106 parsimony informative (Pi) sites and 68 singletons.

**Table 5.4:- Nucleotide Frequencies of *trnL-F* intergenic spacer in percentage**

| Sl No | Name of the Taxa | Percentage of | | | | Total length of the base pairs |
|---|---|---|---|---|---|---|
| | | T | C | A | G | |
| 1. | *Leucas angularis* | 31.7 | 22.1 | 29.0 | 17.2 | 331 |
| 2. | *Leucas biflora* | 31.6 | 22.0 | 29.2 | 17.2 | 332 |
| 3. | *Leucas chinensis* | 31.6 | 22.0 | 29.2 | 17.2 | 332 |
| 4. | *Leucas deodikarii* | 31.6 | 22.0 | 29.2 | 17.2 | 332 |
| 5. | *Leucas dhonimalayensis* | 31.9 | 21.7 | 28.9 | 17.5 | 332 |
| 6. | *Leucas eriostoma* | 31.9 | 21.7 | 28.9 | 17.5 | 332 |
| 7. | *Leucas helianthemifolia* | 31.6 | 22.0 | 28.9 | 17.5 | 332 |
| 8. | *Leucas hirta* | 31.6 | 22.0 | 28.9 | 17.5 | 332 |
| 9. | *Leucas lamiifolia* | 31.6 | 22.0 | 28.9 | 17.5 | 332 |
| 10. | *Leucas candida* | 31.6 | 22.0 | 29.2 | 17.2 | 332 |
| 11. | *Leucas lanceifolia* | 31.9 | 21.7 | 28.9 | 17.5 | 332 |
| 12. | *Leucas lavandulifolia* | 31.2 | 22.8 | 28.8 | 17.1 | 333 |
| 13. | *Leucas lavandulifolia* var. *decipiens* | 31.2 | 22.8 | 28.8 | 17.1 | 333 |
| 14. | *Leucas longifolia* | 31.6 | 22.0 | 28.9 | 17.5 | 332 |
| 15. | *Leucas mambioides* var. *pulneyensis* | 31.6 | 22.0 | 29.2 | 17.2 | 332 |
| 16. | *Leucas martinicensis* | 30.9 | 22.0 | 28.7 | 18.3 | 327 |
| 17. | *Leucas pubescens* | 31.6 | 22.0 | 29.2 | 17.2 | 332 |
| 18. | *Leucas sebaldiana* | 31.9 | 21.7 | 28.9 | 17.5 | 332 |
| 19. | *Leucas suffruticosa* | 31.9 | 21.7 | 28.9 | 17.5 | 332 |
| 20. | *Leucas urticifolia* | 30.8 | 22.2 | 28.3 | 18.8 | 325 |
| 21. | *Leucas wightiana* | 31.6 | 22.3 | 28.9 | 17.2 | 332 |
| 22. | *Leucas zeylanica* | 31.9 | 22.3 | 28.9 | 16.9 | 332 |
| 23. | *Leucas ciliata* | 31.6 | 22.0 | 28.9 | 17.5 | 332 |
| 24. | *Leucas rosmarinifolia* | 31.9 | 21.7 | 28.9 | 17.5 | 332 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 25. | *Leucas mathewiana* | 31.2 | 23.1 | 29.7 | 15.9 | 333 |
| 26. | *Leucas diffusa* | 31.2 | 22.8 | 28.8 | 17.1 | 333 |
| 27. | *Leucas manipurensis* | 31.9 | 21.7 | 28.9 | 17.5 | 332 |
| 28. | *Leucas helicterifolia* | 31.6 | 22.0 | 29.2 | 17.2 | 332 |
| 29. | *Leucas lanata* | 31.9 | 21.7 | 29.2 | 17.2 | 332 |
| 30. | *Leucas macrantha* | 31.6 | 22.0 | 28.9 | 17.5 | 332 |
| 31. | *Leucas deodikarii* | 31.6 | 22.0 | 29.2 | 17.2 | 332 |
| 32. | *Leucas beddomei* | 31.9 | 21.7 | 28.9 | 17.5 | 332 |
| 33. | *Leucas prostrata* | 31.6 | 22.0 | 28.9 | 17.5 | 332 |
| 34. | *Leucas aspera* | 31.5 | 23.0 | 28.3 | 17.2 | 343 |
| 35. | *Leucas nepetifoli*a | 31.1 | 23.7 | 27.8 | 17.4 | 363 |
| 36. | *Leucas martinicensis* | 31.0 | 23.2 | 27.8 | 18.0 | 345 |
| 37. | *Leucas mukerjiana* | 33.0 | 21.2 | 28.1 | 17.7 | 537 |
| 38. | *Leucas decemdentata* var. *decemdentata* | 31.3 | 23.9 | 27.9 | 16.9 | 355 |
| 39. | *Leucas montana* | 31.0 | 23.9 | 27.7 | 17.3 | 364 |
| 40. | *Gomphostemma javanicum* | 30.6 | 22.3 | 29.5 | 17.5 | 359 |
| 41. | *Sideritis macrostachyos* | 26.2 | 19.9 | 33.9 | 19.9 | 221 |
| 42. | *Sideritis romana* | 25.7 | 20.7 | 34.7 | 18.9 | 222 |
| 43. | *Acrotome angustifolia* | 30.3 | 23.7 | 27.6 | 18.4 | 337 |
| 44. | *Acrotome fleckii* | 30.3 | 23.7 | 27.6 | 18.4 | 337 |
| 45. | *Acrotome hispida* | 30.4 | 23.2 | 28.0 | 18.4 | 332 |
| 46. | *Acrotome inflate* | 30.3 | 23.7 | 27.6 | 18.4 | 337 |
| 47. | *Acrotome pallescens* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 48. | *Ballota acetabulosa* | 31.6 | 24.5 | 26.9 | 17.0 | 364 |
| 49. | *Ballota pseudodictamnus* | 31.8 | 24.1 | 27. 3 | 16.8 | 352 |
| 50. | *Isoleucas arabica* | 31.3 | 22.9 | 28.0 | 17.9 | 336 |
| 51. | *Lamium tomentosum* | 32.1 | 22.1 | 28.6 | 17.2 | 308 |
| 52. | *Leonotis grandis* | 30.9 | 22.8 | 28.2 | 18.1 | 337 |
| 53. | *Leonotis leonurus* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 54. | *Leonotis myricifolia* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 55. | *Leonotis myrothamnifolia* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 56. | *Leonotis nepetifolia* var. *Africana* | 30.3 | 22.8 | 28.2 | 18.7 | 337 |
| 57. | *Leonotis nepetifolia* var. *nepetifolia* | 30.3 | 22.8 | 28.2 | 18.7 | 337 |
| 58. | *Leonotis ocymifolia* var. *raineriana* | 30.8 | 22.8 | 28.1 | 18.3 | 338 |
| 59. | *Leonotis ocymifolia* var. *schinzii* | 31.1 | 22.5 | 28.1 | 18.3 | 338 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 60. | *Leucas abyssinica* var. *sidamoensis* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 61. | *Leucas abyssinica* var. *brachycalyx* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 62. | *Leucas aequistylosa* | 30.7 | 23.5 | 27.7 | 18.2 | 336 |
| 63. | *Leucas argentea* | 31.3 | 22.3 | 27.7 | 18.8 | 336 |
| 64. | *Leucas bracteosa* | 30.8 | 22.5 | 28.1 | 18.6 | 334 |
| 65. | *Leucas calostachys* | 31.0 | 22.6 | 27.7 | 18.8 | 336 |
| 66. | *Leucas capensis* | 30.7 | 23.5 | 27.7 | 18.2 | 336 |
| 67. | *Leucas cuneifolia* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 68. | *Leucas deflexa* var. *deflexa* | 31.0 | 22.3 | 28.3 | 18.5 | 336 |
| 69. | *Leucas densiflora* | 30.8 | 22.8 | 28.4 | 18.0 | 338 |
| 70. | *Leucas discolor* | 31.1 | 21.5 | 28.8 | 18.7 | 386 |
| 71. | *Leucas flagellifera* | 30.9 | 23.1 | 27.9 | 18.1 | 337 |
| 72. | *Leucas glabrata* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 73. | *Leucas glabrata* var. *glabrata* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 74. | *Leucas grandis* | 31.0 | 22.5 | 28.6 | 17.9 | 329 |
| 75. | *Leucas inflata* | 31.3 | 22.6 | 27.7 | 18.5 | 336 |
| 76. | *Leucas jamesii* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 77. | *Leucas kishenensis* | 31.3 | 22.9 | 27.7 | 18.2 | 336 |
| 78. | *Leucas martinicensis* | 30.8 | 22.8 | 28.1 | 18.3 | 338 |
| 79. | *Leucas masaiensis* | 31.1 | 21.7 | 28.3 | 18.9 | 322 |
| 80. | *Leucas milanjiana* | 31.3 | 22.6 | 27.7 | 18.5 | 336 |
| 81. | *Leucas minimifolia* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 82. | *Leucas neuflizeana* | 31.0 | 22.9 | 28.0 | 18.2 | 336 |
| 83. | *Leucas nubica* | 31.3 | 23.0 | 27.8 | 17.9 | 335 |
| 84. | *Leucas oligocephala* | 31.1 | 22.0 | 28.6 | 18.3 | 322 |
| 85. | *Leucas sexdentata* | 31.0 | 22.9 | 27.7 | 18.5 | 336 |
| 86. | *Leucas spiculifolia* | 30.9 | 23.1 | 27.9 | 18.1 | 337 |
| 87. | *Leucas stachydiformis* | 31.3 | 22.6 | 28.3 | 17.9 | 336 |
| 88. | *Leucas urticifolia* var. *urticifolia* | 30.7 | 22.9 | 27.7 | 18.8 | 336 |
| 89. | *Leucas usagarensis* | 31.8 | 22.0 | 27.7 | 18.5 | 336 |
| 90. | *Leucas virgata* | 31.3 | 22.9 | 28.0 | 17.9 | 336 |
| 91. | *Leucas volkensii* var. *parviflora* | 30.8 | 22.8 | 28.4 | 18.0 | 338 |
| 92. | *Marrubium vulgare* | 31.9 | 22.8 | 29.1 | 16.2 | 364 |
| 93. | *Otostegia erlangeri* | 31.3 | 22.9 | 27.7 | 18.2 | 336 |
| 94. | *Otostegia fruticosa* | 31.5 | 22.5 | 27.9 | 18.0 | 333 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 95. | *Otostegia hildebrandtii* | 31.0 | 22.6 | 28.3 | 18.2 | 336 |
| 96. | *Rydingia integrifolia* | 30.6 | 23.0 | 28.9 | 17.5 | 343 |
| 97. | *Otostegia modesta* | 31.3 | 22.9 | 27.7 | 18.2 | 336 |
| 98. | *Rydingia persica* | 30.4 | 23.8 | 28.7 | 17.1 | 345 |
| 99. | *Isoleucas somala* | 31.3 | 22.9 | 28.0 | 17.9 | 336 |
| 100. | *Otostegia tomentosa* subsp *ambigens* | 31.3 | 22.9 | 27.7 | 18.2 | 336 |
| 101. | *Phlomis milingensis* | 30.8 | 23.4 | 28.0 | 17.9 | 364 |
| 102. | *Phlomis purpurea* | 29.2 | 24.6 | 27.8 | 18.4 | 342 |
| 103. | *Roylea cinerea* | 30.8 | 23.4 | 28.3 | 17.6 | 364 |
| 104. | *Scutellaria hirta* | 30.6 | 21.0 | 31.1 | 17.2 | 366 |
| 105. | *Scutellaria sieberi* | 30.7 | 21.1 | 31.0 | 17.3 | 365 |
| 106. | *Achyrospermum wallichianum* | 34.2 | 20.9 | 29.8 | 15.1 | 292 |
| 107. | *Eurysolen gracilis* | 32.0 | 20.2 | 30.3 | 17.6 | 347 |
| 108. | *Pogostemon benghalensis* | 31.7 | 20.2 | 31.1 | 16.9 | 366 |
| 109. | *Pogostemon quadrifolius* | 33.1 | 19.8 | 31.8 | 15.3 | 308 |
| 110. | *Pogostemon stellatus* | 31.6 | 20.2 | 31.3 | 16.8 | 351 |
| 111. | *Microtoena delavayi* | 33.9 | 21.4 | 30.0 | 14.6 | 280 |
| 112. | *Colebrookea oppositifolia* | 33.2 | 20.8 | 30.2 | 15.8 | 298 |
| | Average | 31.2 | 22.5 | 28.6 | 17.7 | 336.3 |

### 5. D.3. *rps16* based DNA sequenced data

The shortest length of *rps16* intron gene was noticed in *Leucas nepetifolia* with a base pair length of 437 nucleotides followed by *L. sebaldiana* with 440 base pairs. The longest one in the block was *Leucas hirta* which showed a length of 958 base pairs. After multiple sequence alignment, the total sequence length of 958 was found to be 981 base pairs. Of these, 669 were observed as conserved sites, 285 variable sites, 182 parsimony informative (Pi) sites and 89 singletons.

**Table 5.5:-Nucleotide Frequencies of *rps16* intergenic spacer in percentage**

| Sl No | Name of the Taxa | Percentage of | | | | |
|---|---|---|---|---|---|---|
| | | T | C | A | G | Total length of the base pairs |
| 1 | *Leucas angularis* | 30.8 | 16.0 | 34.2 | 19.0 | 843 |
| 2 | *Leucas biflora* | 31.1 | 15.8 | 33.9 | 19.1 | 852 |
| 3 | *Leucas chinensis* | 31.1 | 15.8 | 33.9 | 19.1 | 852 |
| 4 | *Leucas deodikarii* | 31.4 | 15.4 | 33.9 | 19.4 | 826 |
| 5 | *Leucas dhonimalayensis* | 31.5 | 15.6 | 33.8 | 19.1 | 834 |
| 6 | *Leucas eriostoma* | 31.8 | 15.3 | 33.6 | 19.3 | 824 |
| 7 | *Leucas helianthemifolia* | 31.4 | 15.7 | 33.9 | 18.9 | 834 |
| 8 | *Leucas hirta* | 31.3 | 16.0 | 33.8 | 18.9 | 852 |
| 9 | *Leucas lamiifolia* | 31.6 | 15.5 | 33.9 | 19.0 | 841 |
| 10 | *Leucas candida* | 31.1 | 16.0 | 33.8 | 19.1 | 852 |
| 11 | *Leucas lanceifolia* | 31.5 | 15.8 | 33.6 | 19.0 | 853 |
| 12 | *Leucas lavandulifolia* | 31.5 | 15.7 | 33.7 | 19.1 | 852 |
| 13 | *Leucas lavandulifolia* var. *decipiens* | 31.5 | 15.8 | 33.7 | 19.0 | 852 |
| 14 | *Leucas longifolia* | 31.3 | 15.8 | 34.0 | 18.8 | 852 |
| 15 | *Leucas marrubioides* var. *pulneyensis* | 31.3 | 15.4 | 33.9 | 19.4 | 831 |
| 16 | *Leucas martinicensis* | 31.3 | 16.1 | 33.0 | 19.6 | 818 |
| 17 | *Leucas pubescens* | 31.1 | 16.0 | 33.8 | 19.1 | 852 |
| 18 | *Leucas sebaldiana* | 31.5 | 15.8 | 33.8 | 18.9 | 852 |
| 19 | *Leucas suffruticosa* | 31.6 | 15.4 | 33.7 | 19.3 | 825 |
| 20 | *Leucas urticifolia* | 31.2 | 15.9 | 33.7 | 19.2 | 843 |
| 21 | *Leucas wightiana* | 31.5 | 15.8 | 33.8 | 18.9 | 852 |
| 22 | *Leucas zeylanica* | 31.5 | 15.8 | 33.7 | 19.0 | 852 |
| 23 | *Leucas ciliata* | 31.7 | 15.1 | 33.7 | 19.5 | 816 |
| 24 | *Leucas rosmarinifolia* | 31.7 | 15.2 | 33.7 | 19.5 | 817 |
| 25 | *Leucas mathewiana* | 31.6 | 15.2 | 33.8 | 19.4 | 816 |
| 26 | *Leucas diffusa* | 31.6 | 15.2 | 33.8 | 19.4 | 816 |
| 27 | *Leucas manipurensis* | 31.5 | 15.2 | 33.9 | 19.4 | 816 |
| 28 | *Leucas helicterifolia* | 31.3 | 15.3 | 33.9 | 19.5 | 816 |
| 29 | *Leucas lanata* | 31.4 | 15.3 | 34.1 | 19.2 | 816 |
| 30 | *Leucas macrantha* | 31.4 | 15.2 | 34.2 | 19.2 | 816 |
| 31 | *Leucas deodikarii* | 31.4 | 15.2 | 33.9 | 19.5 | 816 |
| 32 | *Leucas beddomei* | 31.7 | 15.7 | 33.3 | 19.4 | 881 |
| 33 | *Leucas prostrata* | 31.8 | 15.7 | 33.2 | 19.3 | 898 |
| 34 | *Leucas aspera* | 31.6 | 15.8 | 33.5 | 19.1 | 884 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 35 | *Leucas nepetifolia* | 32.5 | 16.2 | 30.4 | 20.8 | 437 |
| 36 | *Leucas martinicensis* | 31.7 | 16.2 | 32.5 | 19.6 | 846 |
| 37 | *Leucas mukerjiana* | 31.5 | 16.1 | 33.3 | 19.2 | 877 |
| 38 | *Leucas decemdentata* var. *decemdentata* | 31.5 | 15.8 | 33.4 | 19.3 | 882 |
| 39 | *Leucas montana* | 31.2 | 16.2 | 33.1 | 19.5 | 897 |
| 40 | *Gomphostemma javanicum* | 30.8 | 16.1 | 33.3 | 19.7 | 882 |
| 41 | *Sideritis macrostachyos* | 30.9 | 15.7 | 33.4 | 20.1 | 881 |
| 42 | *Sideritis romana* | 31.4 | 15.4 | 32.8 | 20.4 | 883 |
| 43 | *Acrotome angustifolia* | 31.5 | 16.1 | 33.4 | 18.9 | 882 |
| 44 | *Acrotome fleckii* | 31.3 | 15.9 | 33.3 | 19.5 | 876 |
| 45 | *Acrotome hispida* | 31.5 | 16.2 | 33.1 | 19.2 | 882 |
| 46 | *Acrotome inflata* | 31.3 | 15.9 | 33.3 | 19.5 | 876 |
| 47 | *Acrotome pallescens* | 31.5 | 16.0 | 33.2 | 19.3 | 882 |
| 48 | *Ballota acetabulosa* | 30.7 | 16.5 | 33.7 | 19.1 | 879 |
| 49 | *Ballota pseudodictamnus* | 30.7 | 16.5 | 33.7 | 19.1 | 879 |
| 50 | *Isoleucas arabica* | 31.1 | 15.9 | 33.6 | 19.4 | 885 |
| 51 | *Lamium tomentosum* | 30.6 | 16.0 | 33.8 | 19.5 | 875 |
| 52 | *Leonotis grandis* | 31.6 | 16.0 | 33.1 | 19.4 | 883 |
| 53 | *Leonotis leonurus* | 30.6 | 16.3 | 33.7 | 19.4 | 849 |
| 54 | *Leonotis myricifolia* | 31.6 | 16.1 | 33.0 | 19.3 | 884 |
| 55 | *Leonotis myrothamnifolia* | 31.6 | 16.0 | 33.1 | 19.3 | 884 |
| 56 | *Leonotis nepetifolia* var. *africana* | 31.5 | 16.1 | 33.1 | 19.4 | 883 |
| 57 | *Leonotis nepetifolia* var. *nepetifolia* | 31.5 | 16.1 | 33.1 | 19.4 | 883 |
| 58 | *Leonotis ocymifolia* var. *raineriana* | 31.5 | 15.7 | 33.4 | 19.5 | 874 |
| 59 | *Leonotis ocymifolia* var. *schinzii* | 31.6 | 16.0 | 33.0 | 19.5 | 883 |
| 60 | *Leucas abyssinica* var. *sidamoensis* | 31.6 | 15.9 | 33.0 | 19.5 | 879 |
| 61 | *Leucas abyssinica* var. *brachycalyx* | 31.5 | 15.9 | 33.0 | 19.5 | 878 |
| 62 | *Leucas aequistylosa* | 31.2 | 15.7 | 33.6 | 19.5 | 885 |
| 63 | *Leucas argentea* | 31.2 | 15.8 | 33.4 | 19.5 | 885 |
| 64 | *Leucas bracteosa* | 31.5 | 16.1 | 32.5 | 19.9 | 850 |
| 65 | *Leucas calostachys* | 31.6 | 15.8 | 33.2 | 19.5 | 874 |
| 66 | *Leucas capensis* | 31.6 | 15.7 | 33.4 | 19.3 | 874 |
| 67 | *Leucas cuneifolia* | 31.5 | 15.9 | 33.0 | 19.5 | 878 |
| 68 | *Leucas deflexa* var. *deflexa* | 31.6 | 16.0 | 32.8 | 19.6 | 883 |
| 69 | *Leucas densiflora* | 31.6 | 15.9 | 32.8 | 19.7 | 883 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 70 | *Leucas discolor* | 31.6 | 15.9 | 33.0 | 19.5 | 879 |
| 71 | *Leucas flagellifera* | 31.7 | 15.9 | 33.2 | 19.3 | 883 |
| 72 | *Leucas glabrata* | 31.2 | 15.8 | 33.4 | 19.6 | 872 |
| 73 | *Leucas glabrata* var. *glabrata* | 31.3 | 15.7 | 33.4 | 19.6 | 872 |
| 74 | *Leucas grandis* | 31.6 | 16.0 | 33.1 | 19.4 | 883 |
| 75 | *Leucas inflata* | 31.8 | 15.9 | 32.9 | 19.4 | 881 |
| 76 | *Leucas jamesii* | 31.5 | 15.9 | 32.8 | 19.7 | 878 |
| 77 | *Leucas kishenensis* | 31.3 | 15.7 | 33.5 | 19.5 | 884 |
| 78 | *Leucas martinicensis* | 31.4 | 16.2 | 32.7 | 19.8 | 848 |
| 79 | *Leucas masaiensis* | 31.5 | 15.8 | 33.3 | 19.5 | 874 |
| 80 | *Leucas milanjiana* | 31.2 | 15.8 | 33.4 | 19.6 | 872 |
| 81 | *Leucas minimifolia* | 31.7 | 15.9 | 33.0 | 19.4 | 878 |
| 82 | *Leucas neuflizeana* | 31.2 | 15.8 | 33.4 | 19.7 | 869 |
| 83 | *Leucas nubica* | 31.4 | 16.1 | 33.1 | 19.5 | 883 |
| 84 | *Leucas oligocephala* | 31.1 | 15.9 | 33.4 | 19.7 | 869 |
| 85 | *Leucas sexdentata* | 31.5 | 16.0 | 33.1 | 19.4 | 882 |
| 86 | *Leucas spiculifolia* | 31.7 | 15.7 | 33.3 | 19.3 | 883 |
| 87 | *Leucas stachydiformis* | 31.2 | 16.0 | 33.4 | 19.5 | 884 |
| 88 | *Leucas urticifolia* var. *urticifolia* | 31.4 | 15.9 | 33.3 | 19.5 | 874 |
| 89 | *Leucas usagarensis* | 31.0 | 16.1 | 33.2 | 19.7 | 870 |
| 90 | *Leucas virgata* | 31.4 | 15.7 | 33.4 | 19.5 | 884 |
| 91 | *Leucas volkensii* var. *parviflora* | 31.5 | 15.9 | 32.8 | 19.8 | 883 |
| 92 | *Marrubium vulgare* | 31.0 | 16.0 | 34.1 | 18.9 | 880 |
| 93 | *Otostegia erlangeri* | 31.3 | 16.1 | 33.4 | 19.3 | 883 |
| 94 | *Otostegia fruticosa* | 31.2 | 16.0 | 33.3 | 19.4 | 885 |
| 95 | *Otostegia hildebrandtii* | 31.1 | 16.1 | 33.5 | 19.3 | 884 |
| 96 | *Rydingia integrifolia* | 31.3 | 16.1 | 33.1 | 19.5 | 882 |
| 97 | *Otostegia modesta* | 31.2 | 16.1 | 33.4 | 19.3 | 884 |
| 98 | *Rydingia persica* | 31.3 | 16.1 | 33.1 | 19.5 | 884 |
| 99 | *Isoleucas somala* | 31.2 | 15.8 | 33.4 | 19.5 | 885 |
| 100 | *Otostegia tomentosa* subsp *ambigens* | 31.2 | 16.0 | 33.3 | 19.4 | 885 |
| 101 | *Phlomis milingensis* | 31.1 | 15.8 | 33.9 | 19.2 | 881 |
| 102 | *Phlomis purpurea* | 30.5 | 15.6 | 33.9 | 20.0 | 881 |
| 103 | *Roylea cinerea* | 31.1 | 16.1 | 33.6 | 19.3 | 882 |
| 104 | *Scutellaria hirta* | 31.0 | 15.2 | 34.6 | 19.3 | 914 |
| 105 | *Scutellaria sieberi* | 31.0 | 15.2 | 34.6 | 19.3 | 914 |
| 106 | *Achyrospermum wallichianum* | 31.4 | 15.4 | 34.0 | 19.2 | 861 |
| 107 | *Eurysolen gracilis* | 30.9 | 15.3 | 35.0 | 18.8 | 823 |
| 108 | *Pogostemon benghalensis* | 32.9 | 14.7 | 33.0 | 19.4 | 788 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 109 | *Pogostemon quadrifolius* | 32.8 | 14.9 | 34.1 | 18.2 | 813 |
| 110 | *Pogostemon stellatus* | 32.4 | 16.7 | 31.5 | 19.4 | 432 |
| 111 | *Microtoena delavayi* | 32.0 | 14.0 | 33.0 | 21.0 | 300 |
| 112 | *Colebrookea oppositifolia* | 31.8 | 15.2 | 33.8 | 19.2 | 828 |
| | Average | 31.4 | 15.8 | 33.4 | 19.4 | 851.8 |

### 5. D.4. Concatanated data set of *trnL, rps16* introns and *trnL-F* intergenic spacer based on DNA sequenced data

The chlororoplast DNA dataset consisted of three chloroplast regions *trnL*, *rps16* introns and *trnL-F* intergenic spacer. The concatenated dataset had 2151 characters of which 1335 was conserved sites, 613 variable, 357 parsimony informative (Pi) sites and 225 singletons. Gaps were treated as 5th character. Four major clades were observed during the analysis.

**Table 5.6:- Nucleotide Frequencies of concatenated data set of *trnL*, *rps16* introns and *trnL-F* intergenic spacer in percentage**

| **Sl No** | **Name of the Taxa** | **Percentage of** | | | | **Total length of the base pairs** |
|---|---|---|---|---|---|---|
| | | **T** | **C** | **A** | **G** | |
| 1 | *Leucas angularis* | 30.1 | 16.9 | 34.5 | 18.5 | 1695 |
| 2 | *Leucas biflora* | 30.2 | 16.9 | 34.3 | 18.5 | 1706 |
| 3 | *Leucas chinensis* | 30.2 | 16.9 | 34.4 | 18.5 | 1705 |
| 4 | *Leucas deodikarii* | 30.3 | 16.7 | 34.4 | 18.5 | 1678 |
| 5 | *Leucas dhonimalayensis* | 30.4 | 16.7 | 34.3 | 18.6 | 1693 |
| 6 | *Leucas eriostoma* | 30.6 | 16.6 | 34.0 | 18.8 | 1678 |
| 7 | *Leucas helianthemifolia* | 30.3 | 16.8 | 34.3 | 18.5 | 1693 |
| 8 | *Leucas hirta* | 30.3 | 16.9 | 34.2 | 18.5 | 1711 |
| 9 | *Leucas lamiifolia* | 30.5 | 16.8 | 34.1 | 18.6 | 1693 |
| 10 | *Leucas candida* | 30.2 | 17.0 | 34.3 | 18.5 | 1705 |
| 11 | *Leucas lanceifolia* | 30.4 | 16.9 | 34.1 | 18.6 | 1712 |
| 12 | *Leucas lavandulifolia* | 30.3 | 17.0 | 34.2 | 18.6 | 1712 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 13 | *Leucas lavandulifolia* var. *decipiens* | 30.3 | 17.1 | 34.2 | 18.5 | 1712 |
| 14 | *Leucas longifolia* | 30.3 | 16.8 | 34.4 | 18.5 | 1711 |
| 15 | *Leucas marrubioides* var. *pulneyensis* | 30.3 | 16.7 | 34.4 | 18.6 | 1684 |
| 16 | *Leucas martinicensis* | 30.0 | 17.0 | 34.0 | 19.0 | 1673 |
| 17 | *Leucas pubescens* | 30.1 | 17.0 | 34.3 | 18.6 | 1711 |
| 18 | *Leucas sebaldiana* | 30.4 | 16.8 | 34.3 | 18.5 | 1716 |
| 19 | *Leucas suffruticosa* | 30.5 | 16.7 | 34.1 | 18.7 | 1684 |
| 20 | *Leucas urticifolia* | 29.9 | 16.9 | 34.3 | 18.9 | 1696 |
| 21 | *Leucas wightiana* | 30.3 | 16.9 | 34.2 | 18.5 | 1711 |
| 22 | *Leucas zeylanica* | 30.4 | 16.9 | 34.2 | 18.5 | 1711 |
| 23 | *Leucas ciliata* | 30.4 | 16.5 | 34.2 | 18.8 | 1675 |
| 24 | *Leucas rosmarinifolia* | 30.5 | 16.5 | 34.2 | 18.8 | 1676 |
| 25 | *Leucas mathewiana* | 30.3 | 16.8 | 34.4 | 18.4 | 1676 |
| 26 | *Leucas diffusa* | 30.3 | 16.8 | 34.2 | 18.7 | 1676 |
| 27 | *Leucas manipurensis* | 30.4 | 16.5 | 34.3 | 18.7 | 1675 |
| 28 | *Leucas helicterifolia* | 30.3 | 16.7 | 34.4 | 18.7 | 1669 |
| 29 | *Leucas lanata* | 30.5 | 16.5 | 34.5 | 18.5 | 1668 |
| 30 | *Leucas macrantha* | 30.3 | 16.5 | 34.4 | 18.8 | 1675 |
| 31 | *Leucas deodikarii* | 30.3 | 16.6 | 34.5 | 18.6 | 1668 |
| 32 | *Leucas beddomei* | 30.5 | 16.8 | 34.0 | 18.8 | 1740 |
| 33 | *Leucas prostrata* | 30.6 | 16.8 | 33.9 | 18.8 | 1757 |
| 34 | *Leucas aspera* | 30.4 | 17.1 | 33.9 | 18.5 | 1753 |
| 35 | *Leucas nepetifolia* | 30.2 | 18.1 | 32.8 | 19.0 | 1329 |
| 36 | *Leucas martinicensis* | 30.2 | 17.4 | 33.5 | 19.0 | 1727 |
| 37 | *Leucas mukerjiana* | 30.8 | 17.4 | 33.2 | 18.6 | 1942 |
| 38 | *Leucas decemdentata* var. *decemdentata* | 30.3 | 17.4 | 33.7 | 18.6 | 1765 |
| 39 | *Leucas montana* | 30.1 | 17.6 | 33.5 | 18.8 | 1791 |
| 40 | *Gomphostemma* | 29.7 | 17.2 | 33.9 | 19.2 | 1783 |
| 41 | *Sideritis macrostachoys* | 28.9 | 16.4 | 35.0 | 19.7 | 1644 |
| 42 | *Sideritis romana* | 29.1 | 16.3 | 34.9 | 19.8 | 1654 |
| 43 | *Acrotome angustifolia* | 30.1 | 17.4 | 33.9 | 18.7 | 1746 |
| 44 | *Acrotome fleckii* | 29.9 | 17.2 | 33.9 | 19.0 | 1740 |
| 45 | *Acrotome hispida* | 30.1 | 17.2 | 33.9 | 18.8 | 1741 |
| 46 | *Acrotome inflata* | 29.9 | 17.2 | 33.9 | 19.0 | 1740 |
| 47 | *Acrotome pallescens* | 30.2 | 17.1 | 33.8 | 18.9 | 1745 |
| 48 | *Ballota acetabulosa* | 29.9 | 17.7 | 33.7 | 18.7 | 1766 |

|  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| 49 | *Ballota pseudodictamnus* | 29.9 | 17.6 | 33.8 | 18.6 | 1754 |
| 50 | *Isoleucas arabica* | 30.1 | 17.1 | 34.0 | 18.8 | 1747 |
| 51 | *Lamium tomentosum* | 30.2 | 17.2 | 33.5 | 19.0 | 1649 |
| 52 | *Leonotis grandis* | 30.2 | 17.1 | 33.9 | 18.8 | 1747 |
| 53 | *Leonotis leonurus* | 29.7 | 17.2 | 34.1 | 18.9 | 1712 |
| 54 | *Leonotis myricifolia* | 30.2 | 17.1 | 33.8 | 18.9 | 1747 |
| 55 | *Leonotis myrothamnifolia* | 30.2 | 17.1 | 33.8 | 18.9 | 1747 |
| 56 | *Leonotis nepetifolia* var. *africana* | 30.1 | 17.2 | 34.0 | 18.7 | 1740 |
| 57 | *Leonotis nepetifolia* var. *nepetifolia* | 30.1 | 17.2 | 34.0 | 18.7 | 1740 |
| 58 | *Leonotis ocymifolia* var. *raineriana* | 30.0 | 17.1 | 34.0 | 19.0 | 1682 |
| 59 | *Leonotis ocymifolia* var. *schinzii* | 30.3 | 17.1 | 33.7 | 18.9 | 1741 |
| 60 | *Leucas abyssinica* var. *sidamoensis* | 30.2 | 17.1 | 33.7 | 19.0 | 1732 |
| 61 | *Leucas abyssinica* var. *brachycalyx* | 30.2 | 17.1 | 33.8 | 19.0 | 1741 |
| 62 | *Leucas aequistylosa* | 30.0 | 17.1 | 34.0 | 18.9 | 1747 |
| 63 | *Leucas argentea* | 30.1 | 16.9 | 33.9 | 19.1 | 1748 |
| 64 | *Leucas bracteosa* | 30.2 | 17.1 | 33.6 | 19.1 | 1711 |
| 65 | *Leucas calostachys* | 30.2 | 17.0 | 33.9 | 19.0 | 1737 |
| 66 | *Leucas capensis* | 30.2 | 17.1 | 33.9 | 18.8 | 1737 |
| 67 | *Leucas cuneifolia* | 30.2 | 17.1 | 33.8 | 19.0 | 1741 |
| 68 | *Leucas deflexa* var. *deflexa* | 30.2 | 17.0 | 33.8 | 19.1 | 1746 |
| 69 | *Leucas densiflora* | 30.2 | 17.0 | 33.8 | 19.0 | 1748 |
| 70 | *Leucas discolor* | 30.3 | 16.9 | 33.8 | 19.0 | 1792 |
| 71 | *Leucas flagellifera* | 30.5 | 17.1 | 33.8 | 18.7 | 1739 |
| 72 | *Leucas glabrata* | 30.0 | 17.1 | 33.9 | 19.0 | 1735 |
| 73 | *Leucas glabrata* var. *glabrata* | 30.1 | 17.0 | 33.9 | 19.0 | 1735 |
| 74 | *Leucas grandis* | 30.3 | 16.9 | 34.1 | 18.7 | 1739 |
| 75 | *Leucas inflata* | 30.4 | 17.0 | 33.7 | 18.9 | 1744 |
| 76 | *Leucas jamesii* | 30.2 | 17.1 | 33.7 | 19.1 | 1741 |
| 77 | *Leucas kishenensis* | 30.4 | 17.0 | 33.8 | 18.9 | 1739 |
| 78 | *Leucas martinicensis* | 30.1 | 17.2 | 33.7 | 19.1 | 1713 |
| 79 | *Leucas masaiensis* | 30.1 | 16.8 | 34.1 | 19.0 | 1723 |
| 80 | *Leucas milanjiana* | 30.1 | 17.0 | 33.9 | 19.0 | 1735 |
| 81 | *Leucas minimifolia* | 30.3 | 17.1 | 33.8 | 18.9 | 1741 |
| 82 | *Leucas neuflizeana* | 30.0 | 16.9 | 34.0 | 19.1 | 1732 |
| 83 | *Leucas nubica* | 30.3 | 17.2 | 33.7 | 18.8 | 1738 |
| 84 | *Leucas oligocephala* | 30.0 | 16.8 | 34.2 | 19.1 | 1718 |
| 85 | *Leucas sexdentata* | 30.2 | 17.1 | 33.8 | 18.9 | 1745 |

|  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| 86 | *Leucas spiculifolia* | 30.5 | 17.0 | 33.8 | 18.7 | 1739 |
| 87 | *Leucas stachydiformis* | 30.1 | 17.1 | 34.1 | 18.7 | 1746 |
| 88 | *Leucas urticifolia* var. *urticifolia* | 30.0 | 17.1 | 33.9 | 19.0 | 1737 |
| 89 | *Leucas usagarensis* | 30.2 | 17.0 | 33.9 | 19.0 | 1733 |
| 90 | *Leucas virgata* | 30.4 | 17.0 | 33.9 | 18.7 | 1739 |
| 91 | *Leucas volkensii* var. *parviflora* | 30.1 | 17.0 | 33.8 | 19.1 | 1748 |
| 92 | *Marrubium vulgare* | 30.1 | 17.2 | 34.4 | 18.4 | 1766 |
| 93 | *Otostegia erlangeri* | 30.2 | 17.1 | 33.9 | 18.7 | 1745 |
| 94 | *Otostegia fruticosa* | 30.2 | 17.0 | 33.9 | 18.9 | 1743 |
| 95 | *Otostegia hildebrandtii* | 30.1 | 17.1 | 34.1 | 18.8 | 1746 |
| 96 | *Rydingia integrifolia* | 30.3 | 17.3 | 33.8 | 18.6 | 1750 |
| 97 | *Otostegia modesta* | 30.1 | 17.1 | 33.9 | 18.8 | 1746 |
| 98 | *Rydingia persica* | 30.2 | 17.4 | 33.8 | 18.6 | 1754 |
| 99 | *Isoleucas somala* | 30.2 | 17.1 | 33.9 | 18.8 | 1747 |
| 100 | *Otostegia tomentosa* subsp *ambigens* | 30.2 | 17.2 | 33.8 | 18.8 | 1734 |
| 101 | *Phlomis milingensis* | 30.1 | 17.2 | 34.0 | 18.7 | 1752 |
| 102 | *Phlomis purpurea* | 29.7 | 17.0 | 34.1 | 19.3 | 1743 |
| 103 | *Roylea cinerea* | 30.0 | 17.3 | 34.0 | 18.7 | 1760 |
| 104 | *Scutellaria hirta* | 29.4 | 16.8 | 34.7 | 19.1 | 1782 |
| 105 | *Scutellaria sieberi* | 29.4 | 16.8 | 34.6 | 19.1 | 1781 |
| 106 | *Achyrospermum wallichianum* | 30.7 | 16.4 | 34.7 | 18.2 | 1623 |
| 107 | *Eurysolen gracilis* | 30.0 | 16.3 | 35.0 | 18.7 | 1692 |
| 108 | *Pogostemon benghalensis* | 31.1 | 16.2 | 34.2 | 18.6 | 1674 |
| 109 | *Pogostemon quadrifolius* | 31.1 | 16.2 | 35.0 | 17.7 | 1597 |
| 110 | *Pogostemon stellatus* | 30.2 | 17.3 | 34.1 | 18.4 | 1298 |
| 111 | *Microtoena delavayi* | 30.3 | 16.9 | 34.8 | 18.1 | 1061 |
| 112 | *Colebrookea oppositifolia* | 30.3 | 16.8 | 34.6 | 18.3 | 1533 |
|  | Average | 30.2 | 17.0 | 34.0 | 18.8 | 1709.7 |

## 5. E. PHYLOGENETIC ANALYSIS

Altogether 112 taxa were used in analysis. Separate analysis were conducted for each genes followed by a concatenated data analysis.

### 5. E.1. Maximum Likelihood Analysis

Maximum Likelihood + Thorough bootstrap were used with 1000 bootstrap replicates. Bootstrap per branch length was selected for the Maximum Likelihood analysis using RAxML.

#### 5. E.1.a. Phylogeny based on *trnL* based DNA sequenced data

The length of the *trnL* varied from 466 base pairs in *Lamium tomentosum* to 580 base pairs in *Leucas sebaldiana.* After trimming and alignment, the data set length was reduced to 574 base pairs. Out of this, 422 were observed as conserved sites, 138 variable sites, 69 parsimony informative (Pi) sites and 68 singletons. Gaps were treated as 5th character.

Maximum Likelihood analysis of *trnL* sequence of 112 taxa was carried out with RAxML GUI3 software package (Figure 5.2). Analysis produced a polytomy with very low bootstrap support due to poor resolution. No conclusion could be drawn from the tree topology. The genus *Leucas* appears paraphyletic as genera like *Leonotis, Acrotome, Otostegia* and *Isoleucas* are nested within it. However a monophyletic nature of annual herbaceous species like *L. aspera, L. lavandulifolia, L. diffusa, L. zeylanica, L. wightiana* and *L. mathewiana* appears with a BS value of 87. The cladogram shows grouping of Asian taxa with ovate leaves and axillary inflorescence at one end and majority of perennial plants with lanceolate leavesand terminal inflorescence at opposite end. Apart from this the analysis did not show well supported clusters. Species level resolution was very poor.

In fact, using *trnL* region alone could not give any insight into infrageneric molecular groups within the genus.

### 5.E.1.b. Phylogeny based on *trnL-F* based DNA sequenced data

Length of the *trnL-F* sequences taken for analysis was shortest in *Sideritis macrostachyos* with 221 base pairs followed by *Sideritis romana* with 222. The *Leucas mukerjiana* extended up to a length of 525 base pairs and it was the longest sequence considered in analysis. After aligning the sequences using Clustal W, the total base pair length was found to be 596. Out of this, 244 were observed as conserved sites, 190 variable sites, 106 parsimony informative (Pi) sites and 68 singletons.

The phylogram obtained from the Maximum Likelihood analysis using RAxML was carried out with *trnL-F* intron from 112 taxa (Figure 5.3). As in previous analysis with *trnL* data, here the analysis produced a polytomy. However Asian *Leucas* from a clade with BS value 52 (Clade A). African *Leucas* formed a paraphyly with a lot of genera like *Leucas, Leonotis Actotome, Otostegia,* and *Isoleucas.* Here the present analysis did not yield a good well supported clustering pattern that reflects relationships among different taxa. The bootstrap support values were not adequate for further discussion.

Within the polytomy, the phylogram resolved Asian species to a group as in previous studies by Scheen & Albert (2009) using Maximum Parsimony method. Our study using Maximum Likelihood methods reveal similar topology with poor Bootstrap Suppport. Selected Lamioideaen members like *Gomphostemma, Scutellaria, Achyrospermum, Eurysolen, Pogostemon, Microtoena, Colebrookea, Sideritis, Phlomis, Lamium, Ballotta, Marrubium* and *Roylea* were clustered as Outgroup.

Deep examination of the clade of Asian *Leucas* showed poor resolution. But three distinct clades, correspond to three morphological groups would be recognized with *trnL* data was used.

Group I consists of the members such as *L. beddomei, L. dhonimalayensis, L. eriostoma, L. lanceifolia, L. sebaldiana, L. suffruticosa, L. rosmarinifolia* and *L. manipurensis* clustered with BS 64. Further resolution obtained poor support values. The species are similar and share certain synapomorphic characters like perennial habit and fan shaped corolla. Results showed that *L. helianthemifolia, L. hirta, L. lamiifolia, L. prostrata,* and *L. ciliata* are not nested within this clade eventhough they are morphologically similar and were included in section *Astrodon* by Bentham.

Group II consists of *L. lavandulifolia, L. lavandulifolia* var. *decipiens, L. mathewiana, L. diffusa, L. wightiana, Leucas aspera,* and *L. zeylanica. L. lavandulifolia, L. lavandulifolia* var. *decipiens, L. mathewiana* and *L. diffusa* with BS value of 85. They are annual herbs having linear lanceolate leaves and fanshaped corolla.

Group III A third group with a BS value 61 is clustered with species like *L. montana, L. decemdentata* var. *decemdentata, L. nepetifolia, L. biflora, L. lanata* var. *candida, L. chinensis, L. pubescense, L. angularis, L. deodikarii, L. lanata, L. mukerjiana, L. helicterifolia* and *L. marrubioides* var. *pulneyensis* share similar morphology of spreading nature, ovate leaves and axillary inflorescence

In this analysis also the two African taxa *L. urticifolia* and *L. martinicensis* clustered with African *Leucas.* Region *trnL-F* appear more variable.

### 5. E.1.c. Phylogeny based on *rps16* based DNA sequenced data

Length of the *rps16* intron gene with the shortest base pair length of 437 for the *Leucas nepetifolia* and the other taxa *Leucas sebaldiana* showed a length of 440 base pairs. The longest one in the block was *Leucas hirta* which showed a length of 958 base pairs. Multiple sequence alignment has been done using Clustal W, which was incorporated in the MEGA 6 software. Total sequence length was found to be 981 base pairs. Out of this, 669 were observed as conserved sites, 285 variable sites, 182 parsimony informative (Pi) sites and 89 singletons.

The results obtained from the ML analysis of *rps16* intron sequences of 112 taxa did not yield a tree (Figure 5.4) with good support value as in the previous analysis with *trnL* and *trnL-F* regions. This analysis yielded a different tree topology than that obtained in earlier analysis with *trnL* and *trnL-F* data. The topology exhibits the entire *Leucas s.l.* form a paraphyletic group with *Leucas, Leonotis, Actotome, Otostegia,* and *Isoleucas*. Poor resolution may be due to lack of variation in this region.

The clade I observed in earlier analysis also clumbed into a group with a BS of just 21. This group is found related to African species than to Indian species in ML analysis. Similarly all other species are clustered together with a BS value of 81 at the other end. Two species *L. deodikarii* and *L. lanata* are found outside this clade. A good supported *L. longifolia* and *L. macrantha* clade with BS 99 could be observed. In all analyses the closeness of *L. macrantha* and *L. longifolia* is evident. Tree resolution using *rps16* alone is very poor when compared to other two regions.

### 5. E.1.d Phylogeny based on concatenated data set of *trnL*, *trnL-F* and *rps16.*

A concatenated plastid DNA of 112 taxa with dataset of three regions *trnL*, *rps16* introns and *trnL-F* intergenic spacer were prepared in MEGA. The concatenated dataset had 2151 characters of which 1335 were conserved sites. 613 variable, 357 parsimony informative (Pi) sites, and 225 were singletons. Gaps were treated as 5th character.

The combined analysis also produced a polytomy (Figure 5.5-5.7) with majority of African *Leucas* together with *Leonotis, Acrotome* and *Otostegia* clustered on one side with a BS of 58 (Clade A). On the opposite end all the Asian species form a cluster (Clade B) with a support value of 69. Meanwhile three species *L. aequistylosa, Isoleucas somala* and *Isoleucas arabica* show independant lineages but with poor support value. The two species *Isoleucas somala* and *Isoleucas arabica* form a cluster with good support value. The feeble support for this three species does not support their status in the polytomy but need to consider these following as independent line of evolution and not part either clade A or B. The tree topology more or less supports earlier observations by Scheen and Albert (2007). *Rydingia persica* and *Rydingia integrifolia* with BS 99 clustered together and formed a basal clade to both *Leucas* groups. Lamioideaen members like *Gomphostemma, Scutellaria, Achyrospermum, Eurysolen, Pogostemon, Microtoena, Colebrookea, Sideritis, Phlomis, Lamium, Ballotta, Marrubium* and *Roylea* form a group on re-rooting the tree as Outgroups.

Within monophyletic Asian clade, four subclades are recognized (Clade I-IV). This Asian clade splits into two Clade-I and a clade consists of all the remaining taxa.

Clade I (BS 52) consisting of 13 species: *Leucas deodikarii, L. lanata. L. pubescens, L. angularis, L. biflora, L. montana, L. chinensis, L. lanata var candida, L. nepetifolia, L. helicterifolia, L. mukerjiana, L. marrubioides* var. *pulneyensis,* and *L. decemdentata* var. *decemdentata* form a monophyletic group with BS 52. Potential synapomorphic characters for this section are found to be the non-ciliated calyx mouth and non-grooved stems. This section shows the characters of the classical taxonomical section *Ortholeucas* proposed by Bentham (1832).

The large clade, sister to clade I bifurcated further to produce Clade II consists of 2 species and the remaining taxa together supported by BS value 89.

Clade-II. This clade consists of *L. longifolia* and *L. macrantha* with only two species share the potential synapomorphic characters such as inflorescence in multiple nodes, annual life-span and linear leaves and clustering of the two species is supported with BS 99. This clade is sister to a large clade consisting of remaining species of Asian *Leucas* which again shows a bifurcation to form clade III and clade IV.

Clade-III consists of the members such as *L. dhonimalayensis, L. eriostoma, L. lanceifolia, L. sebaldiana, L. suffruticosa, L. rosmarinifolia, L. manipurensis, L. beddomei, L. helianthemifolia, L. hirta, L. lamiifolia, L. prostrata,* and *L. ciliata. L. suffruticosa* and *L. rosmarinifolia* are separated as supported by BS 76. Potential synapomorphies of this section include they are spreading herbs, usually the length of the calyx teeth is 2-3 mm, calyx teeth spreading out, fan shaped corolla were also seen.

Clade-IV consists of *L. lavandulifolia, L. lavandulifolia var decipiens, L. mathewiana, L. diffusa, L. wightiana, Leucas aspera,* and *L. zeylanica.* All these share a apomorphic characterof annual herbaceous nature, linear-lanceolate leaves.

Section *Hemistoma* consists of African *Leucas.* Two taxa of *Leucas* of this section are also seen in India. ie. *L. urticifolia* and *L. martinicensis*. We sequenced these taxa and found that taxa collected from India are nested within African lineage.

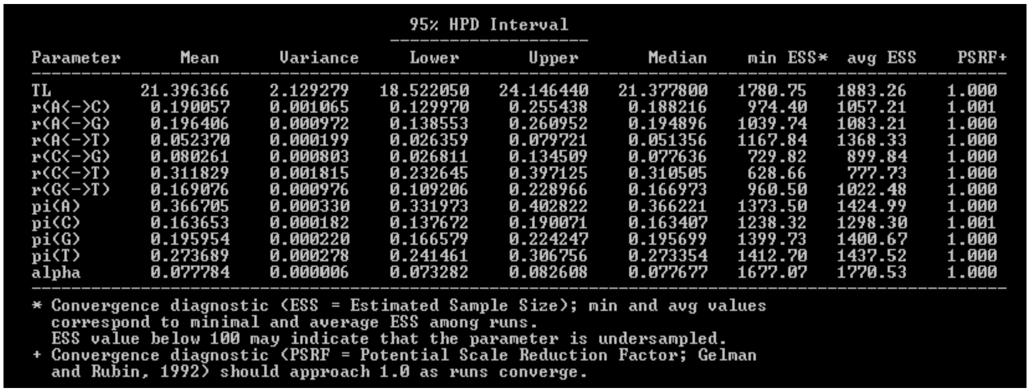
## 5. E.2 BAYESIAN ANALYSIS

### 5. E.2.a Phylogeny based on *trnL* based DNA sequenced data

As in previous analysis herealso we used 112 taxa fo assessing phylogenetic relationship using MrBayes package. The length of the *trnL* varied from 466 base pairs, as in *Lamium tomentosum* and the longest base pair length was 580 in *Leucas sebaldiana.* For the sake of convenience and to get more convincing result; the sequences were trimmed and the final alignment length was maintained as 574 base pairs. Out of these 422 were observed as conserved sites, 138 variable sites, 69 parsimony informative Pi) sites and 68 singletons.

The HPD values obtained in the Bayesian analysis are given below

## Table 5.7 :- 95% HPD Interval



```
                                       95% HPD Interval
                                     --------------------
Parameter      Mean      Variance     Lower       Upper       Median    min ESS*  avg ESS    PSRF+
---------------------------------------------------------------------------------------------------
TL          21.396366   2.129279  18.522050   24.146440   21.377800   1780.75   1883.26    1.000
r(A<->C)     0.190057   0.001065   0.129970    0.255438    0.188216    974.40   1057.21    1.001
r(A<->G)     0.196406   0.000972   0.138553    0.260952    0.194896   1039.74   1083.21    1.000
r(A<->T)     0.052370   0.000199   0.026359    0.079721    0.051356   1167.84   1368.33    1.000
r(C<->G)     0.080261   0.000803   0.026811    0.134509    0.077636    729.82    899.84    1.000
r(C<->T)     0.311829   0.001815   0.232645    0.397125    0.310505    628.66    777.73    1.000
r(G<->T)     0.169076   0.000976   0.109206    0.228966    0.166973    960.50   1022.48    1.000
pi(A)        0.366705   0.000330   0.331973    0.402822    0.366221   1373.50   1424.99    1.000
pi(C)        0.163653   0.000182   0.137672    0.190071    0.163407   1238.32   1298.30    1.001
pi(G)        0.195954   0.000220   0.166579    0.224247    0.195699   1399.73   1400.67    1.000
pi(T)        0.273689   0.000278   0.241461    0.306756    0.273354   1412.70   1437.52    1.000
alpha        0.077784   0.000006   0.073282    0.082608    0.077677   1677.07   1770.53    1.000
---------------------------------------------------------------------------------------------------
* Convergence diagnostic (ESS = Estimated Sample Size); min and avg values
  correspond to minimal and average ESS among runs.
  ESS value below 100 may indicate that the parameter is undersampled.
+ Convergence diagnostic (PSRF = Potential Scale Reduction Factor; Gelman
  and Rubin, 1992) should approach 1.0 as runs converge.
```

* Convergence diagnostic (ESS = Estimated Sample Size); min and avg values correspond to minimal and average ESS among runs. ESS value below 100 may indicate that the parameter is undersampled.

* Convergence diagnostic (PSRF = Potential scale reduction factor [Gelman and Rubin, 1992], uncorrected) should approach 1 as runs converge. The values may be unreliable if you have a small number of samples. PSRF should only be used as a rough guide to convergence since all the assumptions that allow one to interpret it as a scale reduction factor are not met in the phylogenetic context.

r(A<->C), r(A<->G), r(A<->T), r(C<->G), r(C<->T), r(G<->T) - rate pameters.

pi(A), pi(C), pi(G), pi(T) – four stationary nucleotide frequencies.

Alpha - the shape parameter of the gamma distribution of rate variation.

TL - the total tree length (the sum of all branch lengths).

Results show that tree resolution was very poor in *trnL* sequence in Bayesian analysis (Figure 5.8). All *Leucas* species together with *Otostegia, Isoleucas, Acrotome* and *Leonotis* forms a large polytomy, sister to two *Otostegia* species. Within the large polytomy, Asian species form two groups. The Group I consists of 10 species *L. angularis., L. biflora*, *L. chinensis*, *L. candida, L. marrubioides* var. *pulneyensis, L. helicterifolia, L. nepetifolia, L. mukerjiana, L. decemdentata* var. *decemdentata* and *L. montana*. These species clustered in all previous analysis as a cluster. The second group consists of all other Asian *Leucas* (pp 0.97). Within this group, two species *L. longifolia*, and *L. macrantha* form a polytomy and all other Asian species form a cluster Group II. Species in this cluster form a polytomy. Within this cluster all annual species form a sub-cluster Group III with pp=1. Species resolution was very poor. In this analysis *L. deodikarii* appears as very primitive; not clustered with Asian species.

### 5. E.2.b Phylogeny based on *trnL-F* BASED DNA SEQUENCED DATA

Among the 112 taxa taken for the analysis, the length of *trnL-F* intergenic spacer gene was shortest in *Sideritis macrostachyos* with a base pair length of 221 followed by *Sideritis romana* with 222 base pairs. The longest sequence was observed in *Leucas mukerjiana* with a bse pair length of 525 base pairs. After aligning the sequences using Clustal W, total base pair length was found to be 596. Out of these 244 were observed as conserved sites, 190 variable sites, 106 parsimony informative (Pi) sites and 68 singletons.

The HPD value obtained during the Bayesian analysis are given below

## Table 5.8:- 95% HPD interval

| Parameter | Mean | Variance | Lower | Upper | Median | min ESS* | avg ESS | PSRF+ |
|---|---|---|---|---|---|---|---|---|
| TL | 19.966619 | 2.589005 | 16.900990 | 23.268890 | 19.967370 | 315.37 | 1810.78 | 1.007 |
| r(A<->C) | 0.122525 | 0.000523 | 0.077567 | 0.166601 | 0.121164 | 2178.79 | 2239.96 | 1.000 |
| r(A<->G) | 0.207814 | 0.001040 | 0.147566 | 0.273724 | 0.207019 | 1977.16 | 2047.48 | 1.000 |
| r(A<->T) | 0.092964 | 0.000439 | 0.051353 | 0.135500 | 0.092509 | 134.26 | 1458.86 | 1.010 |
| r(C<->G) | 0.119951 | 0.000803 | 0.069610 | 0.174612 | 0.116731 | 174.12 | 1342.39 | 1.007 |
| r(C<->T) | 0.316315 | 0.001128 | 0.251433 | 0.382446 | 0.315209 | 1575.05 | 1711.23 | 1.000 |
| r(G<->T) | 0.140431 | 0.000654 | 0.092597 | 0.190917 | 0.138940 | 2139.33 | 2226.87 | 1.000 |
| pi(A) | 0.286264 | 0.000304 | 0.253152 | 0.321235 | 0.285761 | 1377.29 | 1816.68 | 1.002 |
| pi(C) | 0.218307 | 0.000242 | 0.186389 | 0.247317 | 0.218484 | 255.13 | 1606.81 | 1.004 |
| pi(G) | 0.183531 | 0.000212 | 0.155026 | 0.211584 | 0.183467 | 667.41 | 1552.96 | 1.002 |
| pi(T) | 0.311898 | 0.000302 | 0.277977 | 0.346538 | 0.311409 | 420.11 | 1519.48 | 1.002 |
| alpha | 0.100859 | 0.000480 | 0.088061 | 0.105774 | 0.096633 | 58.89 | 1525.85 | 1.056 |

* Convergence diagnostic (ESS = Estimated Sample Size); min and avg values correspond to minimal and average ESS among runs. ESS value below 100 may indicate that the parameter is undersampled.

* Convergence diagnostic (PSRF = Potential scale reduction factor [Gelman and Rubin, 1992], uncorrected) should approach 1 as runs converge. The values may be unreliable if you have a small number of samples. PSRF should only be used as a rough guide to convergence since all the assumptions that allow one to interpret it as a scale reduction factor are not met in the phylogenetic context.

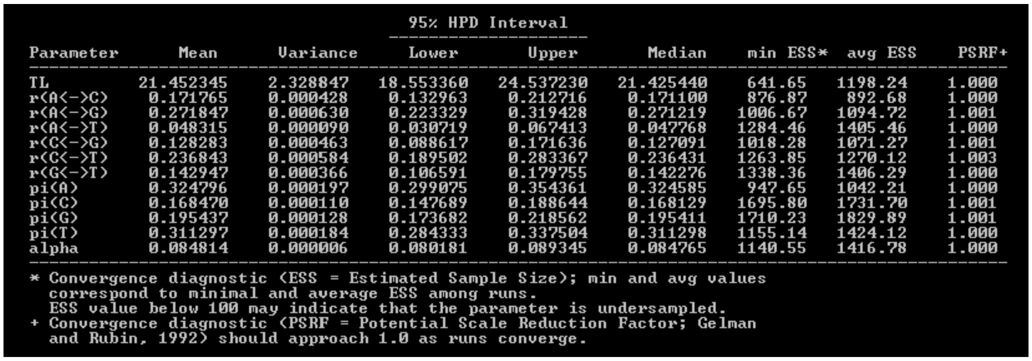
r(A<->C), r(A<->G), r(A<->T), r(C<->G), r(C<->T), r(G<->T) - rate pameters, pi(A), pi(C), pi(G), pi(T) – four stationary nucleotide frequencies, alpha - the shape parameter of the gamma distribution of rate variation. TL - the total tree length (the sum of all branch lengths).

As in the *trnL* data the analysis with *trnL-F* also yielded a polytomy (Figure 5.9). The poor resolution may be due to low variation in the sequences. Two major Clade A and B could be differentiated. The clade A with pp=0.8 show a clustering of all Asian *Leucas* and the cluster B represents African *Leucas.* Asian *Leucas* further diversification also produces a large polytomy. The clade A show further diversification and these Groups (I, II and III) are recognized. Group I show clustering of 13 taxa and this corresponds to the same species which clustered in previous analysis. Group II and III also show clustering of same set of species as in ML analysis. Each cluster shows polytomy and no resolution at species level could be observed. The overall tree topology gives as indication of further line of evolution.

### 5. E.2.c PHYLOGENY BASED ON *rps16* BASED DNA SEQUENCED DATA

Among the 112 taxa taken for the analysis, the length of *rps16* intron gene was shortest in *Leucas nepetifolia* with a base pair length of 437 followed by *Leucas sebaldiana* with 440 base pairs. The longest sequence was observed in *Leucas hirta* with a base pair length of 958 base pairs. After aligning the sequences using Clustal W, total base pair length was found to be 981. Out of these 669 were observed as conserved sites, 285 variable sites, 182 parsimony informative (Pi) sites and 89 singletons were observed.

## Table 5.9:- 95% HPD interval



```
                                       95% HPD Interval
                                     --------------------
Parameter        Mean       Variance      Lower        Upper        Median     min ESS*  avg ESS    PSRF+
----------------------------------------------------------------------------------------------------------
TL           21.452345    2.328847    18.553360    24.537230    21.425440     641.65    1198.24    1.000
r(A<->C)      0.171765    0.000428     0.132963     0.212716     0.171100     876.87     892.68    1.000
r(A<->G)      0.271847    0.000630     0.223329     0.319428     0.271219    1006.67    1094.72    1.001
r(A<->T)      0.048315    0.000090     0.030719     0.067413     0.047768    1284.46    1405.46    1.000
r(C<->G)      0.128283    0.000463     0.088617     0.171636     0.127091    1018.28    1071.27    1.001
r(C<->T)      0.236843    0.000584     0.189502     0.283367     0.236431    1263.85    1270.12    1.003
r(G<->T)      0.142947    0.000366     0.106591     0.179755     0.142276    1338.36    1406.29    1.000
pi(A)         0.324796    0.000197     0.299075     0.354361     0.324585     947.65    1042.21    1.000
pi(C)         0.168470    0.000110     0.147689     0.188644     0.168129    1695.80    1731.70    1.001
pi(G)         0.195437    0.000128     0.173682     0.218562     0.195411    1710.23    1829.89    1.001
pi(T)         0.311297    0.000184     0.284333     0.337504     0.311298    1155.14    1424.12    1.000
alpha         0.084814    0.000006     0.080181     0.089345     0.084765    1140.55    1416.78    1.000
----------------------------------------------------------------------------------------------------------
* Convergence diagnostic (ESS = Estimated Sample Size); min and avg values
  correspond to minimal and average ESS among runs.
  ESS value below 100 may indicate that the parameter is undersampled.
+ Convergence diagnostic (PSRF = Potential Scale Reduction Factor; Gelman
  and Rubin, 1992) should approach 1.0 as runs converge.
```

* Convergence diagnostic (ESS = Estimated Sample Size); min and avg values correspond to minimal and average ESS among runs. ESS value below 100 may indicate that the parameter is undersampled.

* Convergence diagnostic (PSRF = Potential scale reduction factor [Gelman and Rubin, 1992], uncorrected) should approach 1 as runs converge. The values may be unreliable if you have a small number of samples. PSRF should only be used as a rough guide to convergence since all the assumptions that allow one to interpret it as a scale reduction factor are not met in the phylogenetic context.

r(A<->C), r(A<->G), r(A<->T), r(C<->G), r(C<->T), r(G<->T) - rate pameters, pi(A), pi(C), pi(G), pi(T) – four stationary nucleotide frequencies, alpha - the shape parameter of the gamma distribution of rate variation. TL- the total tree length (the sum of all branch lengths)

Results of the *rps16* Bayesian analysis also did not produce a fully resolved tree (Figure 5.10). All *Leucas* species (Asian, African) and members of tribe Leucadeae form a large polytomy with a pp value of 0.59. Within this large polytomy, Asian species did not form a single group as seen some of the earlier analysis. But two distinct sub-cluster (Group I and Group II) could be observed. Group I consists of 11 species which are morphologically similar due to ovate leaves and having axillary clustered inflorescence. All other Asian species cluster in the Group II with a approximate pp value = 1. Polytomy observed within this clade reveal lack of resolution at species level. Close similarity among some species could be seen ( *L. helianthemifolia* and *L. hirta*; *L. lamiifolia* and *L. prostrata*; *L. longifolia* and *L. macrantha*; *L. suffruticosa* and *L. rosmarinifolia*; *L. wightiana* and *L. aspera*).

### 5. E.2.d Phylogeny based on concatenated data set of *trnL*, *trnL-F* and *rps16*

The concatenated plastid DNA dataset consists of three chloroplast regions *trnL*, *rps16* introns and *trnL-F* intergenic spacer. The concatenated dataset had 2151 characters of which 1335 were conserved sites, 613 variable, 357 parsimony informative (Pi) sites and 225 singletons. Gaps were treated as 5th character.

## Table 5.10:- 95% HPD Interval

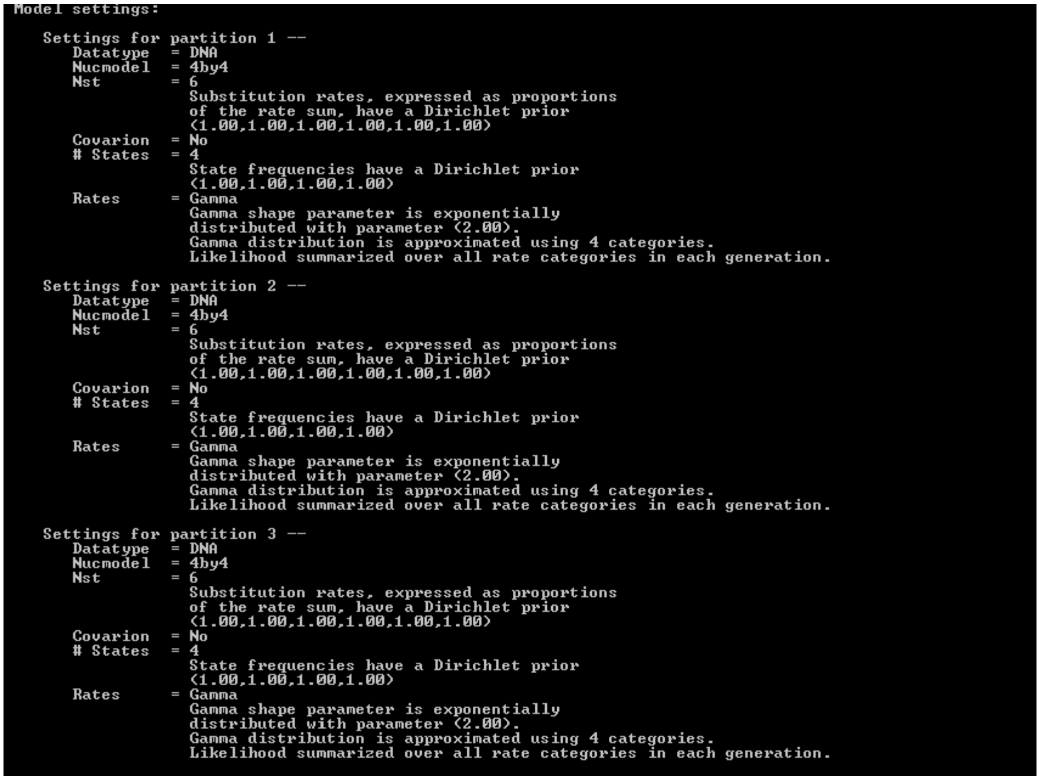
| | Mean | Variance | Lower | Upper | Median | min ESS* | avg ESS | PSRF+ |
|---|---|---|---|---|---|---|---|---|
| TL | 0.889360 | 0.002321 | 0.805780 | 0.990692 | 0.887418 | 2658.03 | 2753.74 | 1.000 |
| r(A<->C) | 0.155974 | 0.000200 | 0.128407 | 0.183408 | 0.155573 | 1218.78 | 1329.09 | 1.000 |
| r(A<->G) | 0.206260 | 0.000280 | 0.174272 | 0.238965 | 0.206005 | 1191.57 | 1304.06 | 1.000 |
| r(A<->T) | 0.049927 | 0.000041 | 0.037679 | 0.062262 | 0.049661 | 1769.89 | 1835.43 | 1.000 |
| r(C<->G) | 0.137897 | 0.000251 | 0.108198 | 0.170530 | 0.137568 | 1416.97 | 1464.45 | 1.000 |
| r(C<->T) | 0.298226 | 0.000406 | 0.260843 | 0.338564 | 0.298282 | 1204.97 | 1223.51 | 1.000 |
| r(G<->T) | 0.151716 | 0.000209 | 0.124977 | 0.180749 | 0.151065 | 1350.19 | 1486.11 | 1.001 |
| pi(A) | 0.334902 | 0.000086 | 0.316350 | 0.353010 | 0.334912 | 1651.81 | 1864.67 | 1.000 |
| pi(C) | 0.174198 | 0.000050 | 0.160387 | 0.188041 | 0.174105 | 1795.30 | 1885.03 | 1.000 |
| pi(G) | 0.189207 | 0.000055 | 0.174919 | 0.203949 | 0.189085 | 1648.76 | 1754.90 | 1.000 |
| pi(T) | 0.301693 | 0.000083 | 0.283864 | 0.318972 | 0.301631 | 1401.67 | 1730.86 | 1.000 |
| alpha | 0.532877 | 0.002745 | 0.435767 | 0.637455 | 0.529043 | 2533.95 | 2578.40 | 1.000 |

* Convergence diagnostic (ESS = Estimated Sample Size); min and avg values correspond to minimal and average ESS among runs. ESS value below 100 may indicate that the parameter is undersampled.

* Convergence diagnostic (PSRF = Potential scale reduction factor [Gelman and Rubin, 1992], uncorrected) should approach 1 as runs converge. The values may be unreliable if you have a small number of samples. PSRF should only be used as a rough guide to convergence since all the assumptions that allow one to interpret it as a scale reduction factor are not met in the phylogenetic context.

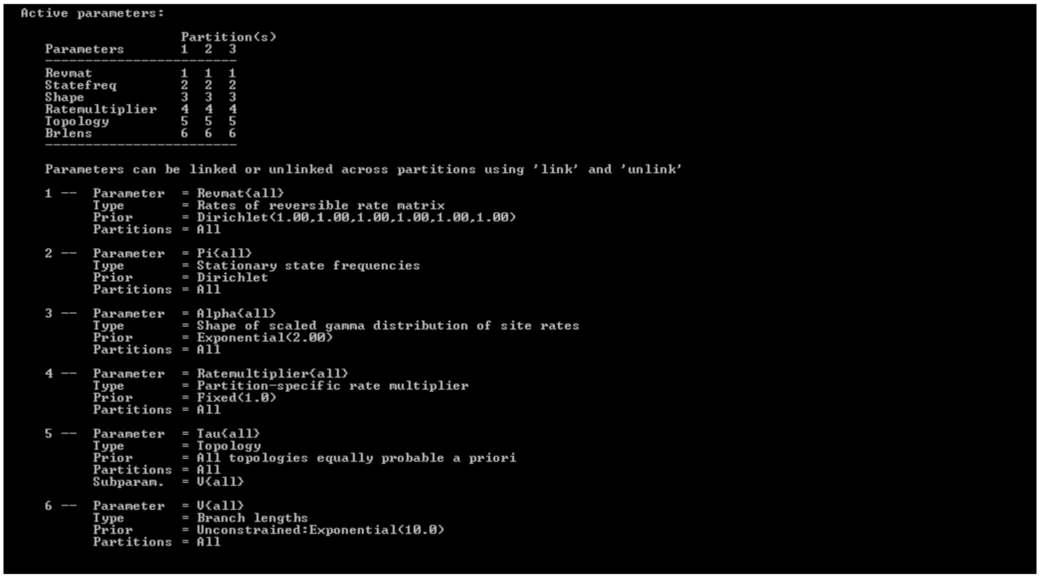
r(A<->C), r(A<->G), r(A<->T), r(C<->G), r(C<->T), r(G<->T) - rate pameters, pi(A), pi(C), pi(G), pi(T) – four stationary nucleotide frequencies, alpha - the shape parameter of the gamma distribution of rate variation. TL - the total tree length (the sum of all branch lengths).

## Table 5.11:- MrBayes Model and partition settings



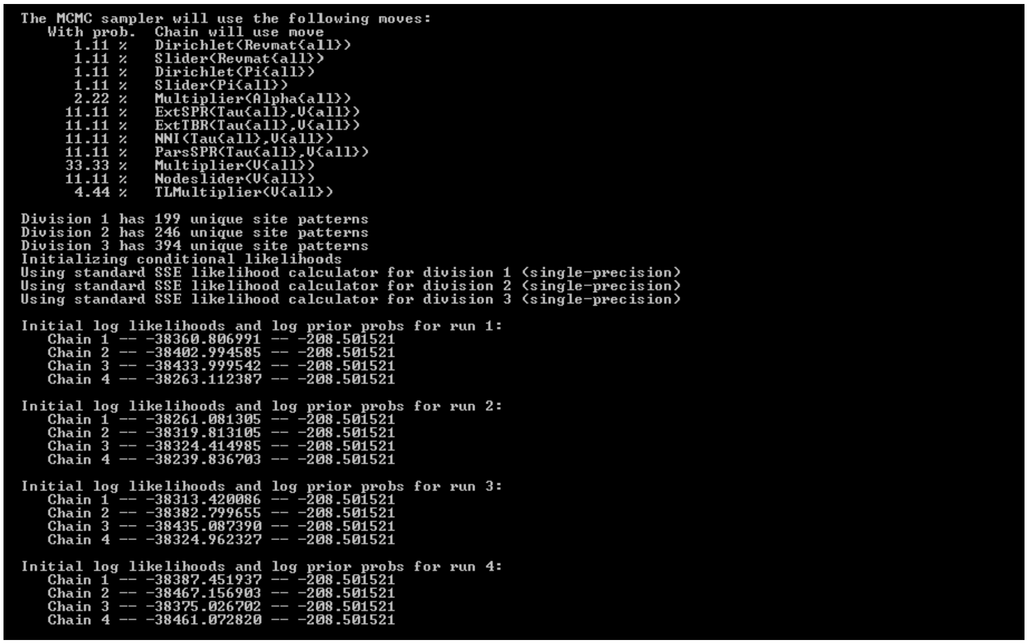
```
Model settings:

   Settings for partition 1 --
      Datatype  = DNA
      Nucmodel  = 4by4
      Nst       = 6
                  Substitution rates, expressed as proportions
                  of the rate sum, have a Dirichlet prior
                  (1.00,1.00,1.00,1.00,1.00,1.00)
      Covarion  = No
      # States  = 4
                  State frequencies have a Dirichlet prior
                  (1.00,1.00,1.00,1.00)
      Rates     = Gamma
                  Gamma shape parameter is exponentially
                  distributed with parameter (2.00).
                  Gamma distribution is approximated using 4 categories.
                  Likelihood summarized over all rate categories in each generation.

   Settings for partition 2 --
      Datatype  = DNA
      Nucmodel  = 4by4
      Nst       = 6
                  Substitution rates, expressed as proportions
                  of the rate sum, have a Dirichlet prior
                  (1.00,1.00,1.00,1.00,1.00,1.00)
      Covarion  = No
      # States  = 4
                  State frequencies have a Dirichlet prior
                  (1.00,1.00,1.00,1.00)
      Rates     = Gamma
                  Gamma shape parameter is exponentially
                  distributed with parameter (2.00).
                  Gamma distribution is approximated using 4 categories.
                  Likelihood summarized over all rate categories in each generation.

   Settings for partition 3 --
      Datatype  = DNA
      Nucmodel  = 4by4
      Nst       = 6
                  Substitution rates, expressed as proportions
                  of the rate sum, have a Dirichlet prior
                  (1.00,1.00,1.00,1.00,1.00,1.00)
      Covarion  = No
      # States  = 4
                  State frequencies have a Dirichlet prior
                  (1.00,1.00,1.00,1.00)
      Rates     = Gamma
                  Gamma shape parameter is exponentially
                  distributed with parameter (2.00).
                  Gamma distribution is approximated using 4 categories.
                  Likelihood summarized over all rate categories in each generation.
```

## Table 5.12:- MrBayes parameter settings



```
Active parameters:

                     Partition(s)
   Parameters        1  2  3
   ------------------------
   Revmat            1  1  1
   Statefreq         2  2  2
   Shape             3  3  3
   Ratemultiplier    4  4  4
   Topology          5  5  5
   Brlens            6  6  6
   ------------------------

   Parameters can be linked or unlinked across partitions using 'link' and 'unlink'

   1 --  Parameter  = Revmat{all}
         Type       = Rates of reversible rate matrix
         Prior      = Dirichlet(1.00,1.00,1.00,1.00,1.00,1.00)
         Partitions = All

   2 --  Parameter  = Pi{all}
         Type       = Stationary state frequencies
         Prior      = Dirichlet
         Partitions = All

   3 --  Parameter  = Alpha{all}
         Type       = Shape of scaled gamma distribution of site rates
         Prior      = Exponential(2.00)
         Partitions = All

   4 --  Parameter  = Ratemultiplier{all}
         Type       = Partition-specific rate multiplier
         Prior      = Fixed(1.0)
         Partitions = All

   5 --  Parameter  = Tau{all}
         Type       = Topology
         Prior      = All topologies equally probable a priori
         Partitions = All
         Subparam.  = V{all}

   6 --  Parameter  = V{all}
         Type       = Branch lengths
         Prior      = Unconstrained:Exponential(10.0)
         Partitions = All
```

### Table 5.13:- Details of number of chains in Mrbayes



```
The MCMC sampler will use the following moves:
   With prob.  Chain will use move
      1.11 %   Dirichlet(Revmat{all})
      1.11 %   Slider(Revmat{all})
      1.11 %   Dirichlet(Pi{all})
      1.11 %   Slider(Pi{all})
      2.22 %   Multiplier(Alpha{all})
     11.11 %   ExtSPR(Tau{all},V{all})
     11.11 %   ExtTBR(Tau{all},V{all})
     11.11 %   NNI(Tau{all},V{all})
     11.11 %   ParsSPR(Tau{all},V{all})
     33.33 %   Multiplier(V{all})
     11.11 %   Nodeslider(V{all})
      4.44 %   TLMultiplier(V{all})

Division 1 has 199 unique site patterns
Division 2 has 246 unique site patterns
Division 3 has 394 unique site patterns
Initializing conditional likelihoods
Using standard SSE likelihood calculator for division 1 (single-precision)
Using standard SSE likelihood calculator for division 2 (single-precision)
Using standard SSE likelihood calculator for division 3 (single-precision)

Initial log likelihoods and log prior probs for run 1:
   Chain 1 -- -38360.806991 -- -208.501521
   Chain 2 -- -38402.994585 -- -208.501521
   Chain 3 -- -38433.999542 -- -208.501521
   Chain 4 -- -38263.112387 -- -208.501521

Initial log likelihoods and log prior probs for run 2:
   Chain 1 -- -38261.081305 -- -208.501521
   Chain 2 -- -38319.813105 -- -208.501521
   Chain 3 -- -38324.414985 -- -208.501521
   Chain 4 -- -38239.836703 -- -208.501521

Initial log likelihoods and log prior probs for run 3:
   Chain 1 -- -38313.420086 -- -208.501521
   Chain 2 -- -38382.799655 -- -208.501521
   Chain 3 -- -38435.087390 -- -208.501521
   Chain 4 -- -38324.962327 -- -208.501521

Initial log likelihoods and log prior probs for run 4:
   Chain 1 -- -38387.451937 -- -208.501521
   Chain 2 -- -38467.156903 -- -208.501521
   Chain 3 -- -38375.026702 -- -208.501521
   Chain 4 -- -38461.072820 -- -208.501521
```

Bayesian analysis with concatenated dataset produced a well resolved tree with good support values (Figure 5.11-5.13). The combined tree topology is different from each single gene tree.

In the analysis the outgroup taxa *Rydingia integrifolia* and *Rydingia persica* formed base to the entire *Leucas* group. Combined analysis produced a polytomy with two sister groups supported by good posterior probability (pp) values 0.94 and 0.92 respectively. These two clades correspond to two distinct groups in *Leucas* ie, Asian group and African group. Good posterior probability values support Asian species to a monophyletic group, whereas the African group consisting of species from Africa and Arabia is paraphyletic consisting of genera like *Actotome, Otostegia, Isoleucas* and *Leonotis.* This observation is at par with that of earlier molecular phylogeny works by Scheen & Albert (2009). The taxa *Isoleucas arabica*, *I. somala* and *Leucas aequistylosa* form polytomy and show different line of evolution, but show more similarity to African groups.

All other lamioideaen members like *Gomphostemma, Scutellaria, Achyrospermum, Eurysolen, Pogostemon, Microtoena, Colebrookea, Sideritis, Phlomis, Lamium, Ballotta, Marrubium* and *Roylea* are clustered as Outgroup.

Close observation of the first major clade of Asian *Leucas* revealed four sub-groups in it. These groups formed four clades supported by good posterior probability values.

## Clade-I:

Clade-I (pp 0.96) consisting of *Leucas deodikarii*, *L. lanata, L. pubescens, L. angularis, L. biflora, L. chinensis, L. lanata* var. *candida, L. nepetifolia, L. montana, L. helicterifolia, L. mukerjiana, L. marrubioides* var. *pulneyensis* and *L. decemdentata* var. *decemdentata*. *L. helicterifolia* and *L. mukerjiana* by (pp 0.97). Tree bifurcation in this clade was satisfactory but cannot fully consider for species relationships. Farely good support value shows robustness of the tree resolution inside this clade. Two species *L.deodikarii* and *L. lanata* form the primitive species in this clade and all other species form sister to these taxa. Further branching shows *Leucas pubescence* on one side and remaining taxa as sisters to it. Poor resolution was noticed among the remaining species in this clade, but close similarity of *L. helicterifolia* and *L. mukerjiana* is noticed as recently evolved clade. Similarly *L. marrubioides* var. *pulneyensis* showed similarity with *L. decemdentata* var. *decemdentata*. All the remaining ingroup taxa formed as sisters lineage to clade-I.

## Clade-II:

All remaining species form a clade and further branching of this clade shows just two species on oneside and remaining species sisters to the species. This branching was supported by a pp value = 1. Clustering of these two species suggests a unique line of evolution in this lineage. Two species *L. longifolia* and *L. macrantha* grouped in this clade form an evolutionary primitive group; the clustering is supported by good posterior probability value = 1. Clustering of these two species suggests a unique line of evolution in this lineage.

**Clade-III:**

After separation of clade II the remaining species form two sister clades clade III and clade IV, supported by pp value of 0.98. The clade III forms a large clade with 13 taxa and is shown as highly evolved clade and is sister to clade-IV. Species group in this clade are *L. dhonimalayensis* sp.nov, *L. eriostoma*, *L. lanceifolia*, *L. sebaldiana*, *L. suffruticosa*, *L. rosmarinifolia*, *L. manipurensis*, *L. beddomei*, *L. helianthemifolia*, *L. hirta*, *L. lamiifolia*, *L. prostrata* and *L. ciliata*.

**Clade-IV:**

Sister to Clade III Clade II shows clustering of wide spread taxa. This clade consists of seven taxa, mostly annual herbaceous forms. Species grouped in this clade are *L. lavandulifolia*, *L.lavandulifolia* var *decipiens*, *L. mathewiana*, *L. diffusa, L. wightiana, L. aspera* and *L. zeylanica*.

Trees obtained in this analysis using concatenated dataset of three genes showed similar topology in both ML and Bayesian analysis. Good bootstrap values and posterior probability values support the robustness of these trees. Thus Asian monophyletic group shows four distinct clades in it represents four different lines of evolution in *Leucas s.str.*

# CHAPTER 6

# DISCUSSION

# DISCUSSION

This work aimed at tracing the evolutionary history of Asian *Leucas* using molecular phylogeny. Total 39 taxa of Asian *Leucas* were collected from different parts of India and used as ingroup taxa. Available DNA sequences of African and Arabian taxa of *Leucas* were downloaded and used in the analysis. Members of tribe Leucadeae were downloaded and used along with selected members of sub-family Lamioideae as outgroups in the analysis. Altogether 112 taxa were used in the analysis of which 39 taxa belonging to Asian *Leucas* were ingroups.

## 6.1. DNA extraction and sequence generation

Thorough reviews of available phylogenetic works in Lamiaceae were conducted and possibilities of different gene regions to incorporate in the analysis were explored. The available molecular phylogenetic works on the genus *Leucas* used four chloroplast regions and similar regions were found giving satisfactory variation in majority of other Lamiacean members. After this survey, three plastid regions: *trnL* intron, *trnL-F* intergenic spacer and *rps16* introns were selected as regions to be sequenced. Genomic DNA was extracted from silica gel dried leaf samples collected afresh from different parts of India. We used DNeasy Plant Mini Kit for isolation of genomic DNA. Amplifications of the three regions were carried out with universal primers and the regions were sequenced following Sanger sequencing methods. Use of field collected tissue yielded good amount of genomic DNA and amplification were carried out with universal primers. Amplicons were cleaned before sequencing. Sanger sequencing yielded good DNA sequences which in turn provided satisfactory results in phylogenetic analysis.

## 6.2 Phylogenetic analysis

Phylogenetic analyses were conducted singly and jointly using concatenated sequences. Maximum Likelihood and Bayesian approaches were performed in RaxML and MrBayes packages respectively. Results showed that the genus *Leucas s.l* is not monophyletic and consists of two clades which correspond to an Asian group and a heterogeneous Afro-Arabian group. These two sister clades (A and B) were well supported (PP=0.99; BS=76) in both the analysis using concatenated data set. These sister clades were found in both Likelihood and Bayesian methods when majority of Asian species are represented in analysis. Topologies of individual trees were not truly identical, and in some, the clade II was not recognized. Incongruence in the topologies of trees was hardly noticeable. The strong support for both sister clades in each gene region data set suggests that the species found in Asia is monophyletic and is genetically distinct from other closely related species in Africa and Arabia. The African species clade form a paraphyletic group with genera like *Acrotome, Isooleucas, Leonotis* and *Otostegia* nested within it. This is in agreement with the earlier works on morphology (Sebald, 1980, Ryding, 1999) and molecular phylogeny (Scheen *et al.,* 2011). Current works supports the contention that Afro-Arabian species of *Leucas* are different from the Asian species. Thus these works, done with majority of *Leucas s.l* taxa from all geographic regions, form an additional support for separating Asian species as *Leucas s.str.* as suggested by Scheen and Albert (2007). The *trnL*, *trnL-F* and *rps 16* chloroplast DNA data could possibly resolve the relationships within *Leucas* in analysis with additional species sampled from India. The combined data set resolved Asian *Leucas* as a distinct genus (clade A) but the generic relationships within *Leucas s.l.* is still unresolved due to paraphyly of African groups (clade B). Again the phylogenetic position of *Isoleucas* spp. and *Leucas aequistylosa* need to be ascertained by using more markers.

The phylogram of the combined data set illustrates that Asian *Leucas* is genetically distinct from the other genera included. The phylogram also shows that there are genetic variations within Asian *Leucas* and four distinct groups could be recognised corresponding to four groups identified morphologically. Almost similar topology is obtained in all the analyses, using single gene or in combined analysis, except in the case of Clade II. This fact supports robustness of these groups, irrespective of slight differences in the support values.

Total four clades are recognised in Asian group. Initially, the Asian species shows two sister clades with a well supported pp value of 0.92 in Bayesian analysis and moderately good BS value (=69) in ML analysis. Among these two sister clades, one forms the clade I and the other a large clade with much internal radiation. The large clade again show bifurcation with just two species (clade II) on one side and all other on the other end supported by a good support value pp=1 in Bayesian analysis and a BS= 89 in ML analysis. The large clade further splits into two clades (clade III and IV) in Bayesian analysis with a pp of 0.98 and in ML analysis with a BS value of 76. Then two clades possess similar characters, but each clade has its on synapomorphic characters narrated below.

The phylogram also shows that there are small branch lengths within each clade (I, III and IV) except sub-clade II, where the sample size for the latter is small. Genetic markers that have found sufficient base pair variation within other closely related genera revealed high variation within Asian *Leucas* clade but little variation within each clade even between morphologically distinct species. No nodes within the section showed significant support using the *trnL, trnL-F* and *rps16* data, either alone or jointly. (Figure 7,8, 15 and 16).

The nodes were supported well with good posterior probabilities, but these had comparatively low bootstrap values. Common characters of these nodes are:

**Clade-I:-** *Leucas* species grouped under this clade possesses certain character combinations like: short lived (1-2 years) herbs; angular stem; leaves generally ovate; inflorescence as loose clusters in almost all nodes; bracteoles few with small size; calyx erect, tubular with 10 distinct teeth and naked mouth; corolla with emarginate lower lip. Species occupied in this section are *L. lanata, L. deodikarii, L. nepetifolia, L. marrubioides, L. decemdentata, L. helicterifolia, L. mukerjiana, L. pubescens, L. angularis, L. biflora, L. montana* and *L. chinensis.* Sequences of *L. lanata* downloaded from genbank are found distantly related to its variety *L. lanata* var. *candida.* This suggests separation of var. *candida* to a species rank as *L. candida*. Members of this clade are mainly distributed in India except species like *L. chinensis*, *L. decemdentata* and *L. lanata.* Most of the members of the section are seen at high altitude.

**Clade-II: -** This clade consists of just two species, a central Indian species *L. longifolia* and a narrow endemic *L. macrantha* found in Rajasthan deserts. Both of them have more or less the same floral characters. In both the inflorescences are in multiple nodes as clusters. Both are annual herbs and having linear leaves. The multi nodal inflorescence in both species is a character observed in members of clade I where as its linear lanceolate leaves are a synapomorphic characters. The typical corolla characters seen in this clade (in both members) are seen in its sister clade III and also in IV derived from it. Appearance of this type of corolla with fan shaped lower lip is a synapomorphic character, evolved first in this clade and shared in all *Leucas* taxa in Clade III & IV.

**Clade III:** Members included in this clade are *L. lamiifolia, L. prostrata, L. ciliata, L. helianthemifolia, L. hirta, L. sebaldiana, L. dhonimalayensis, L. lanceifolia, L. rosmarinifolia, L. suffruticosa, L. eriostoma* and *L. beddomei.* These are mostly perennial herbs or undershrubs with thick sometime woody basal stems; leaves linear-lanceolate to orbicular with coriaceous to chartaceous texture; inflorescence towards terminal 2-3 nodes; bracteoles linear-lanceolate, many, enveloping inflorescence; calyx tubular, mouth ciliate, teeth spreading; corolla lower lip fan shaped but not emarginate. Generally, herbaceous members of this clade produce new branches continuously from the basal stem, as a result it appear woody and amorphous with thick periderm. Growth of these plants from the basal stem may last for a few years. Plants grouped in this clade are found exclusively on the shola grasslands, above 1200 m and are mostly endemic to south India particularly in southern Western Ghats. In species like *L. prostrata* and *L. ciliata* , the branches are herbaceous but the basal stems shows woody nature. Three chloroplast genes have given clear resolution at the sectional level but failed to give an explanation at the species level resulting polytomies in all the lineages.

This clade is sister to clade IV in Bayesian and ML analysis and share common characters in inflorescence nature and flower morphology. The golden yellowish colour for the upper lip (posterior lip) of corolla is a synapomorphic character seen in few members of this clade (*L. ciliata, L. dhonimalayensis, L. beddomei* and *L. lamiifolia*) is a unique characters observed in this clade.

**Clade-IV: -** This consists of plants having annual herbaceous nature; leaves linear lanceolate with serrate margins; inflorescence in terminal 1-3 clusters, often appressed; bracteole linear lanceolate, longer than or as long as calyx; calyx oblique with varying degree; corolla with fan shaped lower lip. Some of the species included in this section are widespread in Asia and even in Africa (*L. lavandulifolia, L. aspera*). Others are widespread in Asian countries. These plants are used as medicinal plants and the species in this clade include: *L. zeylanica, L. aspera, L. lavandulifolia, L. diffusa, L. cephalotes, L. wightiana* and *L. mathewiana*.

Genera like *Gomphostemma, Scutellaria, Achyrospermum, Eurysolen, Pogostemon, Microtoena, Colebrookea, Sideritis, Phlomis, Lamium, Phlomis, Ballotta, Marrubium,* and *Roylea cinerea* included in the analysis served as outgroups.

## 6.3. The paraphyletic African *Leucas* vs the monophyletic Asian *Leucas*

The large and widely distributed genus *Leucas s.l.* is paraphyletic. But the Asian species of *Leucas* do form monophyletic group with well supported posterior probability values. As opined by (Ryding 1998), three morphological characters combinations distinguish Asian species from African species of *Leucas*:

a) Absence of glands on ovaries and nutlets.

b) Anterior corolla lip longer than posterior corolla lip.

c) Dorsal side of calyx exceeding or equaling the ventral sides

These observations of morphologists (Sebald, 1980., Ryding, 1998) were well supported in the present study. The two African members of *Leucas* found in India (*L. martinicensis* and *L. urticifolia*) are grouped within African clade as in previous studies suggested Scheen & Albert (2009). The appearance of glands on nutlets in *L. beddomei*, a narrow endemic species found in a single locality in Wayanad district in Western Ghat is a homoplastic character due to convergent evolution.

According to Ryding (1998), there are few additional anatomical characters that further distinguish Asian and Afro-Arabian species of *Leucas.* The peculiarity of the fruit pericarp of Asian taxa is that it lacks apical ribs and glands, but instead it has a highly thick layer of bone cells and two layers of mesocarp with crystals (Ryding 1993). However there is no apparent unique combination of distinguishing morphological characters that are present in all African species and absent in all Asian species of *Leucas*. The observations of Ryding (1998) based on morphology and Scheen & Albert (2009) based on molecular phylogeny that Asian taxa are different from African taxa is confirmed in the present analysis using more taxa from Asia. The present study supports the consideration that Asian species form a separate genus different from African species and to be given a separate generic name (Scheen & Albert, 2007).

Further, the study conducted by Ryding (1993) on the fruit pericarp structure revealed that *Acrotome*, *Leonotis*, and a large group of African and Arabian species of *Leucas* shared a reduction in the bone cells, resulting in a thin sclerenchyma region in the pericarp. This thin sclerenchyma region is a synopomorphy for a strongly supported group that includes the majority of the African *Leucas, Acrotome* and *Leonotis*.

Species of *Acrotome* are readily distinguished from species of *Leucas* by three characters: inclusion of stamens in the corolla tube, lack of bearded fringe on posterior corolla lip and hairy style (Ryding 1998; Harley *et al.,* 2004). Phylogenetic analysis of the morphological characters revealed that *Acrotome* is derived from within the paraphyletic African *Leucas* (Ryding 1998). Current molecular studies corroborate the result.

The species of *Leonotis* are easily distinguishable by their unique striking inflorescences with dense verticillasters of predominantly orange-haired flowers (Iwarsson & Harvey 2003). The long posterior corolla lip and significantly shorter and early withering anterior lip are considered to be distinguishing characters of the genus *Leonotis* from *Leucas* (Ryding 1998; Iwarsson & Harvey 2003). Despite these striking morphological differences, a previous phylogenetic analysis of morphological data demonstrated that *Leonotis* was derived from within the paraphyletic genus *Leucas* (Ryding 1998), and this is corroborated by the current molecular data.

### 6.4. Classification

The present investigation showed distinct molecular groups within Asian *Leucas*. These groups show further line of evolution within the genus and hence this could be considered for systematic treatments. Morphologists considered different infrageneric sections (Bentham, 1830, 1834, 1848; Hook.f, 1885; Briquet, 1896; Singh, 2001; Sunojkumar, 2005) within the genus based on morphological similarities. Similar approach of defining infrageneric sections based on the results obtained in the present molecular phylogenetic analyses will further refines the existing treatment.

The four clades recognised in the Asian *Leucas* represent four molecular groups, as four sections. Three of these clades (clade, I, II and III) corresponds to three sections recognised by Bentham (1830) and followed by Hooker (1885) and Briquet (1896). Clade I represents the section *Ortholeucas* Benth. (*Leucas*), clade II the section *Astrodon* Benth. and clade III as section *Plagiostoma* Benth. Clade IV is a new group recognised in molecular analysis which can be considered as a new line of evolution and as a new section.

In the infrageneric treatment, the molecular phylogenetic studies well supports Bentham's concept of evolution in the genus and is in contrary with Singh's (2001) treatment under six sections.

In the treatment of Sunojkumar (2005), the sections recognised by Bentham was kept as same but the section Astrodon was splitted in to three to represent further line of evolution in the group. The molecular studies conducted here supports close similarity of *L. suffruticosa* and *L. rosmarinifolia* but *L. helianthemifolia* showed more closeness to *L. hirta.* Similarly closeness of shrubby form of species like *L. eriostoma* and *L. lanceifolia* is evident in molecular analysis. The section Astrodon recognised in molecular analysis did not resolve well at species level but the overall branching shown further line of evolution happening in this group. Thus molecular evidence supports the concept of Sunojkumar (2005) to split the section Astrodon to three sections. But more support from molecular analysis is needed.

Based on the present molecular phylogenetic analysis four infrageneric sections as given below are recognized in *Leucas s.str.*

### 1. Section *Leucas* (Benth.) Singh

The section consists of species like *L. deodikarii, L. lanata, L. pubescens, L. angularis, L. biflora, L. chinensis, L. nepetifolia, L. Montana, L. helicterifolia, L. mukerjiana, L. marrubioides* var. *pulneyensis* and *L. decemdentata.*

### 2. Section *Longifolia* Vimal & Sunojk. sect. nov

This new section consists of two species *L. longifolia* and *L. macrantha*.

### 3. Section *Astrodon* Benth.

Consists of *L. beddomei, L. ciliat, L. dhonimalayensis sp.nov., L. eriostoma, L. lanceifolia, L. sebaldiana, L. suffruticosa, L. rosmarinifolia, L. manipurensis, L. helianthemifolia, L. hirta, L. lamiifolia* and *L. prostrata*.

4. **Section *Plagiostoma* Benth.**

This section consists of *L. aspera, L. diffusa, L. mathewiana, L. lavandulifolia, L. wightiana* and *L. zeylanica*.

Of this four, section *Leucas* is primitive followed by *Longifolia, Astrodon,* and *Plagiostoma*.

## 6.5. Biogeographic History of *Leucas*

It has been postulated that Lamiaceae originated in Indomalaya or Southern China Wu and Li (1982). However based on cladistics analysis of morphological variation, Ryding (1998) suggested a northeast African origin for *Leucas*. The current phylogeny suggests an Asian origin for the genus *Leucas* as well as for the entire *Leucas* group.

The genus *Leucas s.l.* has two disjunct centres of diversity north eastern Africa and the Indian subcontinent (Sebald 1980; Singh 2001). The present study point out that the ancestors of *Leucas* stalk diverged and proceeded independent line of evolution. This ultimately from two sister clades (clade A with Asian species alone and B with African species of *Leucas* together with species of *Acrotome*, *Leonotis* and *Otostegia*) and diversified independently in two separate geographical areas.

Two species of *Isoleucas* and *L. aequistylosa* show similarity to Asian on some characters, but show more similarity to African groups. They follow different line of evolution.

The occurrence of widespread annual weeds *L. martinicensis* and *L. urticifolia* in Asia is the result of secondary migrations or introduction across the African Asian geographic boundary. Similarly, the occurrence of Asian species *Leucas lavandulifolia* and *Leucas aspera* in Madagascar or the

Mascarenes of the eastern coast of Africa is due to introduction (Sebald, 1980; Singh 2001). This view is supported here.

Diversification in Asian *Leucas* (*Leucas s.str.*) suggests that sect. *Longifolia* is a north and central Indian section where as sect. *Leucas* has diversity in all over India. Species like *L. lanata, L. biflora, L. helicterifolia,* and *L. decemdentata* are found towards the north and northeastern side of India whereas species like *L. pubescens* and *L. marrubioides* var. *pulneyensis* are found only in Southern Western Ghats. Other species like *L. mukerjiana*, *L. montana* and *L. nepetifolia* has central to South Indian distribution. *L. decemdentata* is found in Australia too.

Section *Astrodon* are found mostly as south Indian endemic (eg: *L. beddomei, L. dhonimalayensis, L. eriostoma, L. lanceifolia, L. sebaldiana, L. suffruticosa, L. rosmarinifolia, L. hirta, L. helianthemifolia, L. lamiifolia* and *L. prostrata*). Only *L. manipurensis* is found as endemic in north eastern India.

Section *Plagiostoma* has all India distribution and the diversity extend from S.E Asia upto Australia.

## CHAPTER 7

# SUMMARY AND CONCLUSION

# SUMMARY AND CONCLUSION

The present study considered genus *Leucas* in a broad sense (*Leucas s.l.*) to understand the infrageneric lineages within it. Three gene regions of 69 *Leucas s.l.* (out of ca. 100 spp) taxa were considered for the study which is more or less a good sampling to understand the course of evolution within the genus. Existing molecular phylogenetic works gave more emphasis on *Leucas* found in Africa and Arabia and the sampling from Asia was very poor. The present work elaborated this by including maximum species from India (37 taxa) which fairly represent Asian diversity in phylogenetic analysis. The present work thus focused on the phylogeny of Asian *Leucas* to understand the evolutionary lineages and speciation within it. Major conclusions drawn out of this study are:

a) The genus *Leucas s.l.* is not monophyletic and this supports the opinion of clasical taxonomists to include Asian species and Afro-Arabian species in different sections (Bentham, 1830, 1848; Hooker, 1885; Briquet, 1896; Singh, 2001; Sunojkumar, 2005;). The apparent morphological similarity is due to common ancestry.

b) Asian *Leucas* is monophyletic and Afro-Arabian species are paraphyletic. This conclusion further highlights the opinion of Ryding (1998) and Scheen and Albert (2009).

c) As opined by Scheen and Albert (2007) the Asian *Leucas* alone can be considered under the name *Leucas s.str*. Further studies required to circumscribe Afro-Arabian diversity.

d) Four molecular groups are present in *Leucas s.str*. which corresponds to four infrageneric lineages. These lineages represent morphological sections within the genus. The molecular phylogenetic approach supports classical botanists (Bentham, 1834 and Hooker 1885) in the infrageneric classification of genus *Leucas*.

e) Species with ovate leaves and inflorescence in multiple nodes are considered as primitive and annual herbaceous forms with linear lanceolate leaves and terminal inflorescence are advanced.

f) The richest endemic diversity in Western Ghats is due to recent radiation, particularly in section Astrodon.

g) Diversity found in other Asian countries can be attributed to migration and dispersal from India.

h) Low resolution at species level in each clade indicate probable role of hybridisation in bringing out diversity. This can be tested by including nuclear genes in the analysis.

i) Present analysis identifies *Leucas dhonimalaensis* as a new species in section Astrodon.

j) Phylogenetic analysis suggests separation of *Leucas candida* to a species level as it is quite different from the proper species *Leucas lanata*.

k) The glands on nutlets in *L. beddomei*, a character common in African *Leucas*, are a homoplastic character due to convergent evolution.

i) Among the three gene region *trnL-F* appears morevariable than *trnL* and *rps16* regions.

One of the major objectives of this work was to test the monophyletic nature of Asian species as opined by Scheen & Albert (2009) and this is confirmed in the analysis. This in turn supports the opinion of Scheen and Albert (2007) to split the genus into *Leucas s.str.* consists of species from

Asia and the informal 'African *Leucas'* consists of species from Africa and Arabia together with *Acrotome, Ortholeucas, Leonotis*, and *Otostegia*.

Another objective was to identify the molecular groups within *Leucas s.str*. Altogether four molecular groups identified from four clades and this represents the evolutionary lineages within the genus *Leucas s.str*. These groups corresponds to four infrageneric sections in a systematic point of view and supports the view of classical taxonomist Bentham (1830) and rejects the treatment of Singh (2001). Resolution at deep level was poor in the analysis using only chloroplast gene regions and this indirectly point out the probable role of hybridisation in bringing out these diversity in India. To prove this and to get good resolution of nodes, nuclear region can be considered in future analysis.

The evolutionary signals in the three chloroplast regions projects section *Leucas* (*Ortholeucas*) as the primitive group within *Leucas*. All other regions evolved as a group parallel to this and further diversified in three lines. The new section identified appear primitive in the sister clade, where the nodal inflorescence is a plesiomorphic character seen in section *Leucas*.

Section *Astrodon* and section *Plagiostoma* share the floral structure of the new section, but the terminal inflorescence appears a synapomorphic character. The annual herbaceous nature of section *Plagiostoma* is also a synapomorphic feature. The paraphyletic nature of African species necessitates further studies to resolve monophyly.

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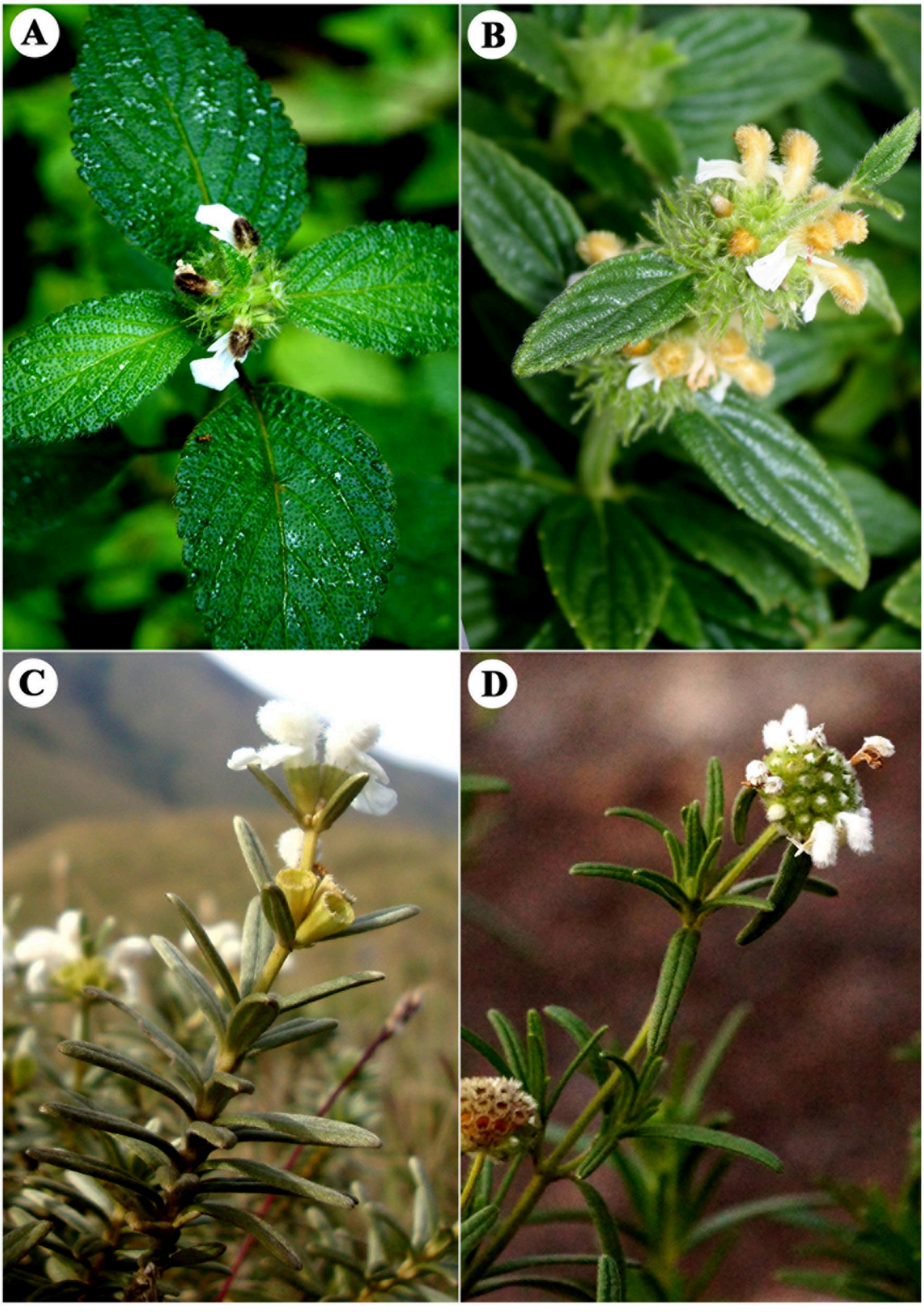

Plate.1. *Leucas ciliata* Benth., B. *Leucas dhonimalayensis*., C. *Leucas helianthemifolia* Desf., D. *Leucas rosmarinifolia* Benth.

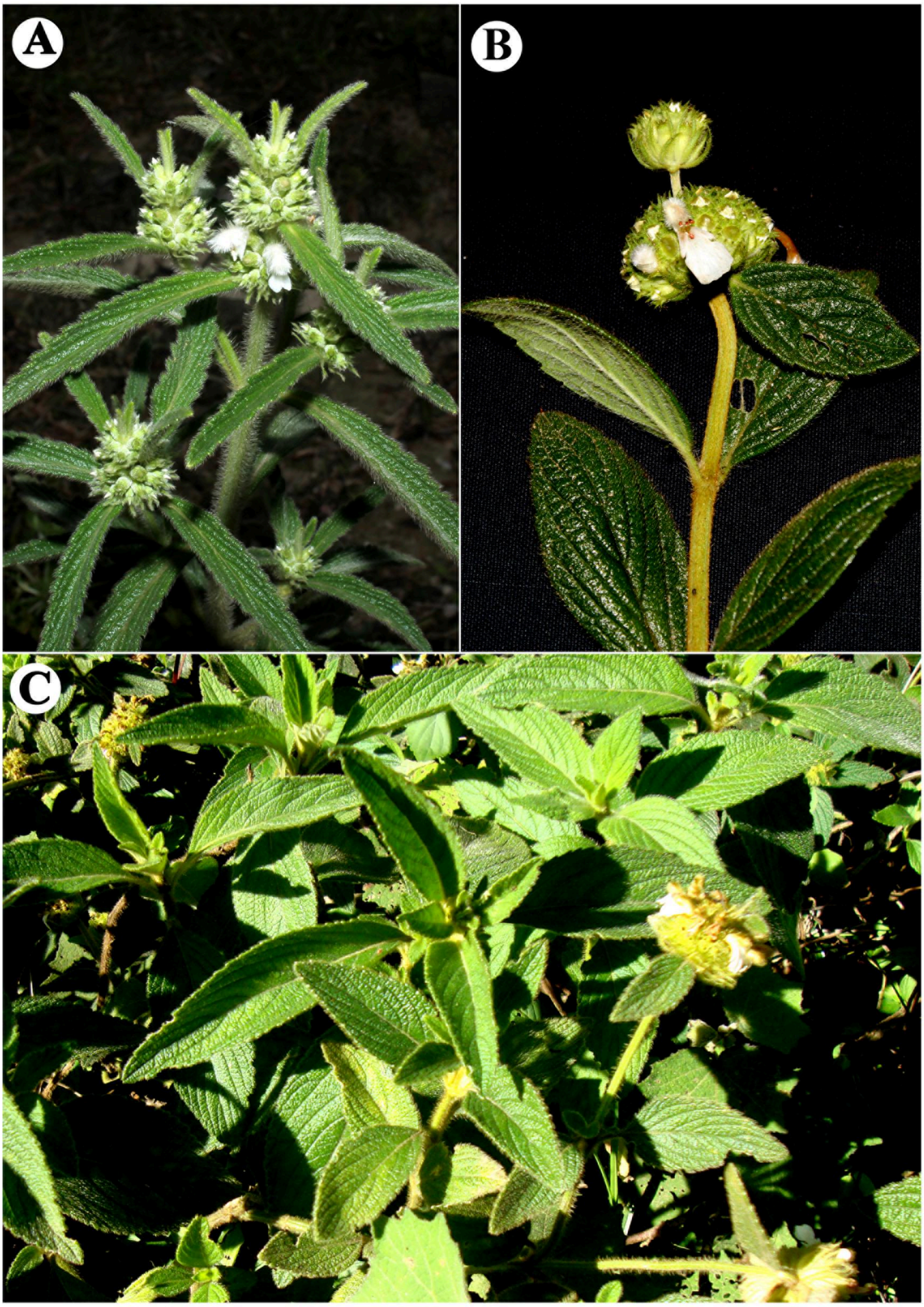

Plate.2. *Leucas eriostoma* Hook.f., B. *Leucas hirta* Spreng., C. *Leucas lamiifolia* Desf.

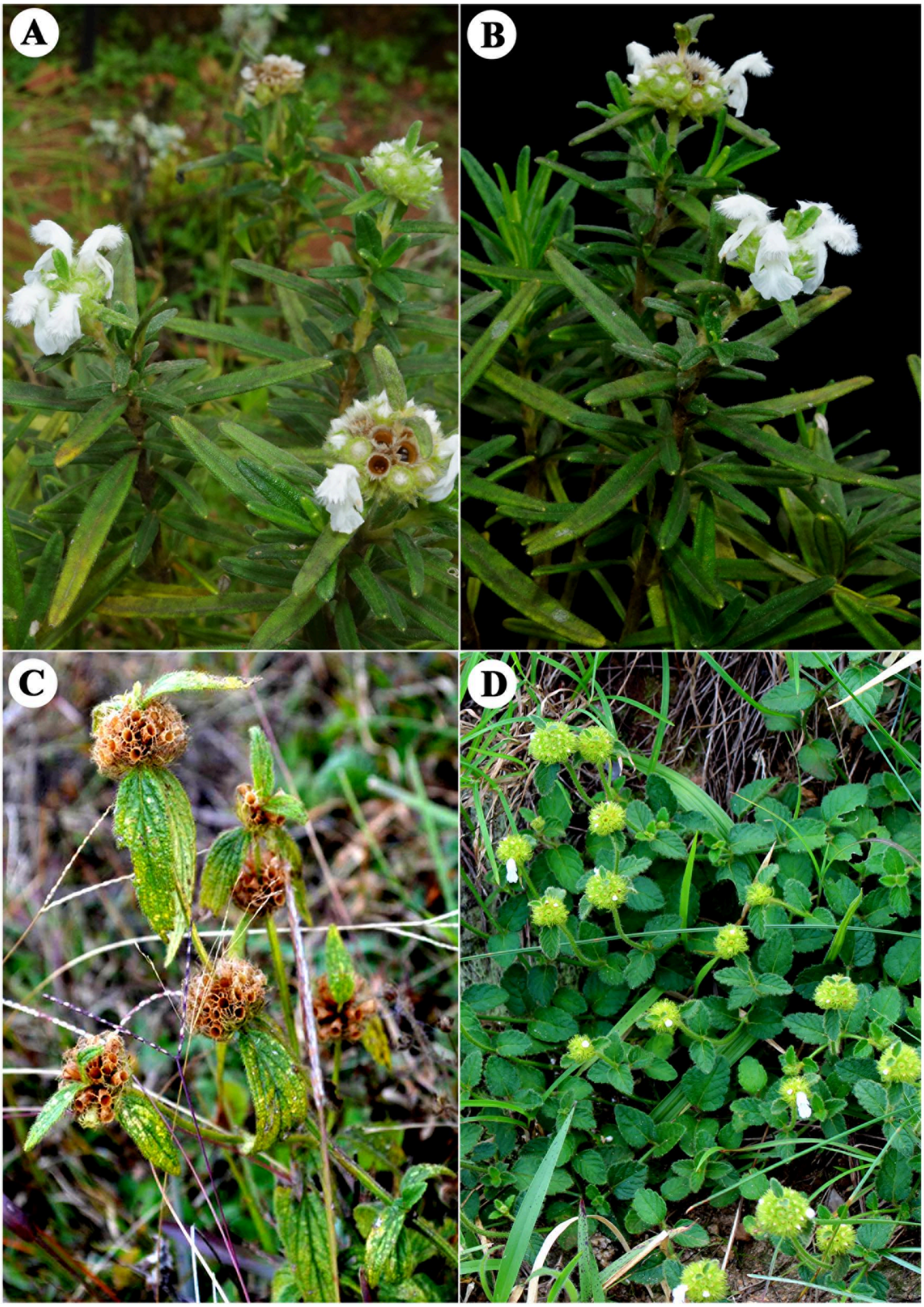

Plate.3 A&B. *Leucas suffruticosa* Benth., C. *Leucas manipurensis* V. Singh., D. *Leucas prostrata* Gamble.

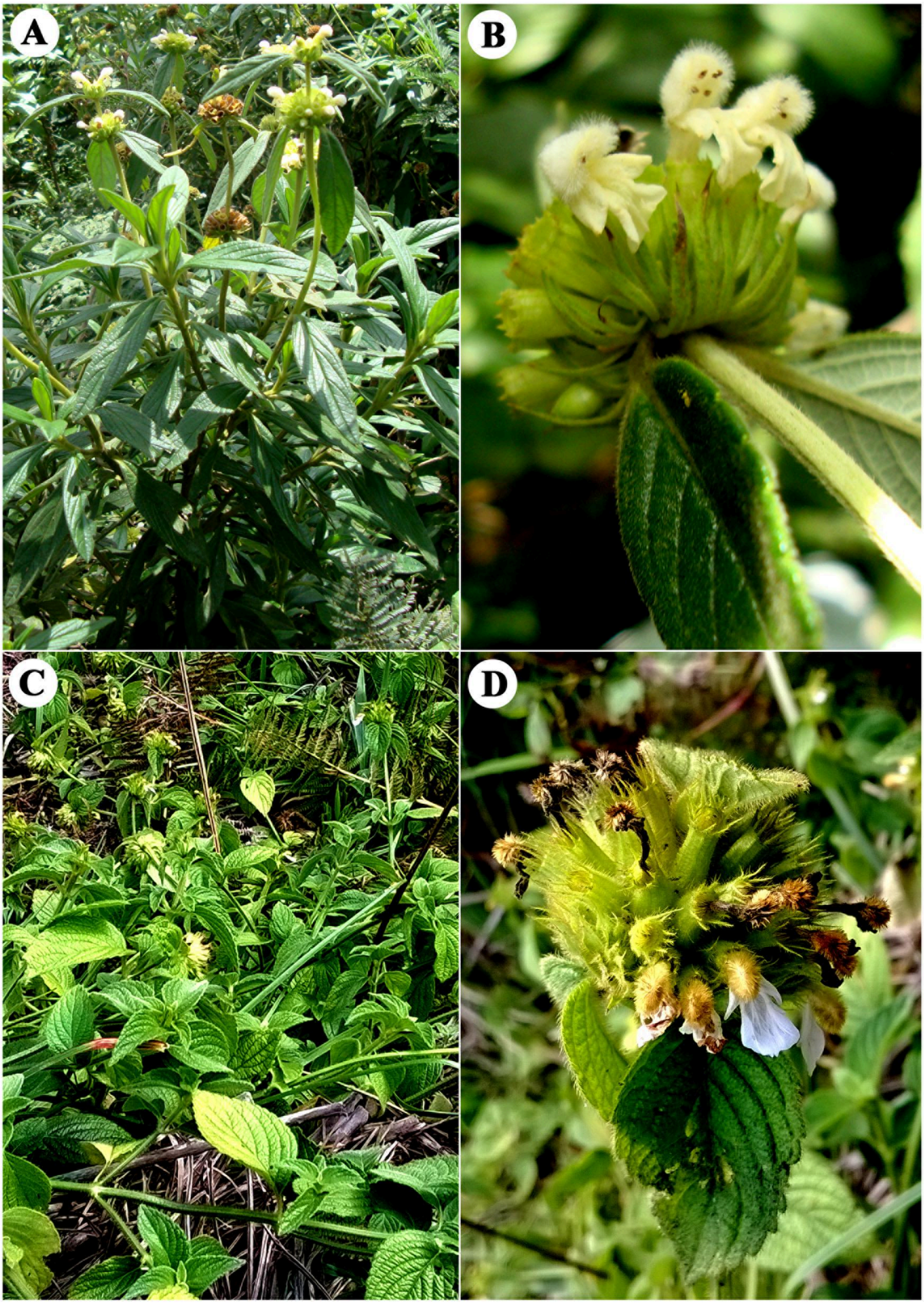

Plate.4. A&B. *Leucas lanceaefolia* Desf., C&D. *Leucas sebaldiana* Sunojk.

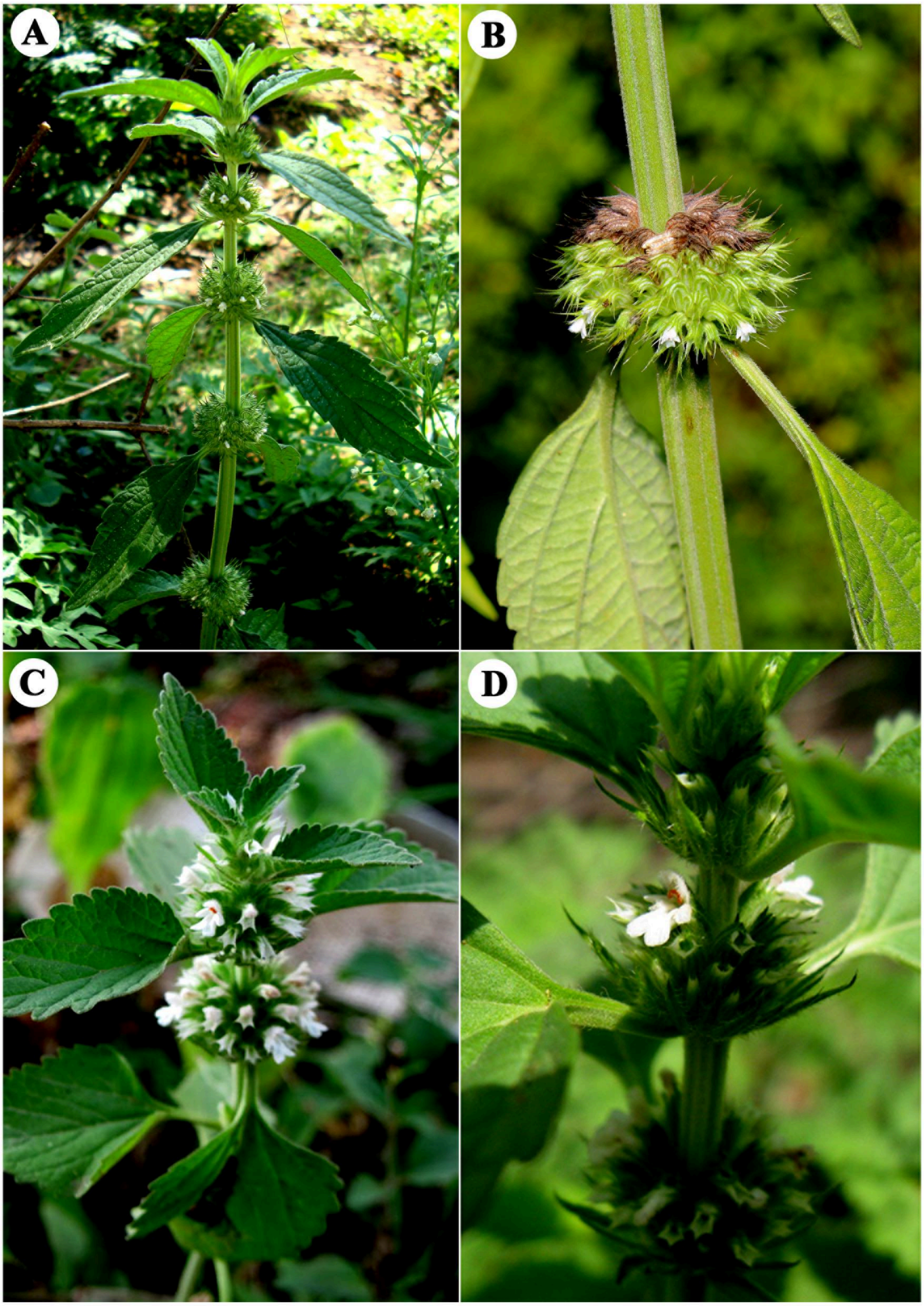

Plate.5. A&B. *Leucas martinicensis* (Jacq.) R.Br., C&D. *Leucas urticifolia* (Vahl) Sm.

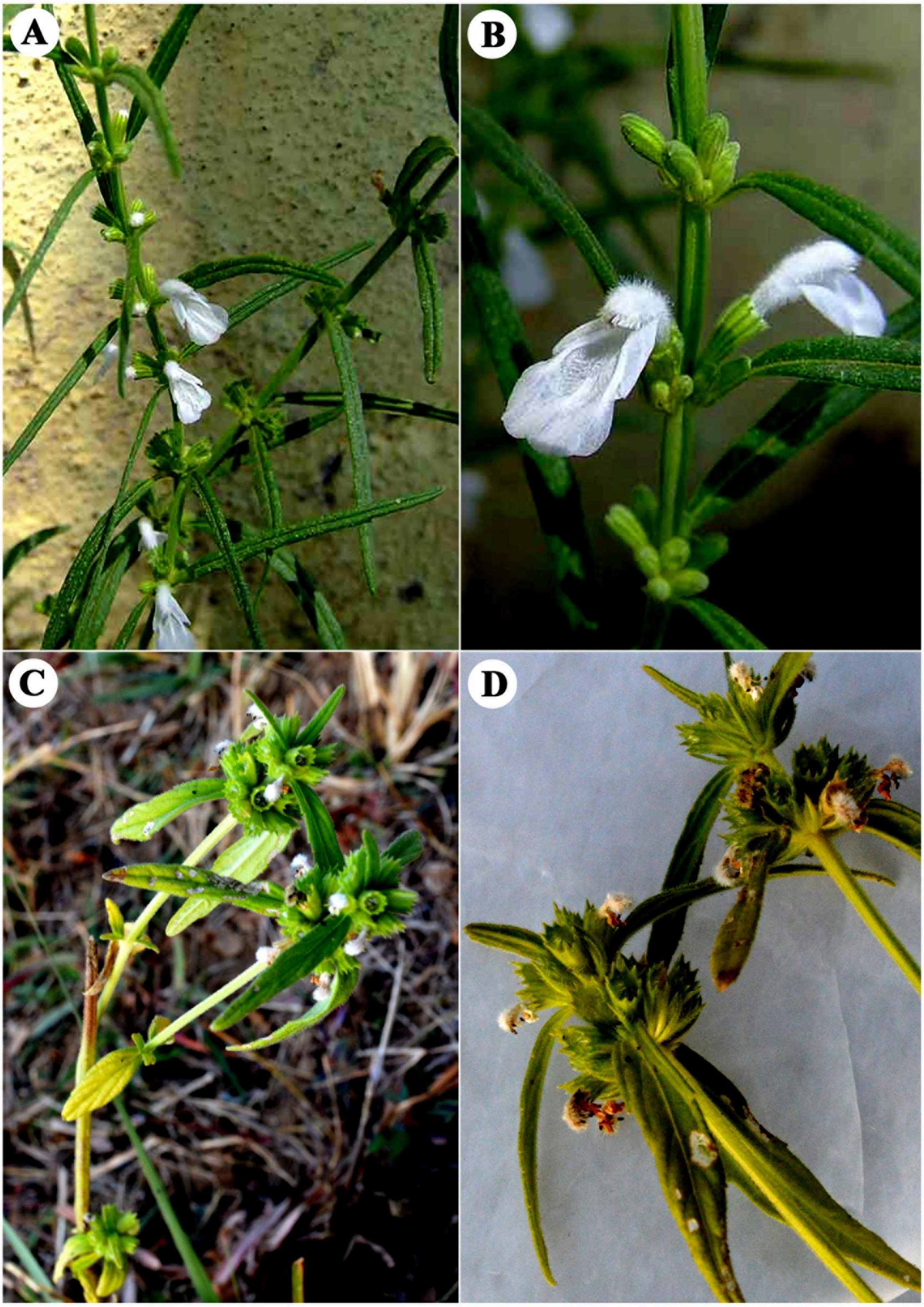

Plate.6. A&B. *Leucas longifolia* Benth., C&D. *Leucas macrantha* Blatt & Hallb.

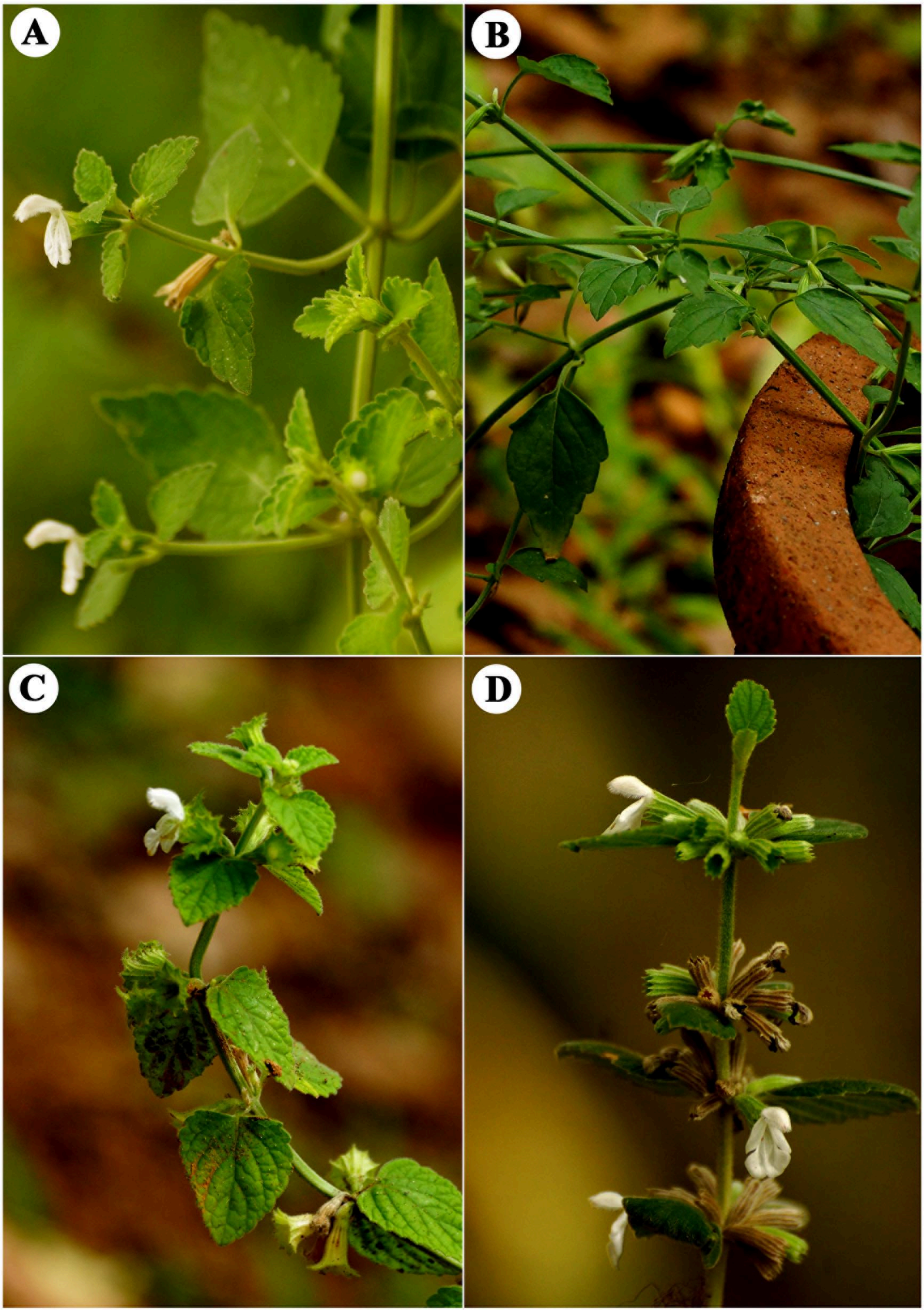

Plate.7. *Leucas angularis* Benth., B. *Leucas biflora* (Vahl) Sm., C. *Leucas chinensis* (Retz.) Sm., D. *Leucas lanata* var. *candida*

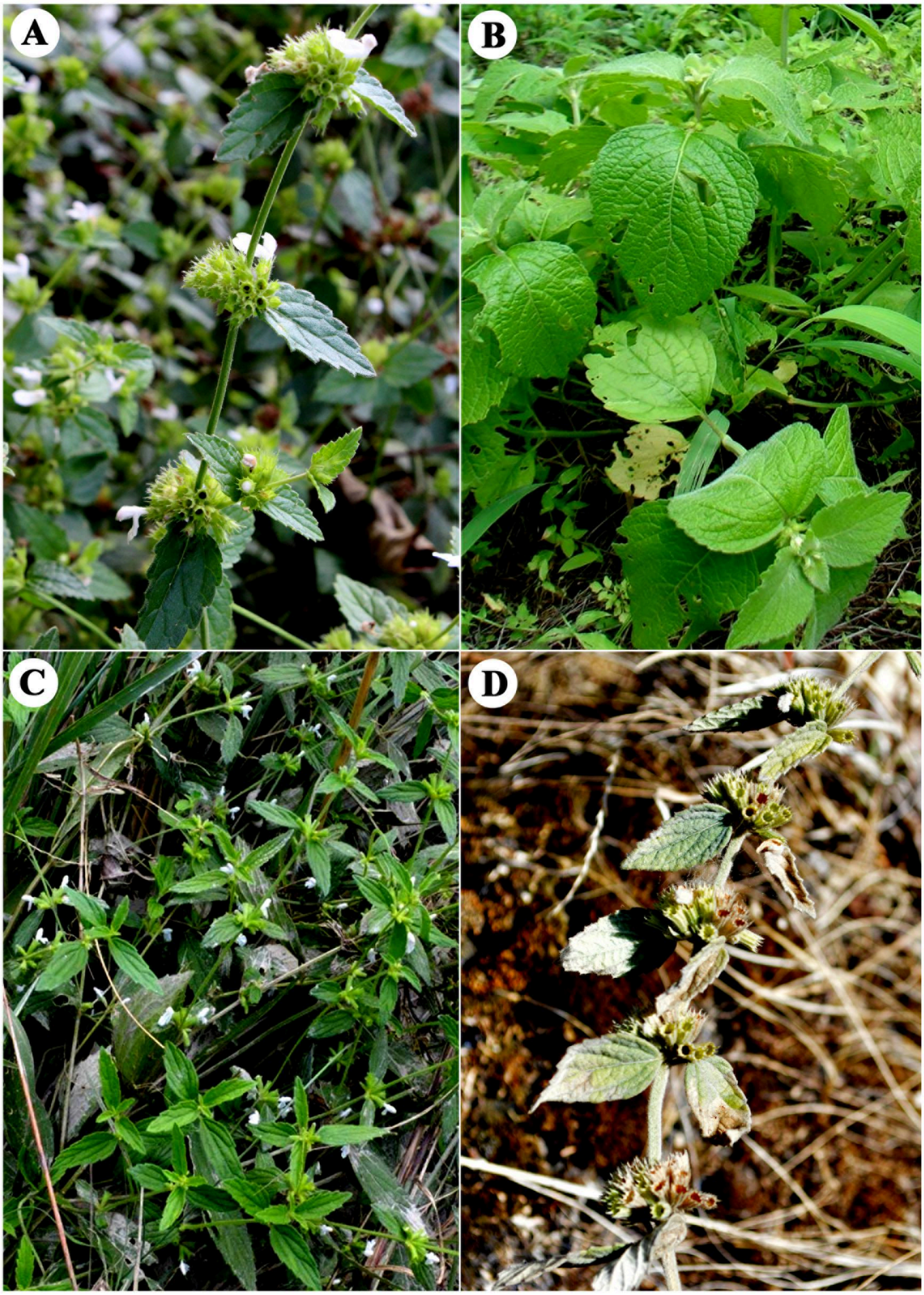

Plate.8. A. *Leucas decemdentata* var. *decemdentata* (Willd.) Sm., B. *Leucas deodikarii* Billimore & Hemadri., C. *Leucas helicterifolia* Haines., D. *Leucas lanata* Benth.

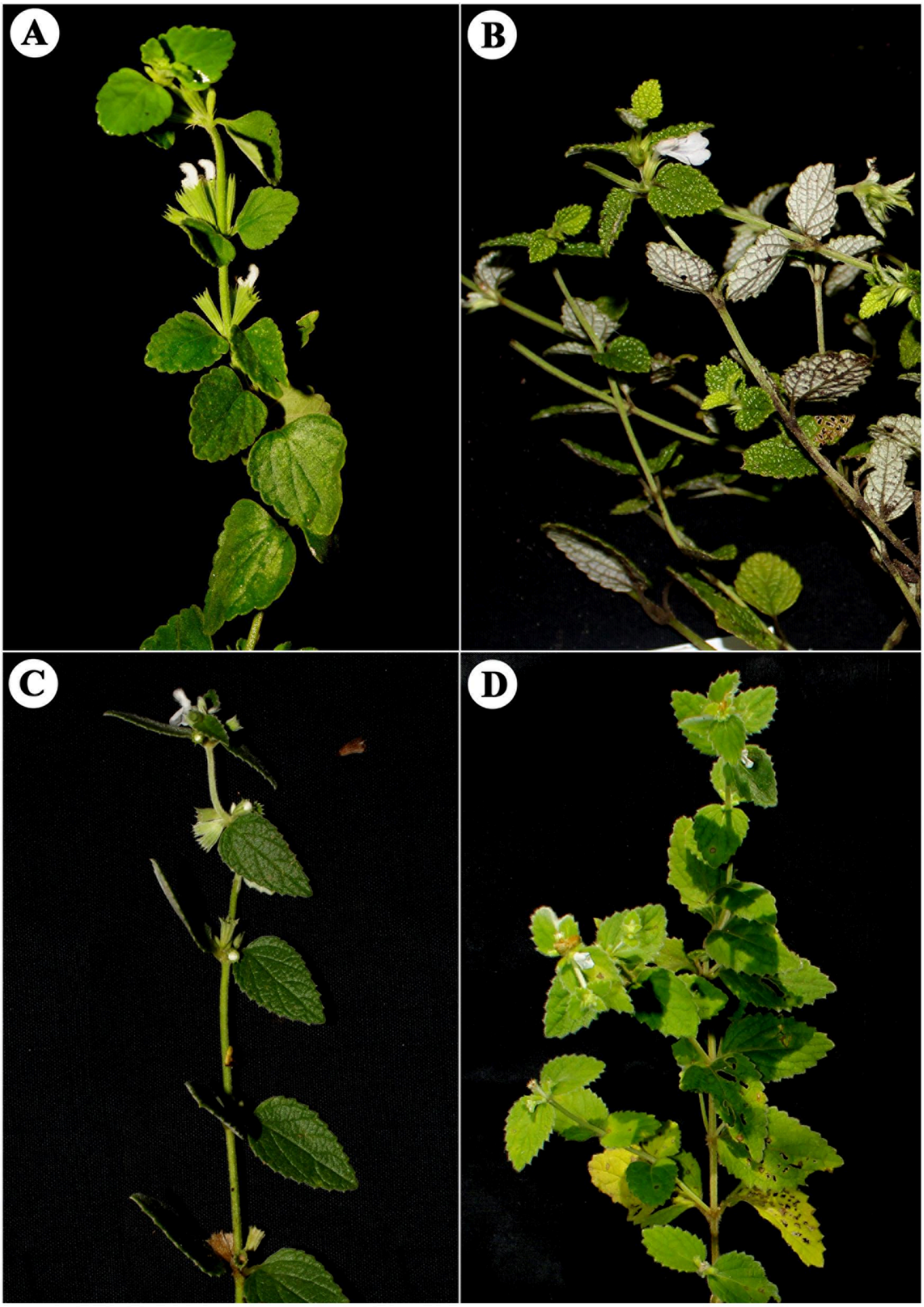

Plate.9. A. *Leucas nepetifolia* Benth., B. *Leucas marrubioides* var. *pulneyensis* Hook.f., C. *Leucas montana* (Roth.) Spreng., D. *Leucas mukerjiana* Subba Rao & Kumari.

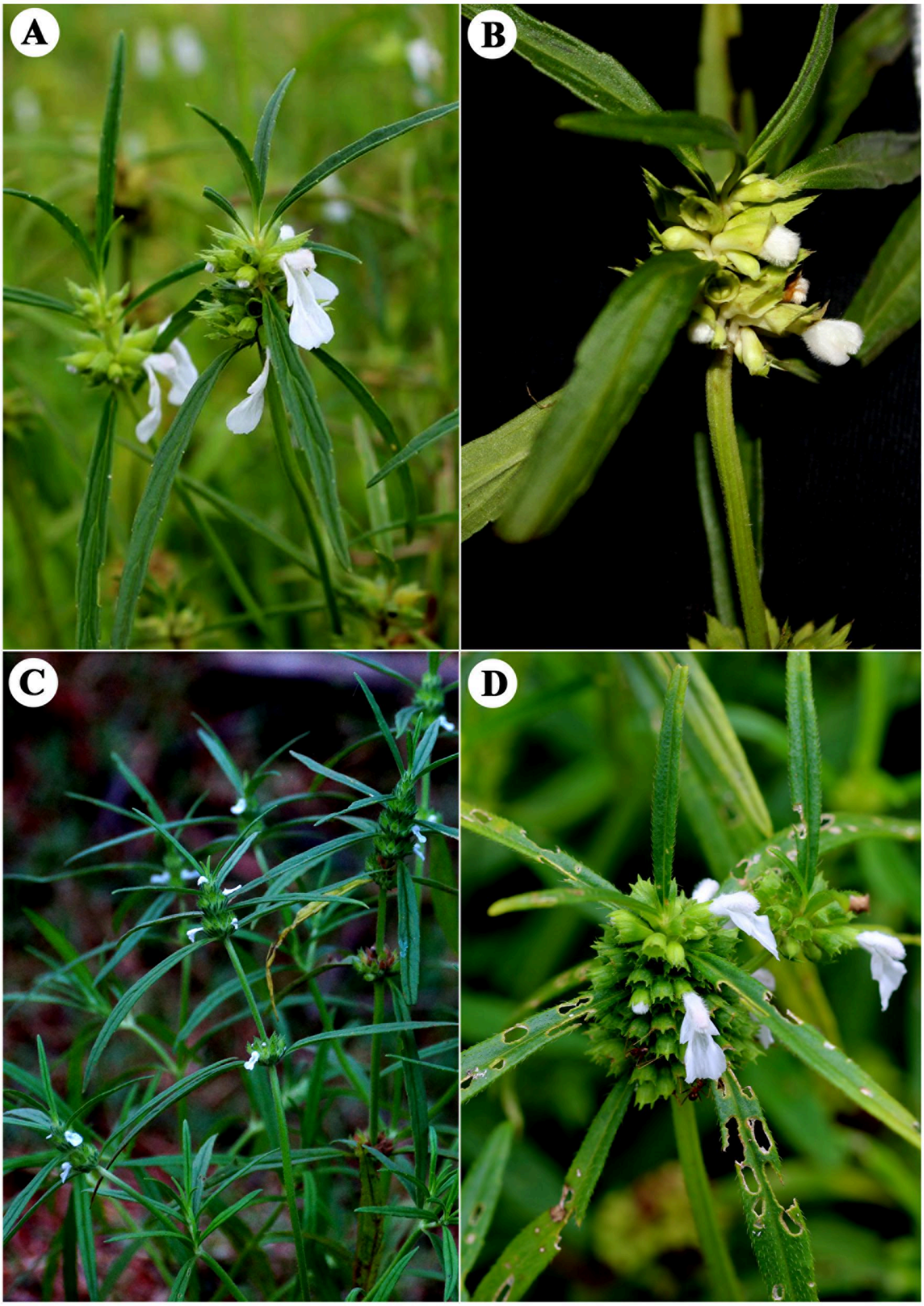

Plate 10 A & B. *Leucas lavandulifolia* Sm.; C. *Leucas wightiana.* C-Wall. ex. Benth.; D. *Leucas zeylanica* (L.).

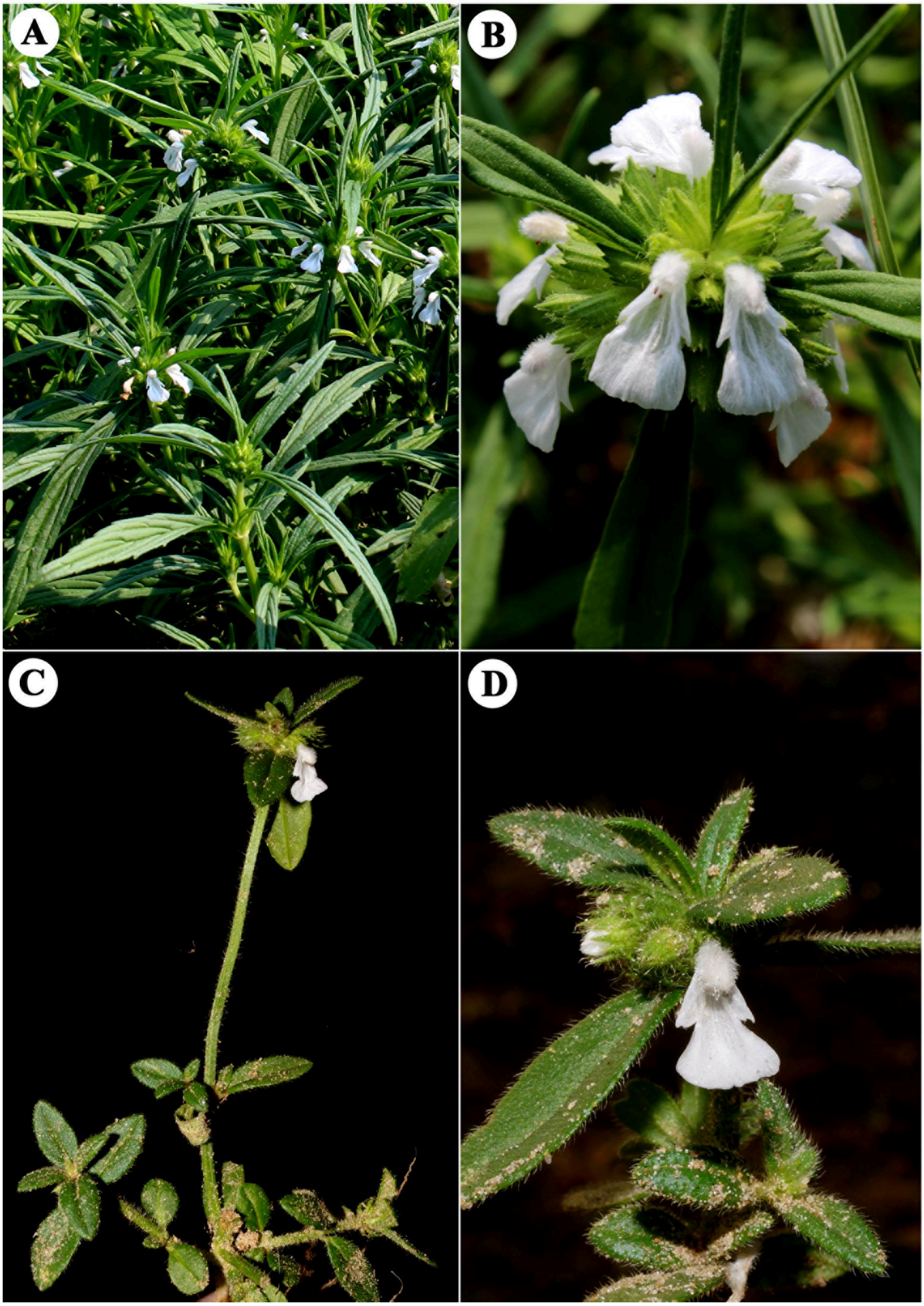

Plate.11. A & B *Leucas aspera* Willd. Link., C & D. *Leucas Diffusa* Benth.

Fig. 5.4. The 50% majority rule consensus phylogram from RAxML analysis of *rps16* intron region of chloroplast genome

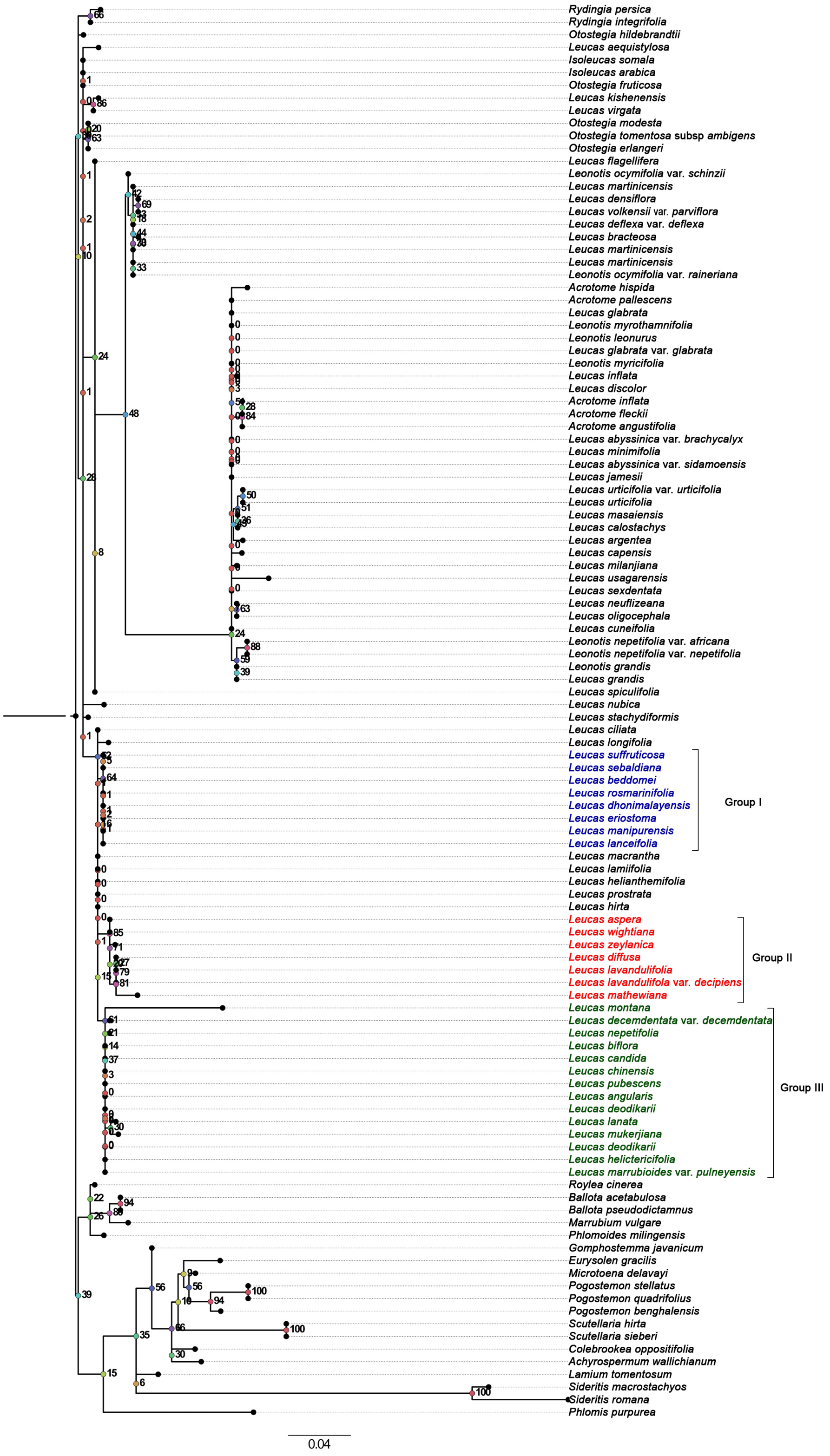

Fig. 5.3. The 50% majority rule consensus phylogram from RAxML analysis of *trnL-F* intergenic spacer region of chloroplast genome

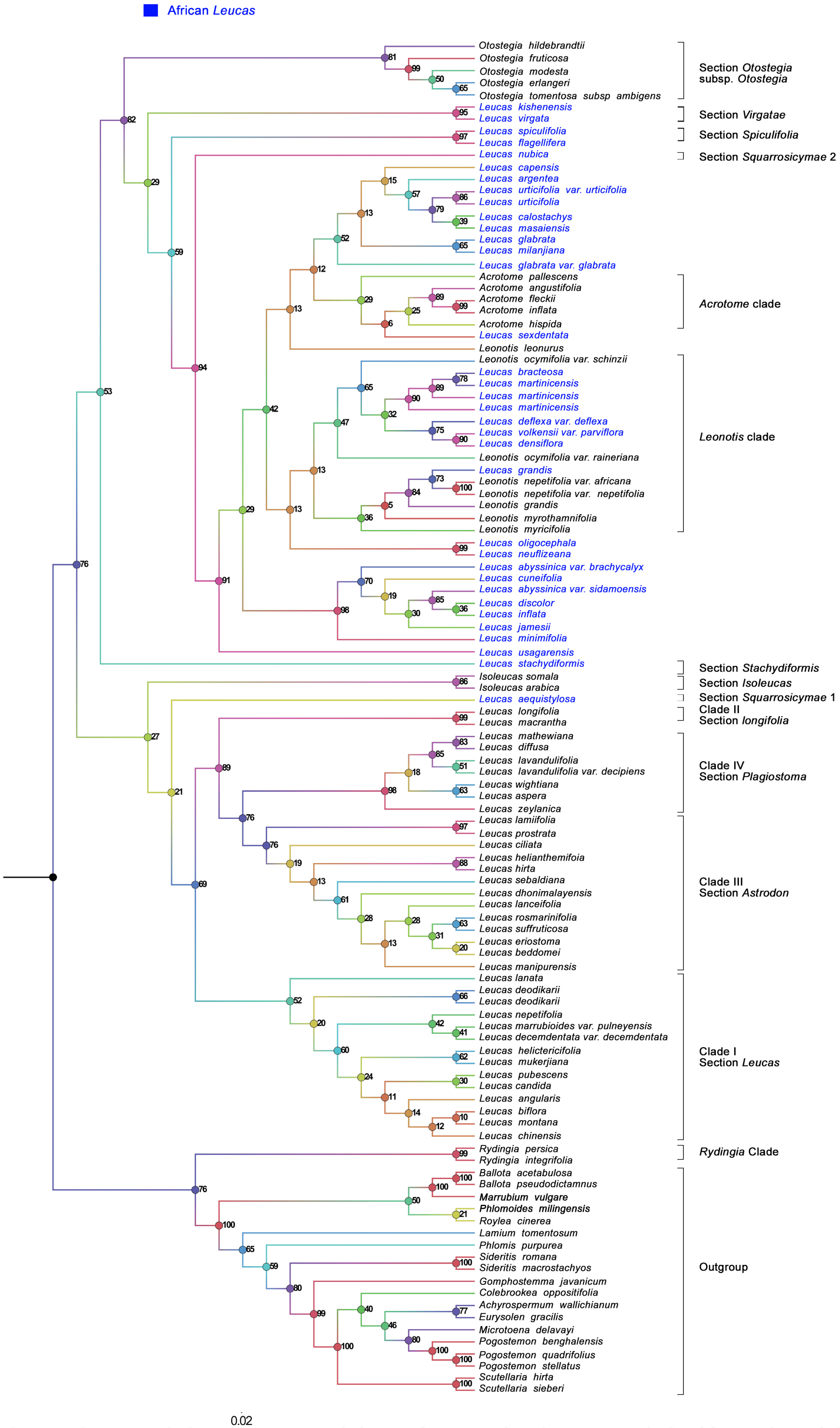

Fig. 5.6. The 50% majority rule consensus cladogram from a partioned RAxML analysis of four regions of chloroplast genome (*trnL* intron, *trnL-F* spacer & *rps16* intron regions)

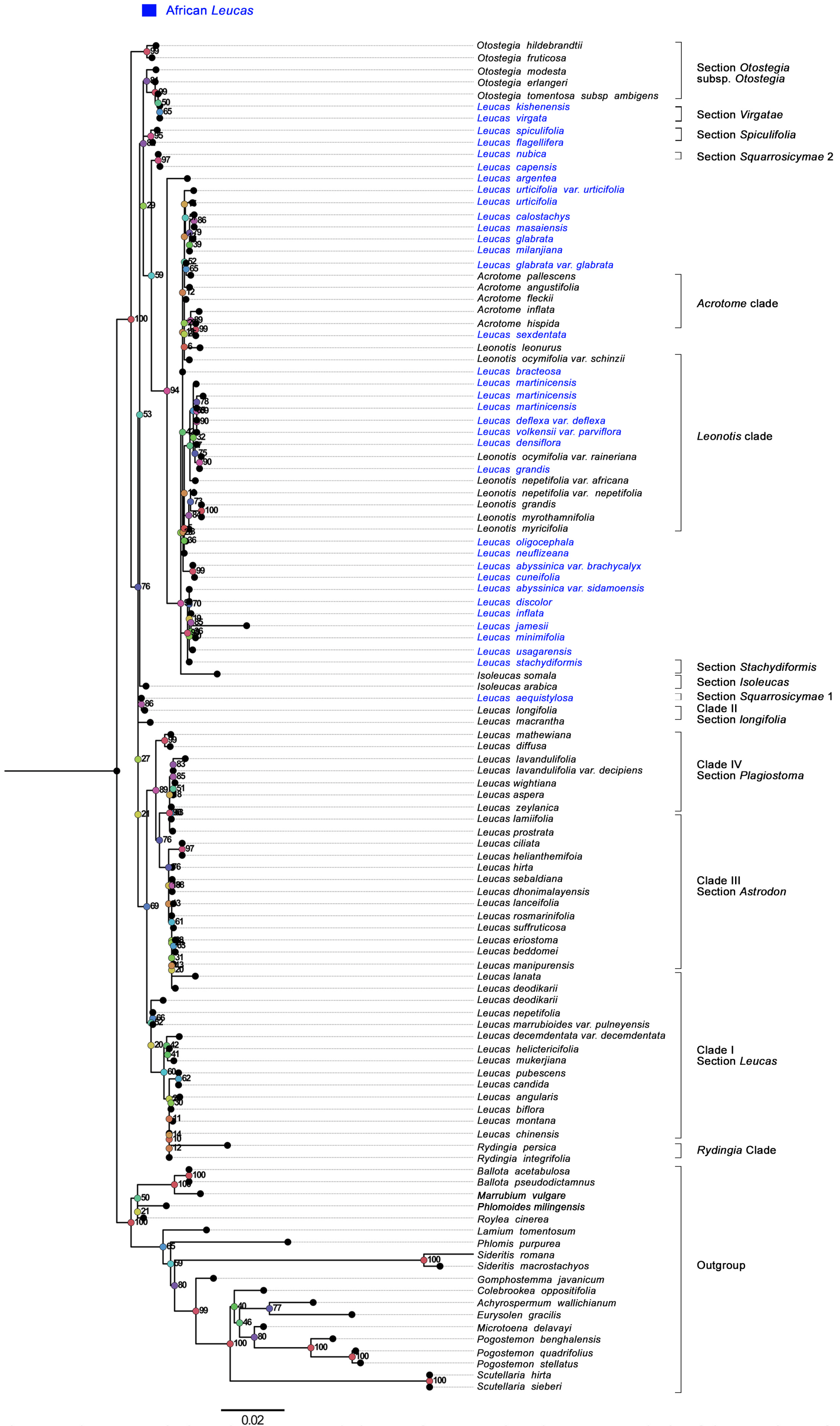

Fig. 5.5. The 50% majority rule consensus phylogram from a partioned RAxML analysis of three regions of chloroplast genome (*trnL* intron, *trnL-F* spacer & *rps16* intron regions)

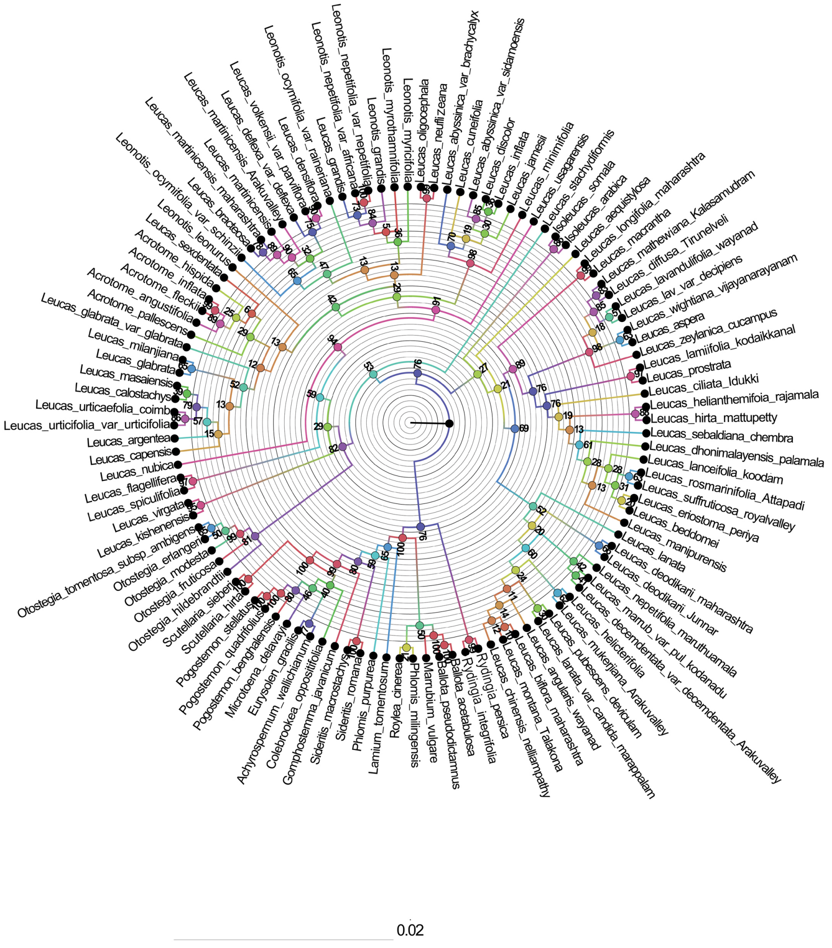

Fig. 5.7. The 50% majority rule consensus polar diagram from a partioned RAxML analysis of three regions of chloroplast genome (*trnL* intron, *trnL-F* intergenic spacer& *rps16* intron )

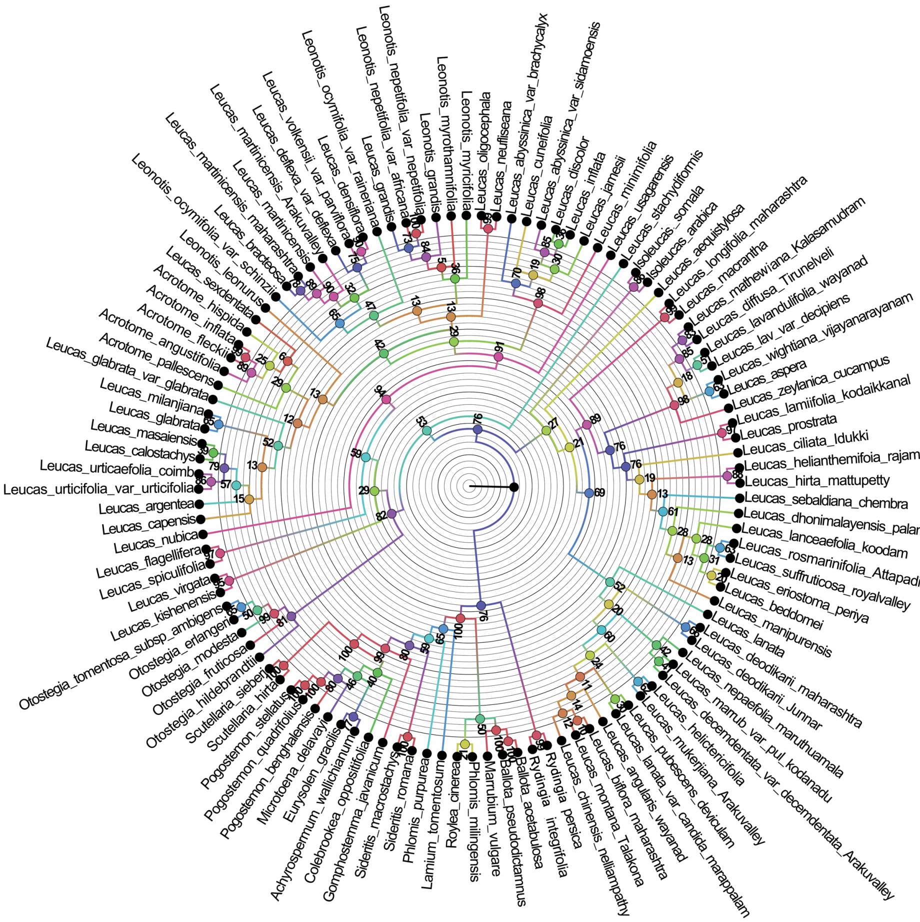

Fig. 5.2. The 50% majority rule consensus polar diagram from RAxML analysis of *trnL* intron of chloroplast genome.

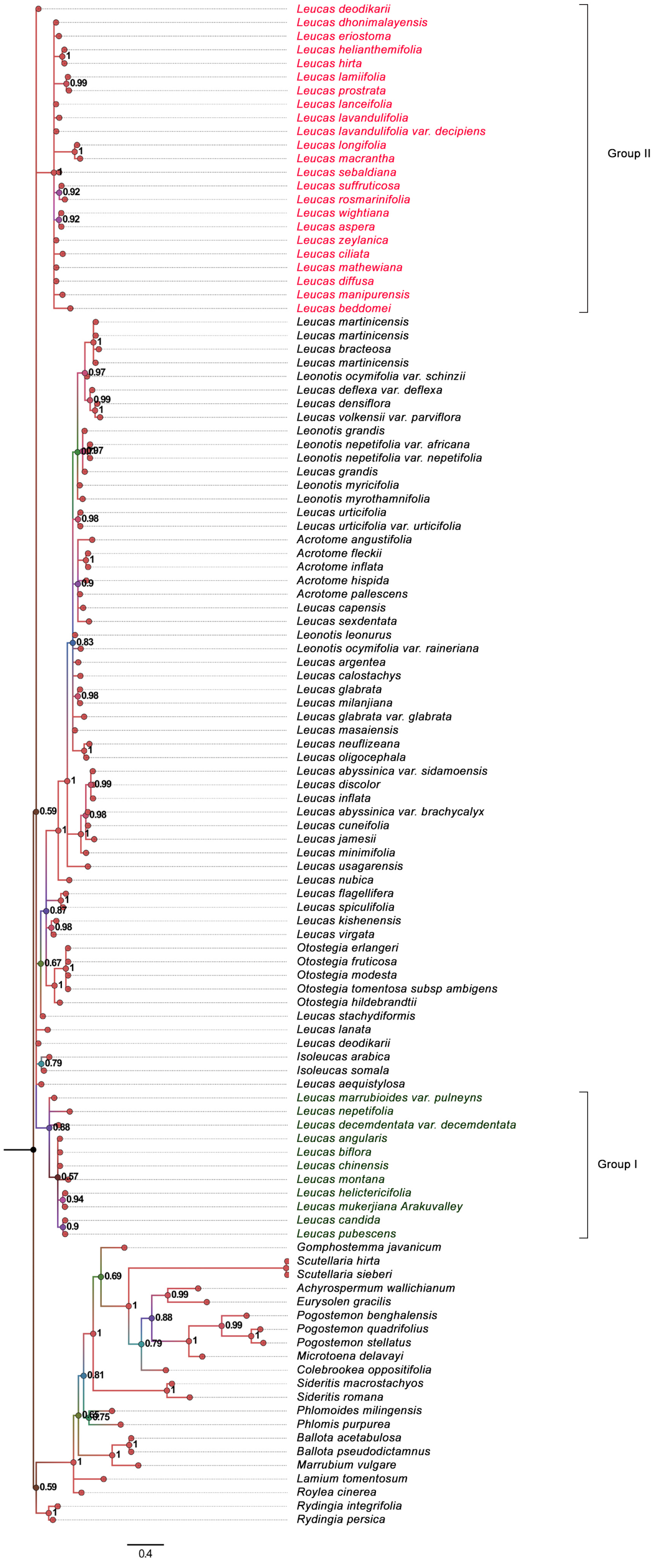

Fig. 5.10. The 50% majority rule consensus phylogram from Bayesian analysis of *rps16* intron region of chloroplast genome

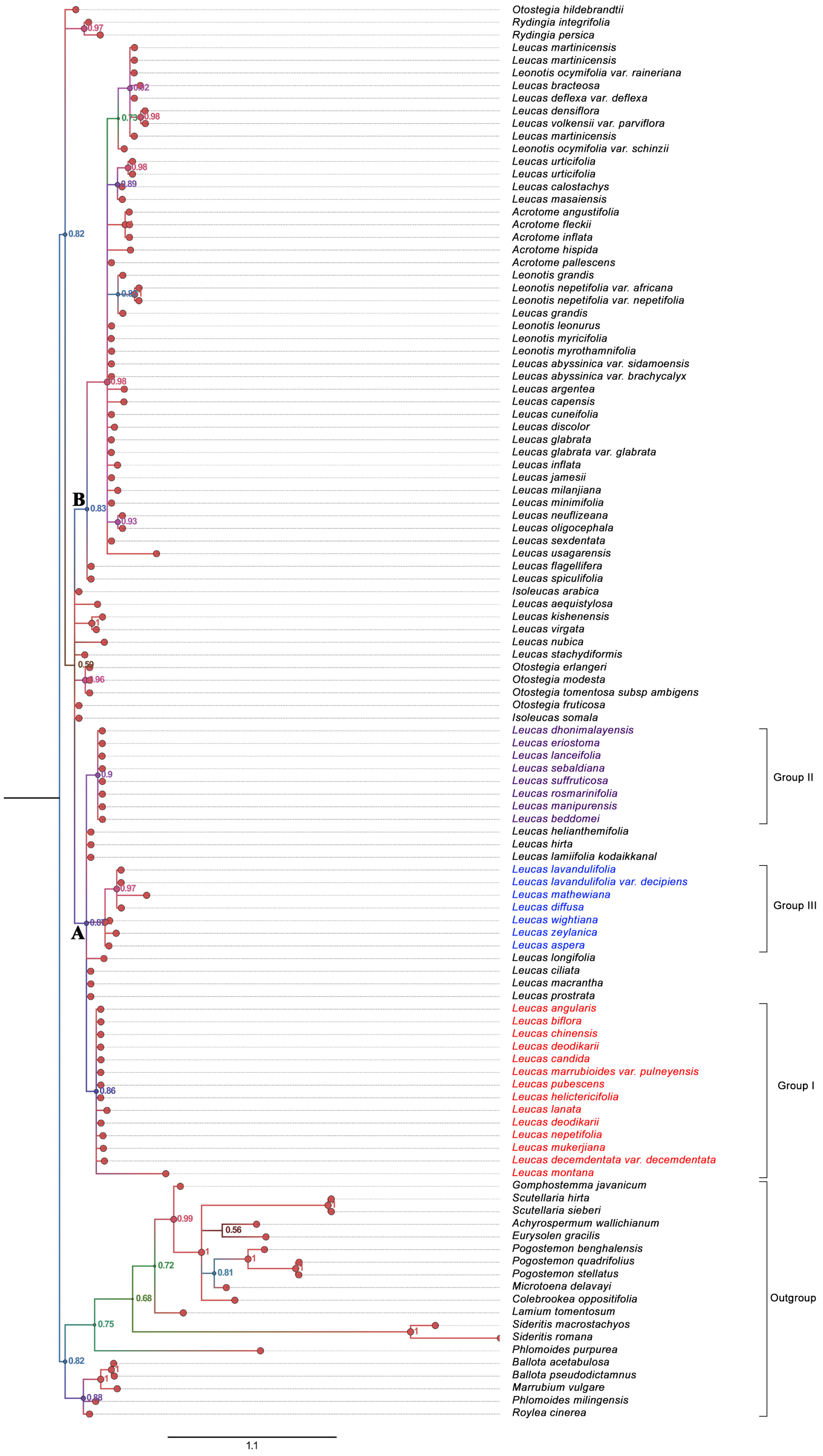

Fig. 5.9. The 50% majority rule consensus phylogram from Bayesian analysis of *trnL-F* intergenic spacer region of chloroplast genome

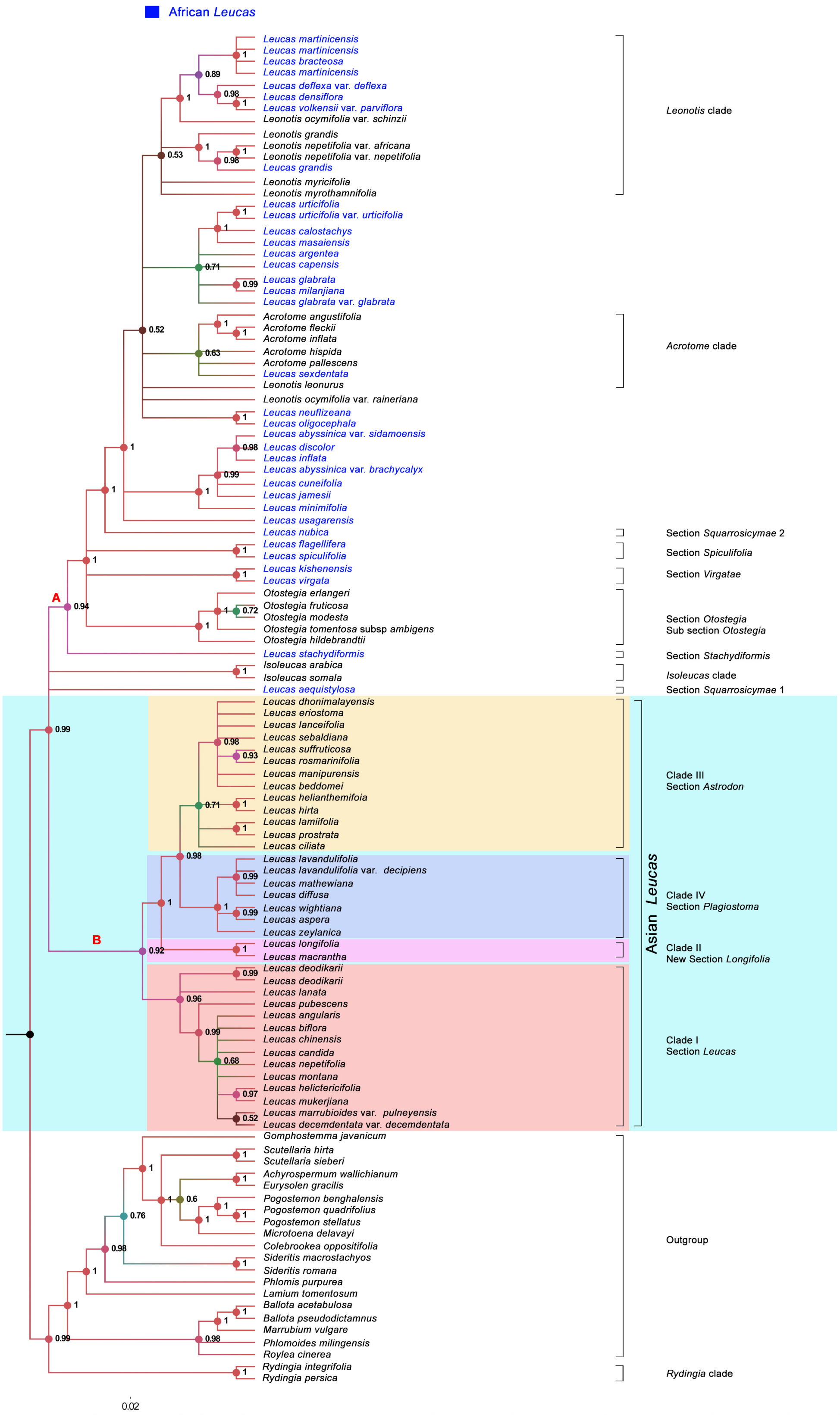

Fig. 5.12. The 50% majority rule consensus cladogram from a partioned Bayesian analysis of three regions of chloroplast genome (*trnL* intron, *trnL-F* spacer & *rps16* intron regions)

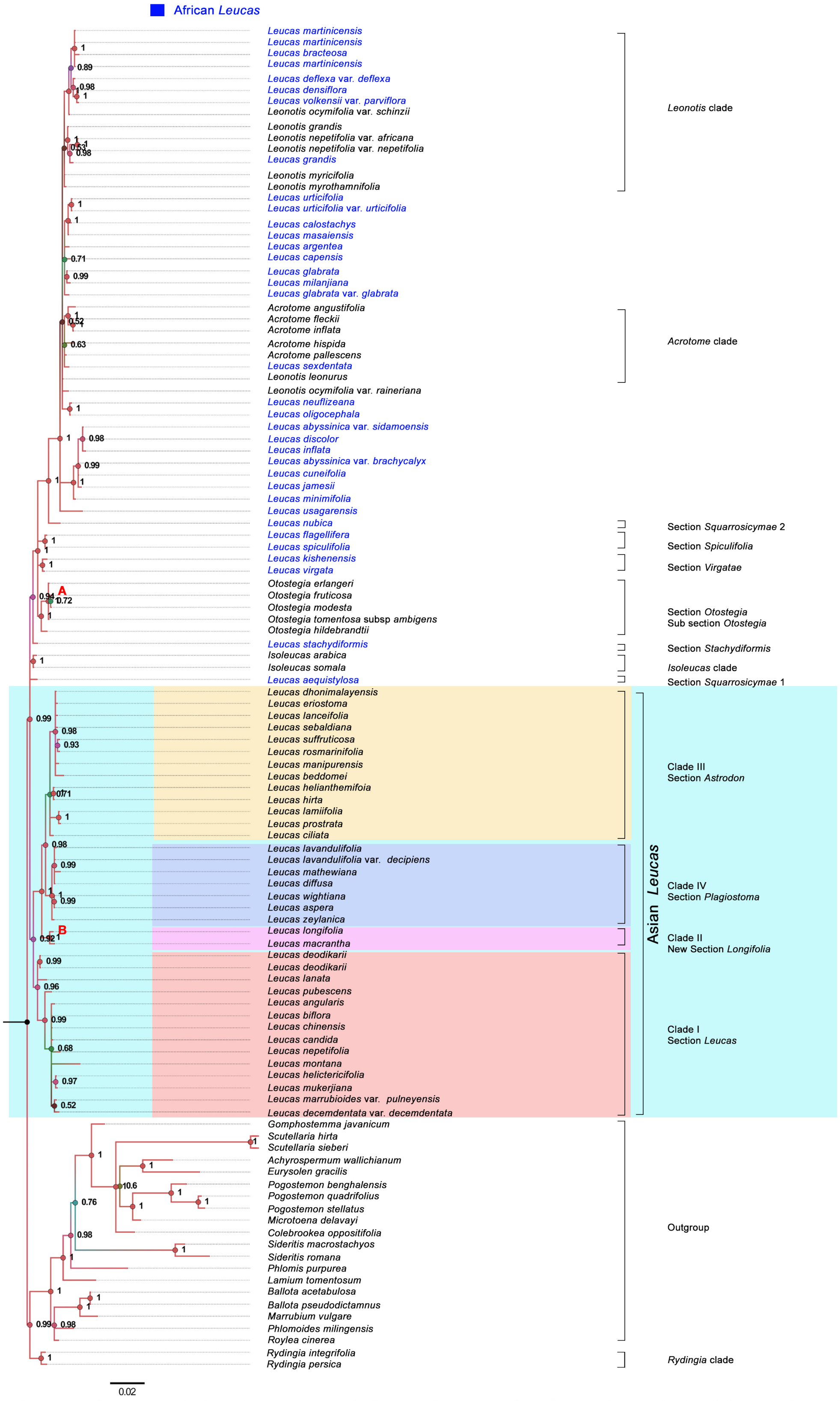

Fig. 5.11. The 50% majority rule consensus phylogram from a partioned Bayesian analysis of three regions of chloroplast genome (*trnL* intron, *trnL-F* spacer & *rps16* intron regions)

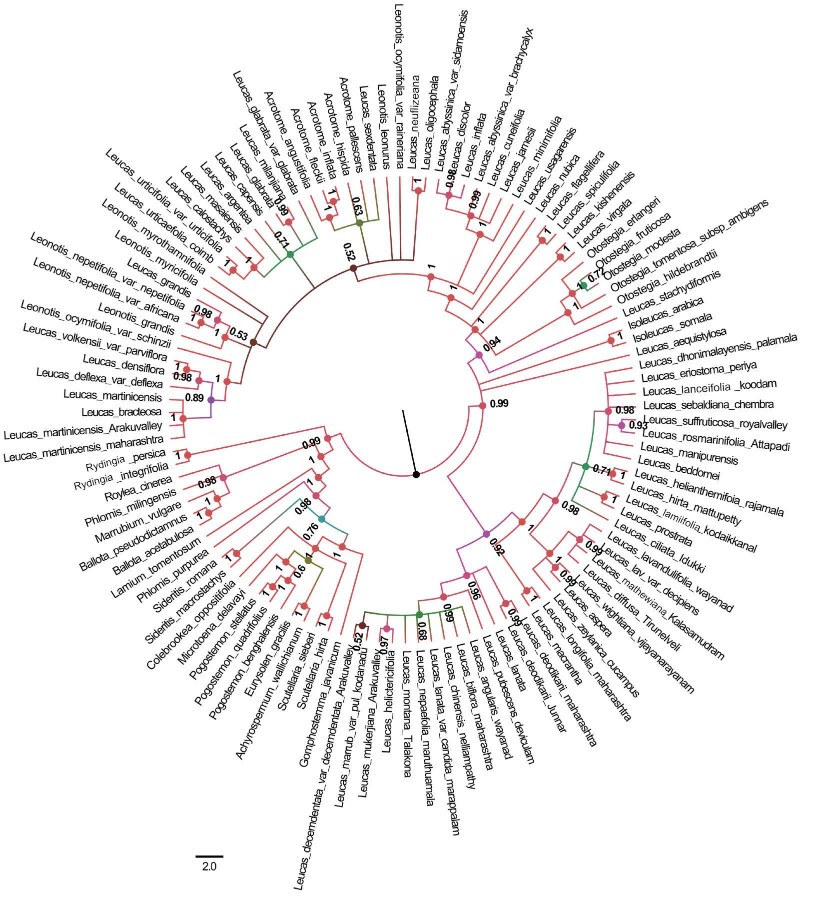

Fig. 5.13. The 50% majority rule consensus polar diagram from a partioned Bayesian analysis of three regions of chloroplast genome (*trnL* intron, *trnL-F* intergenic spacer & *rps16* intron)

Fig. 5.8. The 50% majority rule consensus phylogram from Bayesian analysis of *trnL* intron region of chloroplast genome