Phylogenetics and characterisation of Lamiaceae tribe Ocimeae, subtribe Ociminae

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Phylogenetic analyses of the subtribe Ociminae (Lamiaceae) based on four plastid DNA regions (*trn*L–*trn*F intergenic spacer, *trn*H-*psb*A intergenic spacer, *mat*K and *rps*16 intron) and two nuclear DNA regions (ITS and ETS) are presented. Bayesian inference was utilized for the reconstruction of phylogenies and for the assessment of statistical support for clades. The phylogeny was used for the investigation of the clade in terms of the distribution of morphology and geography. The subtribe Ociminae is monophyletic and can be simply diagnosed by morphological synapomorphies and is of African origin. The results clarify the relationships among *Orthosiphon* and its related genera *Fuerstia* and *Hoslundia. Orthosiphon* is monophyletic with exclusion *Or. fruticosus,* and it is a sister to *Fuerstia* and *Hoslundia.* Because *Fuerstia* and *Hoslundia* formed a monophyletic group with high morphological similarity between them, these genera will be merged under the earliest name *Hoslundia*. *Ocimum* is monophyletic with the inclusion of subgenus *Nautochilus. Ocimum* subgenus *Nautochilus* is a sister to the rest of *Ocimum* (subgenus *Ocimum* and subgenus *Gymnocimum*). Surprisingly, *Orthosiphon fruticosus* is a member of subgenus *Nautochilus* and highly supported, therefore, nomenclatural changes are required if the groups are to remain monophyletic. Analysis displayed strong congruence between the combinable component consensus trees and the traditional classification for most of genera and the results supported by morphological and geographic distribution. However, more taxon sampling to cover some regions of geography missed in this study is required. Moreover, the efficiency of ITS, *mat*K, *trn*H-*psb*A and in combination for species identification of *Orthosiphon* was assessed. For DNA barcode analysis, the determination of intraspecific and interspecific divergence, assessment of barcoding gap, reconstruction of phylogenetic trees and evaluation of barcode regions for species identification (based on best match and best close match) were carried out. The results revealed that ITS is a significant barcode region for identifying *Orthosiphon* species as a single marker and, for the combination, ITS+*mat*K+*trn*H-*psb*A.

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Table of Contents

List of Figures

List of Tables

List of Appendices

Chapter 1 : General introduction

1.1 A brief overview of the position of the Ociminae within the Lamiaceae

The mint family, Lamiaceae, has around 236 genera and 7200 species (Drew et al., 2017), making it the sixth largest of the flowering plant families (Harley et al., 2004). Hedge (1992) identified six geographic regions where there is a high diversity of Lamiaceae, namely the Mediterranean and South West Central Asia; Madagascar and Africa, South of the Sahel; China; Australia; South America; and Northern America and Mexico. Harley et al. (2004) identified an additional region: the Indomalesian region of Southeast Asia.

Morphological (Cantino and Sanders, 1986; Cantino et al., 1992) and the latest molecular results (Wagstaff et al.1995; Wagstaff and Olmstead, 1997; Wagstaff et al., 1998) were used extensively by Harley et al. (2004), who recognised seven subfamilies, known as Ajugoideae, Lamioideae, Nepetoideae, Prostantheroideae, Scutellarioideae, Symphorematoideae, and Viticoideae. Five more subfamilies have been described, known as Cymarioideae, Peronematoideae, Premnoideae, Callicarpoideae and Tectonoideae (Li et al., 2016; Li & Olmstead, 2017), depending on a large-scale phylogenetic reconstruction of Lamiaceae using of chloroplast sequences.

It was found that *Cymaria* (Scheen et al., 2010) or *Acrymia-Cymaria* (Bendiksby et al., 2011; Chen et al., 2014) had a close association with Lamioideae. However, it was asserted by Bendiksby et al. (2011) and Chen et al., (2014) that "an expanded Lamioideae would be more morphologically heterogeneous and difficult to diagnose", and hence, they suggested that *Acrymia* and *Cymaria* should be excluding from Lamioideae. Only moderate support was provided to the *Acrymia-Cymaria* clade in their studies, which is why it was observed by Chen et al. (2014) that if future evidence strongly validated the presence of this clade, a new subfamily could be created to consider them. This hypothesis was strongly validated in the studies of Li et al. (2016), which led to the creation of a new subfamily, Cymarioideae B. Li, R. G. Olmstead & P. D. Cantino.

Petraeovitex, Peronema, Hymenopyramis are sister to the Scutellarioideae- *Acrymia-Cymaria*- Lamioideae (Chen et al., 2014). On the basis of the phylogeny of Li et al. (2016), the clade including *Hymenopyramis, Petraeovitex, Garrettia,* and *Peronema* cannot be allocated to any accepted subfamily, therefore, a new subfamilial name was put forward there: Peronematoideae B. Li, R. G. Olmstead & P. D. Cantino. Conventionally, *Premna, Gmelina* and *Cornutia* are placed in Viticoideae (Harley et al., 2004), the earlier studies (Bendiksby et al., 2011; Li et al., 2012; Chen et al., 2014; Chen et al., 2016) and also the current ones have demonstrated that the *Premna-Gmelina-Cornutia* clade is not sister to the remaining Viticoideae (Harley et al., 2004). In addition, it is not possible to include these three genera in any other given subfamily on

the basis of findings of Li et al. (2016), and hence, the new subfamily Premnoideae B. Li, R. G. Olmstead & P. D. Cantino was formally described.

Callicarpa and *Tectona* were both transferred from Verbenaceae to Lamiaceae (Cantino et al. 1992, Harley et al. 2004). Given morphological distinctiveness and phylogenetic separation from all of the subfamilies delimited by Li et al. (2016) of the *Callicarpa* and *Tectona*, each was assigned to its own subfamily (Callicarpoideae Bo Li & R. G. Olmstead and Tectonoideae Bo Li & R. G. Olmstead) (Li & Olmstead, 2017).

Nepetoideae (Dumort.) is the largest subfamilies of Lamiaceae. It contains about 105 genera and 3,600 species, having morphological characters that are clearly defined: hexacolpate and three-nucleate pollen, and an investing embryo. This includes the tribes Elsholtzieae, Mentheae, and Ocimeae (Harley et al., 2004).

The tribe Ocimeae is characterised by synthecous anthers and, in most species, declinate stamens (Paton & Ryding, 1998). This tribe contains about 1,000 species in 35 genera, and is found in the tropical regions of Asia, China, Madagascar and South America (Paton & Ryding, 1998). Broader biogeographic analysis across the tribe by Paton et al. (2004) suggests that the ancestral area may be in Africa. After updates were made by Paton et al. (2004), Otieno et al. (2006), Pastore et al. (2011), and Harley and Pastore (2012) and Paton et al. (2018) the number of genera increased to 42. Table 1.1 shows the number of species for each genus of Ocimeae according to the *WCSP* (2019). The tribe Ocimeae contains seven subtribes, Harley et al. (2004) recognised subtribes Lavandulinae, Plectranthinae, Ociminae, Hyptidinae and subtribe Hanceolinae; the latter to accommodate *Isodon*, *Hanceola* and *Siphocranion*. Subtribes Isodoninae and Siphocranioninae have been more recently recognised as a new subtribe by Zhong et al. (2010) based on the combined molecular and morphological evidence.

The subtribe Ociminae contained 12 genera, characterised by a small, flat anterior corolla lip, and the stamens and style rise towards the posterior lip of the corolla (Harley et al., 2004; Paton et al., 1999). However, this was changed to 11 genera when *Hemizygia* and *Syncolostemon* were merged into one genus (Otieno et al., 2006). Many genera in subtribe Ociminae include species that are found in India and Africa, e.g. *Basilicum*, *Platostoma* and *Orthosiphon* (Paton et al., 1994), Madagascar and Tropical or Subtropical Asia for *Orthosiphon* as well (Harley et al., 2004). It is apparent that Ociminae is a diverse and widespread subtribe in India, Africa, Madagascar and Asia, with a few species of *Ocimum*, and one *Orthosiphon* found in the New World (Harley & Paton, 2012).

Table 1.1 Tribe Ocimeae: subtribes following the concept of the cited authors, genera and number of species.

Subtribe	Genus	No. of species *WCSP*
Hanceolinae (C.Y. Wu) A.J. Paton & Harley (Zhong et al., 2010)	*Hanceola* Kudo	8
Hyptidinae Endl. (Pastore et al., 2011)	*Asterohyptis* Epling	4
	Cantinoa Harley & J.F.B.Pastore	25
	Condea Adanson	27
	Cyanocephalus (Pohl ex Benth.) Harley & J.F.B.Pastore	25
	Eplingiella Harley & J.F.B.Pastore	3
	Eriope Humb. & Bonpl. ex Bentham	30
	Eriopidion Harley	1
	Gymneia (Benth.) Harley & J.F.B.Pastore	7
	Hypenia (Mart. ex Benth.) Harley	25
	Hyptidendron Harley	17
	Hyptis Jacquin	165
	Leptohyptis Harley & J.F.B.Pastore	5
	Marsypianthes Mart. ex Bentham	5
	Martianthus Harley & J.F.B.Pastore	4
	Medusantha Harley & J.F.B.Pastore	8
	Mesosphaerum Browne	23
	Oocephalus (Benth.) Harley & J.F.B.Pastore	17
	Physominthe Harley & J.F.B.Pastore	2
	Rhaphiodon Schauer	1
Isodoninae J.S. Zhong, J. Li & H.W. Li (Zhong et al., 2010)	*Isodon* (Schrad. ex Benth.) Spach (Lamiaceae)	102
Lavandulinae Endl. (Harley et al., 2003)	*Lavandula* L.	39
Ociminae (Dumort.) Schmidt (Harley et al., 2004; Otieno et al., 2006)	*Basilicum* Moench	1
	Benguellia G. Taylor	1
	Catoferia (Benth.) Benth.	4
	Endostemon N.E.Br	20
	Fuerstia T. C. E. Fries	9
	Haumaniastrum P. A. Duvign. & Plancke	35
	Hoslundia Vahl	1
	Ocimum L.	64
	Orthosiphon Benth.	44
	Platostoma P.Beauv.	46
	Syncolostemon E. Mey. ex Benth.	45
Plectranthinae Endl. (Paton et al., 2018)	*Aeollanthus* Spreng.	42
	Alvesia Welw.	3
	Capitanopsis S.Moore	6
	Coleus Lour.	271
	Equilabium Mwanyambo, A.J.Paton & Culham	39
	Plectranthus L'Hér.	65
	Tetradenia Benth.	23
	Thorncroftia N.E.Br.	6
Siphocranioninae J.S. Zhong, J. Li & H.W. Li (Zhong et al., 2010)	*Siphocranion* Kudo	2

WCSP = World Checklist of Selected Plant Families (*WCSP*, 2019).

1.2 The importance of Lamiaceae

Lamiaceae is an economically important plant family as the majority of plants within the group have aromatic qualities (Harley et al., 2004). Many of them produce essential oils that are used in traditional and modern medicine, and in the food, cosmetics, and pharmaceutical industry (Mamadalieva et al., 2017). Some of the valuable products of the family are patchouli (*Pogostemon*), peppermint oil (*Mentha*), teakwood (*Tectona*), and a number of herbs employed for cooking such as basil (*Ocimum*), sage (*Salvia*), oregano (*Origanum*), thyme (*Thymus*), rosemary (*Rosmarinus*) and spearmint/peppermint (*Mentha*) (Li et al., 2016).

The Ociminae species have been particularly widely used in medicine, e.g., *Orthosiphon* species provide significant numbers of chemical compounds, some of which were shown to have dynamic pharmacological properties (Sundarammal et al., 2012). These plants have an important place in medicine, particularly herbalism, and are believed to possess medicinal qualities (Singh et al., 2015).

Ocimum is another commercially important genus of subtribe Ociminae (Lamiaceae) well known for medicinal properties and also for commercially important essential oils. Plants of the genus are commonly known as basil and are an important source of aromatic compounds, essential oils, spices, ornamentals, and medicines (Simon et al., 1990), as well as having a traditional medicinal use in many parts of the world (Telci et al., 2006).

1.3 Taxonomic history and problems of subtribe Ociminae

The subtribe Ociminae was divided, using nutlet anatomy, into three informal groups by Ryding (1992): *Acrocephalus* (renamed *Platostoma* by Paton (1997b)), *Ocimum* and *Orthosiphon*. Ryding (1992) reported that the exocarp often has both mucilaginous and non-mucilaginous cells. Underneath which there are soft cells (mesocarp), a vertical layer of bone cells and a thinner central cell layer. Variations found in the pericarp anatomy generally concur with the traditional Ocimeae subdivisions (Ryding, 1992).

1.3.1 The *Platostoma* group

The *Platostoma* group comprises the following previously recognised genera: *Haumaniastrum* (Duvigneaud & Plancke, 1959), *Acrocephalus* Benth. *Benguellia*, *Ceratanthus* F. Muell. ex G. Taylor, *Geniosporum* Wall. ex Benth., *Mesona* Blume, *Nosema* Prain, *Octomeron* Robyns and *Platostoma* P. Beauv. *Limniboza* R. E. Fr. was later added to this group (Ryding, 1993). However, Paton, (1997b) suggested that the previously recognised generic delimitations in the *Platostoma* group were unworkable. He placed *Acrocephalus*, *Geniosporum*, *Ceratanthus*, *Octomeron*, *Mesona*, *Nosema* and *Limniboza* as synonyms of *Platostoma* which was divided into subgenera and sections by using parsimony analysis of morphological data. *Platostoma* is a paraphyletic genus due to the exclusion of *Haumaniastrum* based on study of Paton (1997b). He also pointed out the need for a thorough revision of this group. *Benguellia* is considered to be more closely related to *Orthosiphon* than to the *Platostoma* group (Paton, 1997b). Paton (1997a) worked on a revision of *Haumaniastrum* and found that the relationships within *Haumaniastrum* based on strict consensus tree are uncertain. Therefore, much more information is needed in order to resolve the phylogenetic relationships within *Haumaniastrum* and between *Haumaniastrum* and *Platostoma*.

1.3.2 The *Ocimum* group

Ryding (1992) placed *Hemizygia*, *Syncolostemon* and *Ocimum* together with *Becium* Lindl. [now treated as *Ocimum*], *Catoferia* and *Erythrochlamys* Gürke in his "*Ocimum* group" of the subtribe Ociminae based on the results of a parsimony analysis of *Ocimum* and its close allies. Paton (1992) suggested that *Orthosiphon* subgenus *Nautochilus* (Bremek) should be added to this group as it possesses a number of characteristics of *Ocimum.*

A close relationship between *Hemizygia* and *Syncolostemon* was suggested by Ashby (1935): "in general appearance and in the nature of the floral bracts, signifying the genus *Syncolostemon*". Subsequent studies validated Ashby's observations (Codd, 1976a, 1976b; Paton, 1998; Paton & Balkwill 2001), showing how the two genera were similar in corolla and androecial

features. The shape of the mature calyx is the only attribute that has been used frequently to separate *Hemizygia* and *Syncolostemon.* It was suggested by Codd (1976b) that *Hemizygia* and *Syncolostemon* should be merged; however, he did not do so. It was demonstrated by Paton et al. (1999) that *Syncolostemon* and *Hemizygia* were closely related to each other, as determined from a parsimony analysis. The two genera were united as a monophyletic group and appeared as sister to an extended monophyletic *Ocimum,* however, it was distinct from the latter as it had an unequal lobed disk and fused anterior stamens (Paton et al., 1999). However, they did not deal with the question of whether *Syncolostemon* and *Hemizygia* were congeneric; rather, they stressed on the need to have a study for addressing this issue. The latest studies carried out by Paton et al. (2004) and Otieno et al. (2006) show strong support for the two genera as a monophyletic group, and the genera were officially merged under the earliest name *Syncolostemon* by Otieno et al. (2006).

Syncolostemon species were detailed by Codd (1976a) as falling into a pair of distinct groupings, with the first group containing *S. parviflorus, S. concinnus, S. comptonii, S. argenteus,* and *S. eriocephalus*. These have a small calyx and corolla, with the mature calyx being eight mm long at most, campanulate in shape, frequently becoming subrotund, and setose at the throat, with a corolla tube between 6–10 mm, lax inflorescence, and nutlets having a frill, around the base. The second group comprises *S. densiflorus, S. rotundifolius, S. macranthus,* and *S. latidens*, all of which have a sizeable and conspicuous calyx and corolla, with a mature calyx generally being cylindrical and in excess of 10 mm long, having a corolla tube between 14 and 30 mm long, having a dense inflorescence, and nutlets lacking a frill around the base.

Ocimum is only monophyletic when *Becium*, *Erythrochlamys* and *Orthosiphon* subgenus *Nautochilus* are incorporated; recognising any of the mentioned taxa at the generic level would make *Ocimum* a paraphyletic group (Paton et al., 1999). Sebald (1988; 1989) stated that *Ocimum* and *Becium* may be separated because the latter possesses a gland that releases nectar at the base of the cymes in inflorescence; it also has elongated anthers with parallel thecae instead of orbicular or reniform anthers with diverging thecae; the distinction is not clear as some *Ocimum* species also have divergent anther thecae and a gland at the base of their cymes (Paton et al., 1999). Thus *Becium* is not acknowledged as a separate genus but included in sect. *Ocimum* subsect. *Hiantia* (Paton et al., 1999). Gürke described *Erythrochlamys* in 1894; it contains a pair of species present in Northeast Tropical Africa. It has usually been distinguished from *Ocimum* because it possesses an expanded upper lip on the calyx (Baker 1900), but this characteristic can also be found in *O. circinatum* A.J.Paton (Paton, 1992) and *O. transamazonicum* Pereira (Pereira, 1972). This raises questions over the generic delimitations for *Erythrochlamys*; Paton et al. (1999) placed *Erythrochlamys* as a subsection of *Ocimum*. It would appear that recognising *Erythrochlamys* as a

stand-alone genus would be problematic because it would hide the similarities between that species and certain species of *Ocimum.*

Catoferia was first described as a section of *Orthosiphon* (Bentham, 1848). Later, Bentham (1876) raised sect. *Catoferia* to generic rank. This genus, part of the *Ocimum* group, differs significantly in appearance to the group's other species in that the cyme branches are fused to the inflorescence axis, instead of spreading freely, has a capitate style apex with rounded lobes rather than subulate spreading style branches, and possessing a bent rather than straight embryo (Paton et al., 1999). These characters also separate it from the *Orthosiphon* group though in that case, the style apex is clavate with adpressed lobes (Paton et al., 1999). *Catoferia* is closely related to *Orthosiphon*, which it resembles greatly; this suggests a common origin for the two genera (Ramamoorthy, 1986). Paton et al. (2004) argue, *Catoferia* has a close relationship with *Platostoma* P. Beauv. This genus requires more study in order to clarify its relationship.

1.3.3 The *Orthosiphon* group

The *Orthosiphon* group contains *Orthosiphon*, *Hoslundia Fuerstia*, *Basilicum* and *Endostemon* (Paton et al., 1994). *Endostemon* can not be linked to a particular genus within the *Orthosiphon* group by a putative synapomorphy. The situation will become clear once the relationship between the *Orthosiphon* group is better to understood (Paton et al., 1994).

There are conflicts regarding the placement of all the species in *Nautochilus* Brem. Bremekamp (1933) typified his genus using *N. labiatus* (N. E. Br.) Brem. and described three more species, called *N. urticaefolius* Brem., *N. breyeri* Brem. and *N. amabilis* Brem. A review of the African species of *Orthosiphon* was carried out by Ashby (1938); however, the genus *Nautochilus* Brem was not assessed. Phillips (1951) imposed synonymy on *Nautochilus* under *Orthosiphon* without commenting on reasons. Regarding the description of Bremekamp (1933), *N. urticaefolius* and *N. breyeri* are now regarded as being forms of *N. labiatus* and *N. amabilis* forms a link with *Orthosiphon pseudoserratus* M. Ashby (Codd, 1964). *Nautochilus*, which comprises *Nautochilus labiatus* has similarities with *Orthosiphon* sect. *Serrati*, comprising *O. serratus* Schltr, *O. pseudoserratus* M. Ashby and *O. tubiformis* R. Good in two characters as noted by Codd (1964). First, the pair of upper filaments is attached close to the base of the corolla tube, with the lower part of the filaments a little swollen and pubescent. Second, the disk, which surrounds the ovary, and reaches the lower half or top of the ovary, is annular. On this basis, sect. *Serrati* Ashby would be included in *Nautochilus* Brem. and he preferred to place *Nautochilus* as a subgenus of *Orthosiphon* (Codd, 1964). Paton (1992) moved subgenus *Nautochilus* to the *Ocimum* group for many reasons: in both these taxa, the disk is equally four-lobed, whereas in the remainder of *Orthosiphon* the anterior lobe is usually larger than the others; the style is bifid as in *Ocimum*,

rather than clavate as in *Orthosiphon*; the posterior stamens are adnate near the base of the corolla tube and are basally ciliate, although the cilia are not concentrated into a basal clump as in *Ocimum* sect. *Hierocymum*, whereas the stamens in *Orthosiphon* usually attach around the middle of the tube and are glabrous. *Orthosiphon* subgenus *Nautochilus* shares many of the characters of *Ocimum lamiifolium* in particular (Paton et al., 1999). It seems that *Ocimum lamiifolium* has a close relationship with *Orthosiphon* subgenus *Nautochilus*. Furthermore, Paton et al. (1999) considered it problematic if *Orthosiphon* subgenus *Nautochilus* was recognised as having generic rank, because *Ocimum lamiifolium* has certain, but not all, shared characteristics with the group, but sect. *Hierocymum* has no phylogenetic support and is polyphyletic, based on parsimony analysis, with its members dispersed across a number of clades.

Ashby (1938) divided *Orthosiphon* into three sections: sect. *Serrati* M. Ashby, sect. *Orthosiphon* (*Euorthosiphon* M. Ashby) and sect. *Pallidi* M. Ashby. Ashby described sect. *Pallidi* for species with a supposedly funnel-shaped corolla, with the anterior corolla lip longer than the posterior. *Or. hanningtonii*, which has a funnel-shaped corolla and equal corolla lips, is an intermediate between sect. *Pallidi* and sect. *Orthosiphon*. Only *Or. hanningtonii* has a properly funnel-shaped corolla tube because the extended sides of the exserted part of the tube are not parallel. Therefore, in *Orthosiphon,* there seems to be a continuous variation in the tube shape between parallel-sided/dilating at the throat versus funnel-shaped on the other (Paton et al., 2009).

Paton (1992) suggested that *Orthosiphon* subgenus *Nautochilus* (now *Ocimum* subgenus *Nautochilus*) was closely related to *Ocimum* sect. *Hierocymum* and *Orthosiphon* subgenus *Orthosiphon* was closely related to *Basilicum*. Paton (1992) pointed out that as the delimitation of genera now stands, there is more diversity and larger discontinuities within genera than between them. Further research on *Orthosiphon* group is therefore needed.

Several vital points have been put forward by Paton (1993) which raised doubts of morphology on the generic delimitation of *Fuerstia* from *Orthosiphon*. There is a close relationship between *Orthosiphon* and *Fuerstia* as they have an identically shaped calyx and corolla. Within *Orthosiphon*, *Fuerstia* shows the greatest similarity to subgenus *Orthosiphon* that also exhibits a clavate style with small rounded lobes, a 4-lobed disk having the largest anterior lobe and posterior stamens. These are adnate to the corolla around the middle of the tube. In 1929, Fries separated *Fuerstia* from *Orthosiphon* on the basis of the fact that *Fuerstia* possesses sterile posterior stamens.

The number of fertile stamens is either two or four in flowers of the family Lamiaceae (El-Gazzar & Watson, 1970). The four stamens are often within Ocimeae but two sterile stamens are rarely e.g. in one species in *Plectranthus* (Codd, 1975) and *Ocimum* (Paton et al., 1999), however,

two sterile stamens are common in Mentheae **(**Drew & Sytsma, 2012), *Salvia* (Walker & Sytsma, 2006) and *Pogogyne* (Silveira, 2010). Paton (1993) suggested the *Fuerstia* and *Orthosiphon* should be in the same genus and even within the subgenus *Orthosiphon* because of the resemblance between them but at the time did not make formal changes. He argued against using two sterile posterior stamens as a feature to identify genera because of the number of fertile stamens is not consistent in Lamiaceae. This generic complex may also include *Hoslundia*. There are identical nutlets in this species (Ryding, 1992), as well as corolla, inflorescence structure, and sterile posterior stamens, in comparison with *Fuerstia*. However, *Hoslundia* has a fleshy mature calyx that distinguishes it from other genera. Evolutionary trends of the morphology of the androecium prior to its usage as the basis for classification must be understood.

The use of floral characters in generic delimitation in Lamiaceae is problematic. These have been used for classification since before Linnaeus in 1753; however, they do not always work, particularly in Lamiaceae. It has become clear that morphological classification alone is not successful in Lamiaceae. Work that explores molecular data in order to provide new insights into these complex taxonomic issues is now underway.

1.4 Morphological characters used in classification of genera within subtribe Ociminae

The characters important in generic level delimitation are two fertile stamens in *Fuerstia*, *Hoslundia* (Fries, 1929; Paton, 1993), and one species of *Ocimum* (*O. circinatum*)(Paton, 1992), calyx shape (Paton, 1992; Paton, 1993), style apex (Paton, 1992), shield-like (Paton et al., 1994), lobed disk (Paton et al., 1999) and posterior stamens attached to the corolla (Paton et al., 1999; Harley et al., 2004).

There are several morphological characteristics used in infrageneric classification of *Ocimum, Platostoma, Haumaniastrum* and *Endostemon* (Table 1.2), fruiting calyx (Paton et al., 1994), stamens attached by corolla tube (Paton, 1997b), posterior lobe of calyx (Paton et al., 1999) and corolla tube shape (Paton et al., 2009). However, *Orthosiphon* is not in the table because there is no single key to the species in this genus and no global study since Ashby (1938).

Table 1.2 Morphological characteristics used in infrageneric classification.

Genus	Subgenus	Section	Sub section	Reference
***Ocimum* L.**	***Ocimum*** Corolla tube usually gibbous at midpoint, dilating towards mouth; posterior lip regularly 4-lobed or with median lobes exceeding lateral lobes. Posterior stamens usually bent and appendiculate, very rarely straight and inappendiculate, basally pubescent.	***Ocimum*** Bracts persistent. Calyx throat open in fruit; throat with a hairy annulus; lateral lobes lanceolate, symmetrical. Pollen with angled muri of primary reticulum.		(Paton et al., 1999).
		Gratissima (Benth) A.J.Paton Bracts persistent. Calyx throat closed in fruit by median lobes of lower lip pressing towards upper lip; throat glabrous; lateral lobes asymmetric. Pollen with rounded muri of primary reticulum.	***Gratissima*** Benth. Posterior lobe of calyx not membranous, expanded or not. Median lobes of anterior lip of calyx denticulate. Nutlets spherical. Pollen with an ellipsoid polar outline with isodiametric lacunae formed by primary muri.	
			Erythrochlamys (Gürke) A.J.Paton Posterior lobe of calyx membranous, expanded. Median lobes of anterior lip of calyx lanceolate. Nutlets obovate. Pollen with a circular polar outline with elongate.	
		Hiantia (Benth.) A.J.Paton Bracts caducous, scar developing into an auxiliary nectary. Calyx throat open or laterally compressed in fruit; throat glabrous; lateral lobes asymmetrically lanceolate or truncate. Pollen with rounded or angled muri of primary reticulum.		
		Nudicaulia (Briq.) A.J.Paton Anthers rotund to reniform with divergent thecae. Pollen with ellipsoid polar outline and with rounded muri of primary reticulum.		

Table 1.2 cont. Morphological characteristics used in infrageneric classification.

Genus	Subgenus	Section	Sub section	Reference
***Ocimum* L.**	***Nautochilus*** (Bremek.) A.J.Paton Corolla tube gibbous or not at midpoint, parallel-sided distally, not dilating towards mouth; posterior lip with median lobes exceeding lateral lobes. Posterior stamen straight or basally bent, inappendiculate, basally pubescent.			(Paton et al., 1999).
	Gymnocimum (Benth.) A.J.Paton Corolla tube not gibbous at base, dilating towards mouth; posterior lip regularly 4-lobed. Posterior stamens straight, inappendiculate, glabrous or basally pubescent.	***Gymnocimum*** Benth. Posterior stamens glabrous. Anther thecae unequal.		
		Hierocymum Benth. Posterior stamens basally pubescent. Anther thecae equal.		
***Platostoma* P.Beauv.**	***Acrocephalus*** (Benth.) A.J.Paton Throat of calyx open, anterior lip 4 or 7-toothed with 2 or 5 median lobes, anterior lip calyx 4-lobbed.	***Acrocephalus*** (Benth.) A.J.Paton Nutlets apically glabrous.		(Paton, 1997b).
		Heterodonta (Briq.) A.J.Paton Nutlets apically pubescent or tuberculate.		
	Octomeron (Robyns) A.J.Paton Throat of calyx open, anterior lip 4 or 7-toothed with 2 or 5 median lobes, anterior lip calyx 7-lobbed			
	Platostoma (P. Beauv.) A.J.Paton Throat of fruiting calyx closed by median lobed of anterior lip 3-lobed with one median lobe, entire, denticulate or emarginate at apex.	***Platostoma*** Stamens attached near midpoint of corolla tube; corolla never spurred, nutlets apically rounded; posterior stamens never appendiculate, posterior lip of fruiting calyx rounded, decurrent		
		Ceratanthus (G. Taylor) A.J.Paton Stamens attached near base of corolla tube, corolla often spurred.		

Table 1.2 cont. Morphological characteristics used in infrageneric classification.

Genus	Subgenus	Section	Sub section	Reference
Platostoma **P.Beauv.**		***Mesona*** (Blume) A.J.Paton Stamens attached near midpoint of corolla tube; corolla never spurred, nutlets apically acute; posterior stamens often appendiculate.		(Paton, 1997b).
		Limniboza (R. E. Fr) A.J.Paton Stamens attached near midpoint of corolla tube; corolla never spurred, posterior lip of fruiting calyx acurrent.	***Limniboza*** (R. E. Fr) A.J.Paton Stamens spreading. ***Rotundifolia*** A.J.Paton Stamens declinate.	
Haumaniastrum **P.A.Duvign. & Plancke**		***Arborescentia*** A.J.Paton Plant with a perennial corky trunk terminating in a rosette of leaves from which are produced annual stems.		(Paton, 1997a).
		Botridiocephalum **(**Briq.) A.J.Paton Plant annual or perennial with a tap root or underground woody rootstock lacking a perennial trunk, with or without a rosette of leaves at ground level; plant with a basal rosette of leaves; fully mature calyx with entire anterior and posterior rounded lips anterior weakly emarginate.		
		Haumaniastrum Plant annual or perennial with a tap root or underground woody rootstock lacking a perennial trunk, with or without a rosette of leaves at ground level; plant with a basal rosette of leaves; fully mature calyx posterior lip usually 3-angled or 3-toothed, anterior lip emarginate or 2-toothed.		
		Caudata A.J.Paton Plant lacking basal rosette of leaves; upper leaves subtending heads caudate.		

Table 1.2 cont. Morphological characteristics used in infrageneric classification.

Genus	Subgenus	Section	Sub section	Reference
***Haumaniastrum* P.A.Duvign. & Plancke**		***Revoluta*** A.J.Paton Plant lacking basal rosette of leaves; upper leaves similar in shape to leaves below; leaf margin tightly revolute.		(Paton, 1997a).
		Dubia A.J.Paton Plant lacking basal rosette of leaves; upper leaves similar in shape to leaves below; leaf margin flat or weakly revolute; fully mature calyx posterior lip usually 3-toothe.		
***Endostemon* N.E.Br**		***Leucosphaeri*** **(**Briq.) A.J.Paton, Harley & M.M. Harley Fruiting calyx membranous, spherical, posterior lip with coiled side lobes; verticils 2-flowered.		(Paton et al., 1994).
		Diffusi **(**Briq.) M. Ashby Fruiting calyx thick-textured, tubular or tubular-campanulate, posterior accrescent, not forming coiled side lobes; verticils 2-6 flowered, fruiting and flowering calyx densely villous at throat.		
		Endostemon M. Ashby Fruiting calyx thick-textured, tubular or tubular-campanulate, posterior accrescent, not forming coiled side lobes; verticils 2-6 flowered, fruiting and flowering calyx glabrous at throat, throat of fruiting calyx open; anterior lip of fruiting calyx with 4 lanceolate lobes, median lobes slightly longer than the lateral; pedicels terete.		

Table 1.2 cont. Morphological characteristics used in infrageneric classification.

Genus	Subgenus	Section	Sub section	Reference
Endostemon **N.E.Br**		***Oblongi*** Ayob. ex A.J.Paton, Harley & M. M. Harley Fruiting calyx thick-textured, tubular or tubular-campanulate, posterior accrescent, not forming coiled side lobes; verticils 2-6 flowered, fruiting and flowering calyx glabrous at throat, throat of fruiting calyx closed by the anterior lip pressing against the posterior; anterior lip of fruiting calyx composed of linear, obliquely truncate to rounded lateral lobes and lanceolate median lobes; pedicels flattened.		(Paton et al., 1994).

1.5 DNA sequencing in taxonomy and its role in phylogenetic analysis

DNA sequences can play a significant part in systematics. Phylogenetic analysis of DNA sequence data can offer insights into generic delimitation and allows researchers to obtain almost limitless possibilities for relationship resolution (Goodman et al., 1987; Sibley & Ahlquist, 1987; Goodman, 1989). DNA sequencing could frequently add to the required information for deciding a species boundary (Meier, 2008). The variation in information provided by a DNA sequence, which occurs either in the nucleus of the cell, or in organelles such as mitochondria or chloroplasts, is derived from mutations, which are slowly but constantly accumulating in lineages of organisms over time. This has had a major effect on accelerating taxonomic approaches away from being simply descriptive and towards a more evolutionary, or phylogenetic approach (Harley, 2009). Thus, the DNA sequences are often rich in information. Sequences of different gene markers from the same organisms can be concatenated to produce a phylogenetic tree from more than one dataset. Phylogenies can then be constructed using the primary techniques of distance, parsimony, likelihood, and Bayesian analysis (Harrison & Langdale, 2006). Thus, there are many methods to infer the phylogenetic tree.

DNA sequences have significant differences from traditional morphological data and have become an important focus of research. Both systematic and evolutionary biologists have employed molecular biology techniques alongside anatomical and morphological investigations into problems regarding plant group relationships and ancestry (Miyamoto & Cracraft, 1991). As molecular phylogenetic data provided valuable information, angiosperms were the first significant group of organisms to undergo higher level reclassification largely on the basis of molecular data (APG, 1998; APG, 2003; APG, 2009; APG, 2016). To sum up, DNA sequences have led to profound modifications in the classification of plants and they are important sources of data for phylogenetic analysis.

1.6 Phylogeny of Lamiaceae

In recent times a large-scale high-level phylogenetic analysis has seen the division of Lamiaceae into twelve subfamilies (Li et al., 2016), based on five chloroplast DNA markers (*mat*K, *ndh*F, *rbc*L, *rps*16, and *trn*L-F). The Nepetoideae are clearly distinguished from members of the subfamily Lamioideae using *rbc*L (Kaufmann & Wink, 1994). The subfamily Nepetoideae is monophyletic as reported by Wagstaff et al. (1995) used cpDNA, by Wagstaff & Olmstead (1997) used *rbc*L sequences and by Wagstaff et al. (1998) used sequences of *rbc*L and *ndh*F.

Nepetoideae includes three tribes (Elsholtzieae, Mentheae and Ocimeae) (Harley et al., 2004). A number of molecular studies have been conducted within these tribes. The tribe Elsholtzieae is monophyletic based on a combined *ndh*F and *rbc*L dataset (Chen et al., 2016) and based on a five chloroplast (*rbc*L, *mat*K, *trn*L-F, ycf1 and *ycf*1-*rps*15) and two nuclear (ETS and ITS) dataset (Li et al., 2017).

There have been a number of phylogenetic studies of Mentheae. *Monarda* L. based on the internal transcribed spacer (ITS) region (Prather et al., 2002), *Mentha* L. based on DNA sequences of the chloroplast (cp) *rpl*16 intron and *trn*L-*trn*F region (Bunsawat et al., 2004), *Bystropogon* L'Her. based on sequences of ITS, of the nuclear ribosomal DNA, and of the *trn*L gene and *trn*L - *trn*F intergenic spacer of the chloroplast DNA (Trusty et al., 2004 & 2005), *Salvia* L. using the chloroplast DNA regions *rbc*L and *trn*L-F (Walker et al., 2004), *Micromeria* used *trn*K intron sequence (Bräuchler et al., 2005), *Salvia* used the chloroplast *trn*L-F and the nuclear nrDNA ITS regions (Walker & Sytsma, 2006), *Conradina* A. Gray used sequencing ITS and plastid regions (Edwards et al., 2006; Edwards et al., 2008), *Killickia* (Bräeuchler et al., 2008), *Minthostachys* used nrITS data (Schmidt-Lebuhn, 2008), Menthinae based on chloroplast: *trn*K intron; *trn*L-F; nuclear: ITS (Bräuchler et al., 2010), *Pogogyne* based on chloroplast DNA *trn*Q-*rps*16 and nuclear DNA ITS and ETS (Silveira, 2010), *Lepechinia* based on cpDNA (*ycf*1 and *trn*L-F) and nrDNA (ITS and ETS) markers (Drew & Sytsma, 2011), tribe Mentheae based on cpDNA regions (*ycf*1, *ycf*1-*rps*15 spacer, *trn*L-F, and *rp*l32-*trn*L [UAG]) and nrDNA regions (ITS and ETS) (Drew & Sytsma, 2012), Mentheae based on *ycf*1, ITS and ETS region (Ryding, 2010), *Salvia* based on nuclear ribosomal DNA (ITS and ETS) and plastid DNA (*ycf*1-*rps*15 spacer, *rbc*L, and *trn*L-F) sequences (Takano, 2017), *Salvia* based on ITS region and plastid *psb*A–*trn*H intergenic spacer (Fragoso-Martínez et al., 2018), *Salvia* based on two nuclear (ITS and ETS) and four chloroplast (*psb*A–*trn*H, *ycf*1–*rps15*, *trn*L–*trn*F and *rbcL*) DNA markers (Hu et al., 2018), and *Lallemantia* using nuclear (ITS) and plastid (*trn*L, *trn*L-F, *trn*S-G, *rpl*32, and *rpl*32-*trn*L) DNA sequences (Kamrani & Riahi, 2018).

A number of molecular studies have been conducted within the tribe Ocimeae. *Lavandula* L. based on sequencing of the nuclear nrDNA (ITS 1 and 2) (Upson, 1997). Ocimeae based on sequences of the *trn*L intron, *trn*L-*trn*F intergenic spacer and *rps*16 intron of plastid DNA (Paton et al., 2004). *Plectranthus* group based on chloroplast markers *trn*L intron, *trn*L 3' exon, *trn*L-*trn*F intergenic spacer; *rps*l6 intron, and *trn*S-*trn*G intergenic spacer (Mwanyambo, 2008), *Isodon* using sequence variations in 11 chloroplast DNA regions (Maki, et al., 2010), *Isodon* using the nuclear ribosomal internal transcribed spacer (nrITS), cpDNA regions (*trn*L-*trn*F region and *rps*16 intron) (Zhong et al., 2010), subtribe Hyptidinae based on nuclear ITS and ETS, and plastid *trn*L-F, *trn*S-G, *trn*D-T, and *mat*K (Pastore et al., 2011), *Isodon* using sequences of three plastid markers, the nuclear ribosomal internal transcribed spacer (nrITS), and a low-copy nuclear gene (*LEAFY* intron II) (Yu et al., 2014). The genus *Lavandula* L. based on plastid *trn*K and *mat*K genes (Moja et al., 2016), *Plectranthus* based on the *mat*K and *rbc*L gene sequences (Musila et al., 2017), *Plectranthus, Coleus* and allies based on *rps*16, *trn*L-F and *trn*S-G regions of the plastid genome (Paton et al., 2018).

Within subtribe Ociminae, *Hemizygia* and *Syncolostemon* Otieno et al. (2006) used sequences of the *trn*L intron, *trn*L-*trn*F intergenic spacer and the *rps*16 intron, *Ocimum* L. based on noncoding *psb*A-*trn*H intergenic region (Kumar et al., 2016a).

Phylogenetic studies have been employed to determine generic delimitation in the Lamiaceae by many authors, resulting in significant revision within its constituent taxa. The genera *Wrixonia* and *Prostanthera* have been distinguished on androecial morphology (Carrick, 1976) and Conn (1992) are considered that *Wrixonia* was closely related to *Prostanthera*, while Cantino, (1992) considered it to be included within *Prostanthera*. Phylogenetic study based on chloroplast DNA sequence (*trn*T–F and *ndh*F–*rpl*32) and nuclear (ETS) sequence showed *Prostanthera* to be paraphyletic to *Wrixonia*, and suggested that *Wrixonia* is synonymous with *Prostanthera* (Wilson et al., 2012).

There are serious doubts on the generic delimitation in *Fuerstia*, *Hoslundia* and *Orthosiphon*. The genera *Fuerstia, Hoslundia* and *Orthosiphon* are closely linked but differ in their stamen number, *Fuerstia* and *Hoslundia* have two fertile stamens but *Orthosiphon* has four fertile stamens. Paton (1993) suggested the *Fuerstia* and *Orthosiphon* should be in the same genus, however, the importance of the staminal character as the only feature delimiting genera comes into doubt. Another issue also the position of *Nautochilus* which has been placed in *Orthosiphon* previously by Codd (1964) but Paton (1992) moved this taxon into the *Ocimum* based on morphological grounds. Therefore, phylogenetic study will investigate this issue.

1.7 Mapping morphological and geographic characters using molecular phylogeny

Mapping characters on phylogenies plays a significant part in the comprehension of molecular and morphological evolution (Felsenstein, 1985; Brooks & McLennan, 1991), and biogeographical history (Roncal et al., 2012). Hypotheses regarding morphological trait evolution have been tested with success (Huelsenbeck et al., 2003; Renner et al., 2007). This methodology involves the reconstruction of historical trait changes onto a phylogenetic tree that relates species possessing those traits (Irvahn & Minin, 2014). Moreover, for reconstruction of the taxon, molecular phylogenies and species distribution data must be used (Roncal et al., 2012). Thus this technique has become central to the analysis of evolutionary biology.

Tribe Ocimeae is a well supported monophyletic group with a morphological synapomorphy of dorsifixed, synthecous anthers, but the declinate stamens are not a synapomorphy for this tribe when mapping morphological characters on phylogenetic tree of Ocimeae (Paton et al., 2004). This paper also found an Asiatic origin for Ocimeae by mapping geographic distribution on to a phylogenetic tree. Within the tribe Mentheae there have been a minimum of four transitions between four and two stamens, this occurs a minimum of two times within the subtribe Menthinae, and once each in the subtribes Salviinae and Lycopinae, when using BayesTraits for the inference of the evolution of stamina within the tribe **(**Drew & Sytsma, 2012). North-Central India is the geographical origin of *Ocimum tenuiflorum* (Bast et al., 2014).

1.8 DNA barcoding and species delimitation

DNA barcoding aims to find a single sequence to identify all species (Li et al., 2015). Barcoding for animal life identified that the cytochrome c oxidase subunit 1 (COI) region of mitochondrial DNA has enabled discrimination of some closely allied species in all animal phyla (Hebert et al., 2003b). *matK* and *rbc*L were accepted as a two-locus DNA barcode to classify plant species and *mat*K region of plastid DNA has shown success in plant species discrimination, though it often requires additional identifying regions to enable definite identification (Group et al., 2009). The objective cannot be achieved by any single-locus barcode because of the inadequate variation and low PCR efficiency. In addition, it is presumed that multi-locus markers are more successful in determining species; however, they are not appropriate for universal plant identification (Li et al., 2015). Li et al. (2015) reported that "different research groups have tested different combinations using different taxa while attempting to select a universal barcode, however, universal agreement is yet to be reached". However, Fazekas et al. (2008) compared these barcode combinations using the same large-scale taxonomic samples, but none could identify more than 70% of tested species.

A lot of potential has been demonstrated by whole-plastid-based barcodes in the discrimination of species, particularly for the taxa that are closely linked (Li et al., 2015). Nonetheless, there is a lack of acceptance for this concept at the global level due to the extensive costs of sequencing and the difficulties that involved in acquiring complete plastid genome as compared to using the single-locus barcodes (Li et al., 2015). However, in 2016 Coissac et al. argued that it is capable of surmounting the limitations of some traditional barcodes because of the lack of a PCR phase, suggesting that the sequence data can be recovered when the herbarium specimens containing degraded DNA are used, increasing the possibility of restoring data from type specimens.

DNA barcoding is the first step checking species delimitation *Orthosiphon* especially as there is no single key to the species in this genus and no global study since Ashby (1938). *Orthosiphon rubicundus* from Asia is morphologically very close to the African *Orthosiphon schimperi* and *Orthosiphon thymiflorus* morphology (Paton et al., 2009). Therefore, DNA barcoding is used here to test the delimitation of these taxa.

1.9 Aims and objectives

There has been no molecular phylogeny focusing on the Ociminae except some genera such as *Syncolostemon* (Otieno et al., 2006), and *Ocimum* (Kumar et al., 2016a). Therefore, the relationships within Ociminae are still in question. The medicinal uses of these plants support the case of revision the subtribe Ociminae. This will, therefore, be the focus of the thesis.

The aims of this thesis are first, to provide the first phylogenetic tree of the subtribe Ociminae based on four plastid DNA regions (*trn*L intron, *trn*L–*trn*F intergenic spacer, *trn*H-*psb*A intergenic spacer, *mat*K and *rps*16 intron) and two nuclear DNA regions (ITS and ETS) to evaluate the monophyly of the subtribe; second, to use phylogeny to investigate the distribution of morphological characters and geographical distribution of the clades and third, to evaluate the utility of DNA barcoding in species delimitation of *Orthosiphon*.

The objectives

1. To develop a phylogenetic hypothesis for subtribe Ociminae.
2. To investigate morphological character changes in the context of phylogeny.
3. To investigate geographical distribution in the context of phylogeny.
4. To examine the degree of matching of the molecular data from this study with the existing morphological classifications based on the vegetative (leaf) and floral characters and propose changes to the classification of the group if not so.
5. Develop barcode-based species identification tools for *Orthosiphon*.

Chapter 2 : Molecular phylogeny of cpDNA regions

2.1 Introduction

2.1.1 Lamiaceae molecular phylogeny

There has been an increasing number of phylogenetic studies of Nepetoideae, particularly Mentheae this century. All of the research into Mentheae was limited to particular genera, including *Bystropogon* L'Her. (Trusty et al., 2004 & 2005), *Conradina* A. Gray (Edwards et al., 2006; Edwards et al., 2008), *Mentha* L. (Bunsawat et al., 2004), *Micromeria* (Bräuchler et al., 2005), *Minthostachys* (Schmidt-Lebuhn, 2008), *Monarda* L. (Prather et al., 2002), *Salvia* L. (Walker et al., 2004; Walker & Sytsma, 2006) and *Pogogyne* (Silveira, 2010). Ryding (2010) and Drew and Sytsma (2012) claimed, in relation to these researches, that various genera have either not been placed or are misplaced in the current classification.

Few taxonomic studies have been published on the phylogenetics of tribe Ocimeae, but the phylogenetic position of the tribe Ocimeae has long been controversial. Paton et al. (2004) proposed a new classification system using molecular data after he published a phylogeny of this tribe based on sequences of the *trn*L intron, *trn*L-*trn*F intergenic spacer and *rps*16 intron of plastid DNA. The study led to a better understanding of the relationships among members of the tribe Ocimeae. However, some relationships were still unresolved, including those between Isodoninae, Hyptidinae, Plectranthinae and Ociminae. According to Paton et al. (2004), Ociminae and Plectranthinae are both demonstrably monophyletic; Plectranthinae is a sister of the subtribe Ociminae that includes *Ocimum* L, but its relationships to the other subtribes is unresolved. Mwanyambo (2008) supported monophyly for both the Plectranthinae and *Coleus* clades while support is variable for the *Plectranthus* group based on chloroplast markers *trn*L intron, *trn*L 3' exon, *trn*L-*trn*F intergenic spacer; *rps*l6 intron, and *trn*S-*trn*G intergenic spacer. However, based on a recent study by Paton et al. (2018) *Plectranthus* has been redefined to be a smaller monophyletic group, a clade that contains the *Coleus* type and that includes *Solenostemon*, *Pycnostachys* and *Anisochilus* is sister to others in the group on the basis of the *rps*16, *trn*L-F and *trn*S-G regions of the plastid genome.

Isodon did not obviously belong to any of the existing subtribes according to Paton et al. (2004) on the basis of sampling of five *Isodon* species, however, Harley et al., (2003 & 2004) classified *Isodon* as belonging to the subtribe Hanceolinae, along with *Hanceola* and *Siphocranion.* Zhong et al. (2010) resolved this issue by suggesting two new subtribes: Isodoninae J. S. Zhong, J. Li, & H.W. Li, and Siphocranioninae J. S. Zhong, J. Li, & H.W. Li. and suggested there is a close relationship between *Hanceola* and *Hyptis* based on evidence from combined nrITS and chloroplast DNA *trn*L–*trn*F and rps16. However, the relationships of *Isodon* within tribe Ocimeae

remain unresolved, with the correct placement of subtribe Isodoninae uncertain related to the Ociminae + Plectranthinae and *Hanceola* + *Hyptis* clades, therefore, more research is needed for Ociminae require a larger number of samples to resolve generic/subtribal relationships as well as species evolution within *Isodon* (Zhong et al., 2010).

The phylogenetics of the subtribe Hyptidinae, based on nrITS and nrETS, and plastid *trn*L–F, *trn*S–G, *trn*D–T and *mat*K, were investigated by Pastore et al. (2011) who also explored the relationships among the internal groups of Hyptidinae, Nevertheless, the internal relationship within *Hyptis* was not completely resolved. A significant genus-level revision of Hyptidinae was undertaken by Harley and Pastore (2012) which recognised 12 genera previously unrecognised by Harley et al. (2004).

According to Ociminae, there is still an unresolved relationship between *Ocimum* and *Nautochilus* (Paton et al., 2004), the current recognition of *Ocimum* is polyphyletic (Paton, 1997b). By separately recognising the monophyletic subgenus *Nautochilus* and the monophyletic remainder of *Ocimum* we produce a pair of monophyletic groups (Paton et al, 2004). Al-Qassabi (2014) reported that *Ocimum* can be divided into three subclades based on the cp DNA (*trnL-F* and *rps* 16) gene sequence. One of them contains *O. serratum* and *O. labiatum,* the other contains *O. lamiifolium, O. masaiense* and *O. pseudoserratum,* and the third subclade includes the rest of the *Ocimum* genus. This study recommended placing *Nautochilus* into a new generic account because of differences between it and the Asian clade.

Regarding *Orthosiphon,* Paton et al. (2004) stated: "*Orthosiphon* is monophyletic with the inclusion of *Fuerstia* and *Hoslundia*, but the *Orthosiphon* clade lacks BS (bootstrap support <50%) and clear non-molecular synapomorphies. It may be better to merge these taxa under the earliest name *Hoslundia* Vahl. However, as this will cause many changes in existing nomenclature, we prefer to wait until further evidence for this relationship is available". Until now no such research has been carried out. This may be because of the difficulty in DNA extraction of this group (Harley & Paton, 2012).

The relationships between species of *Platostoma* were unresolved by Paton et al. (2004) who suggested that *Platostoma* is monophyletic with the inclusion of *Basilicum* and *Haumaniastrum*, although *Basilicum* was put in the ''*Orthosiphon* Group'' group by Ryding (1993), therefore, further work is also required on these genera. *Haumaniastrum* is more closely linked to *Platostoma* sect. *Limniboza* rather than *Acrocephalus* (Paton, 1997b). *Catoferia* is related to *Platostoma* P. Beauv (Paton et al., 2004). Paton (1997b) reported that *Benguellia* is considered more closely related to *Orthosiphon* than to the *Platostoma* group. Ryding et al. (2003) merged *Puntia* into *Endostemon*, this was also confirmed by Paton et al. (2004), when they suggested that there is a

close relationship between the two. It is clear that molecular characters support the morphological study.

Paton et al. (2004) reported that *Syncolostemon* is monophyletic only with the inclusion of *Hemizygia* but the relationships within Ociminae were not supported by bootstrap values over 50% because Ryding (1992) placed *Hemizygia*, *Syncolostemon* and *Ocimum* in his "*Ocimum* group" of the subtribe Ociminae and in a parsimony analysis of *Ocimum* and its close allies. Otieno et al. (2006) grade them as *Syncolostemon*, when they used molecular sequences of the *trn*L intron, *trn*L–*trn*F intergenic spacer and the *rps*16 intron giving strong support for *Syncolostemon* and *Hemizygia* as only one monophyletic genus, neither *Hemizygia* nor *Syncolostemon* is monophyletic, therefore, they formally merged the genera under the earliest name *Syncolostemon*. Moreover, Otieno et al. (2006) suggested the monophyly of *S. macranthus S. parviflorus S. argenteus* and *S. concinnus* with 89% bootstrap support (BS). However, the relationships between *S. canescens, Hemizygia petrensis* and *H. linearis* are unresolved and the three emerge in a clade with only 51% BS. Therefore, their relationships require more study.

The tribe Ocimeae is still poorly known and relationships within this tribe are still in question. No report has been published since Paton et al. (2004) on the phylogenetics of the tribe Ocimeae or to update the phylogenetic tree after local changes were described by Otieno et al. (2006) for *Hemizygia* and *Syncolostemon*; by Zhong et al. (2010) for *Isodon;* and by Pastore et al. (2011) for Hyptidinae. Thus, there is no current phylogenetic tree for Ocimeae.

Although the study by Paton et al. (2004) provided a skeleton phylogenetic tree of the tribe the Ocimeae, it left relationships between some genera and species unresolved. For example, as shown in Figure 2.1, the relationships among *Hoslundia*, *Fuerstia,* and *Orthosiphon* require further study because the markers used by Paton et al. (2004) were not sufficiently variable. *Hoslundia*, *Fuerstia*, and *Orthosiphon* were included in the same clade of the maximum parsimony tree of a consensus tree, but the *Orthosiphon* clade lacks adequate bootstrap support (BS <50%) (Table 2.1) and uses successive weighting, an approach whose validity has since been questioned. Moreover, the relationship between *Ocimum* and *Nautochilus* in the equally weighted analysis is unresolved. Because of this, more phylogenetic studies of the tribe Ocimeae and subtribe Ociminae are required, in order to investigate and explore the relationships between some genera.

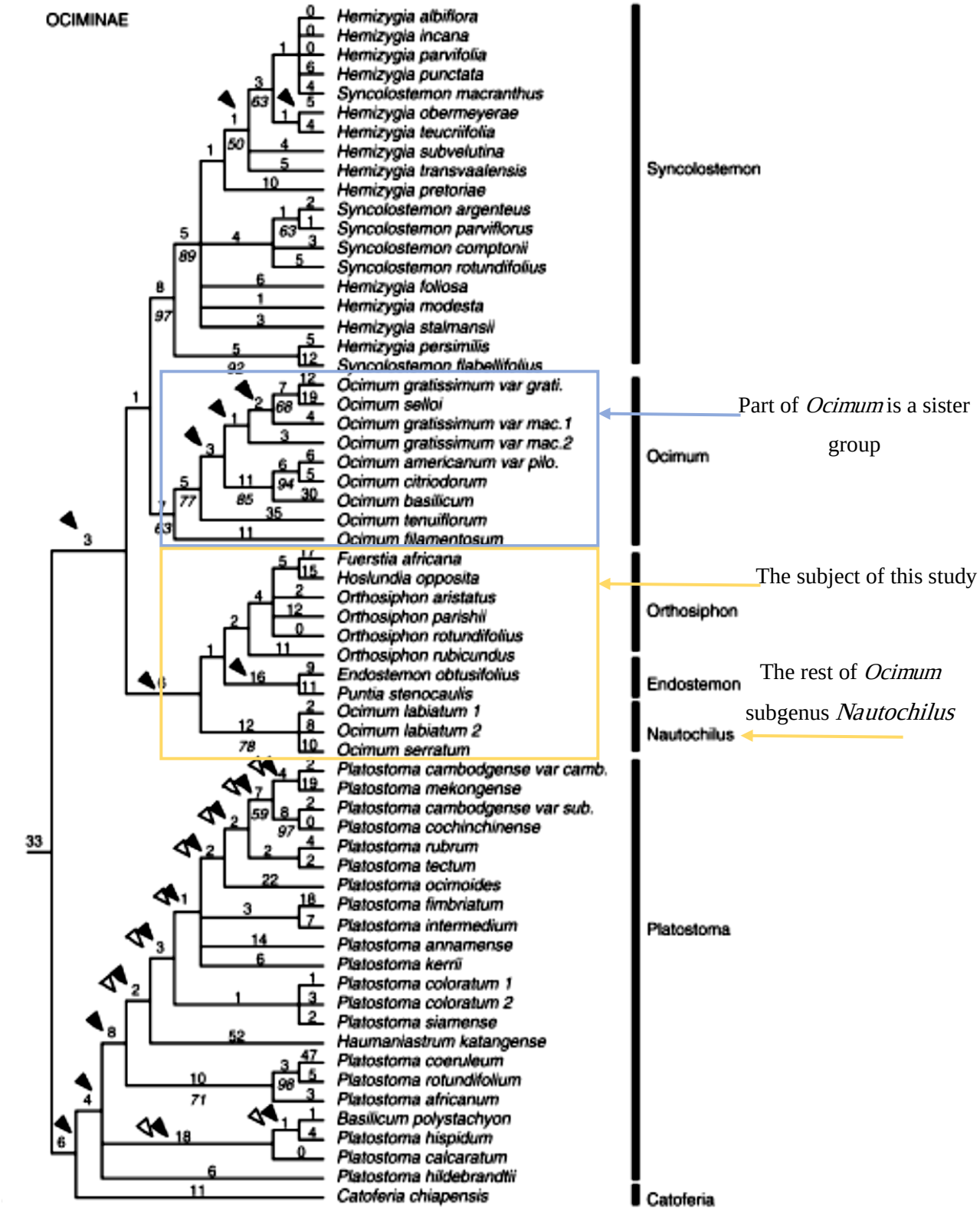


Figure 2.1 The most parsimonious tree after successive weighting of molecular data, showing branch length above branches and bootstrap percentages below branches, italicised, equal weights. Arrows indicate branches collapsing in the strict consensus trees with successive (unfilled arrows) and equal (black arrows) weights (Paton et al., 2004).

Table 2.1 The percentage of maximum parsimony and posterior probability in the Ocimeae phylogenetic tree (Paton et al., 2004).

Genus	Bootstrap support equally weighted maximum parsimony analysis, gaps included (%)	Bayesian posterior probability
Lavandula	100	0.53
Isodon	96	0.35
Hyptidinae	89	0.84
Plectranthinae	92	0.43
Syncolostemon+Hemizygia	97	-
Ocimum	63	0.95
Ocimum subgenus Nautochilus	78	0.46
Orthosiphon + Fuerstia + Hoslundia	<50	0.48 0.61
Pycnostachys, Holostylon, Anisochilus* & species *Plectranthus	97	-
Pycnostachys	96	0.97
Plectranthus + Tetradenia, Thorncroftia and Aeollanthus	<50	0.88 0.97 0.99 0.88
Tetradenia + Thorncroftia	100	0.97/0.99
Capitanopsis and Dauphinea	76	0.88

2.1.2 Selection of DNA regions for phylogenetic study

Schäferhoff et al. (2010) showed that rapid evolution of the *trn*K/*mat*K, *trn*L-F, and *rps*16 chloroplast regions permit the inference of accurate phylogenetic hypotheses for the Lamiales. Plastid genes, such as the *trn*L intron, *trn*L-*trn*F intergenic spacer, *trn*H–*psb*A intergenic spacer, *rps*16 intron and coding genes as such as MaturaseK (*mat*K) have been widely used in phylogenetic studies in Lamiaceae.

The plastid *mat*K region was used by Bramley (2009) for *Callicarpa* L.; it was used by Pastore et al. (2011) for subtribe Hyptidinae; Yao et al. (2016) used it for *Pogostemon* sl; and Li et al. (2017) for Elsholtzieae. The *trn*L-F region, together with *rps*16 were used by Paton et al. (2004) for tribe Ocimeae; Otieno et al. (2006) used it for *Hemizygia* and *Syncolostemon*; Zhong et al. (2010) used it for *Isodon* and allies; Bendiksby et al. (2011) for the subfamily Lamioideae; Xiang et al. (2013) for *Chelonopsis*; Chen et al. (2014) for *Holocheila*; Salmaki et al. (2015) for *Stachys persepolitana*; Berumen Cornejo et al. (2017) for *Stachys coccinea;* and Li et al. (2017) for *Heterolamium*. The *trn*H–*psb*A were used by Jenks et al. (2013) in *Salvia* subgenus *Calosphace*; by Xiang et al. (2013) for *Chelonopsis*; by Krawczyk et al. (2013) for *Galeobdolon luteum*; by Yao et al. (2016) for *Pogostemon* sl; and by Hu et al. (2018) for *Salvia*. For this study, four regions from chloroplast DNA (cpDNA) *trn*L–*trn*F, *trn*H–*psb*A, *rps*16 and *mat*K were selected.

2.2 Aims and objectives

The main aim in this chapter is to determine the generic delimitation of, and phylogenetic relationships among, the genera *Orthosiphon, Fuerstia, Hoslundia, Ocimum* and *Nautochilus* using cpDNA evidence (*trn*L intron, *trn*L-*trn*F intergenic spacer, *trn*H–*psb*A intergenic spacer, *rps*16 intron and *mat*K).

The objectives

1. To test if the subtribe Ociminae is a monophyletic group.
2. To test if *Orthosiphon, Ocimum,* and *Fuerstia* are monophyletic groups.
3. To investigate the relationship among *Orthosiphon, Fuerstia, Hoslundia, Ocimum,* and subgenus *Nautochilus.*

2.3 Materials and methods

2.3.1 Plant materials

Ninety-nine samples were used in this study, 95 samples from 131 species of the subtribe Ociminae collected in the Herbarium at the Royal Botanical Gardens Kew (The Herbarium Catalogue, 2016) (Appendix 1), some specimens in Figure (2.2), excluding three samples (*Hoslundia opposita*, *Orthosiphon serratus* and *Orthosiphon pallidus*), were from the Herbarium at the University of Reading (RNG). The samples were collected from Kew and RNG because most of the samples could be found there. The chosen samples were validated by the researcher and considered to be in a suitable condition as they had green leaves, however, this is sometimes difficult in some specimens because of their age range from 2 to 119 years (Appendix 1). Other sequences were taken from the NCBI-Genbank (Table 2.2), which provided close matches to the sequence of this study in sequence databases by using a BLAST search. Four species of *Plectranthus* were selected as an outgroup for analyses from the DNA Bank of the Royal Botanic Gardens, Kew (Table 2.3).

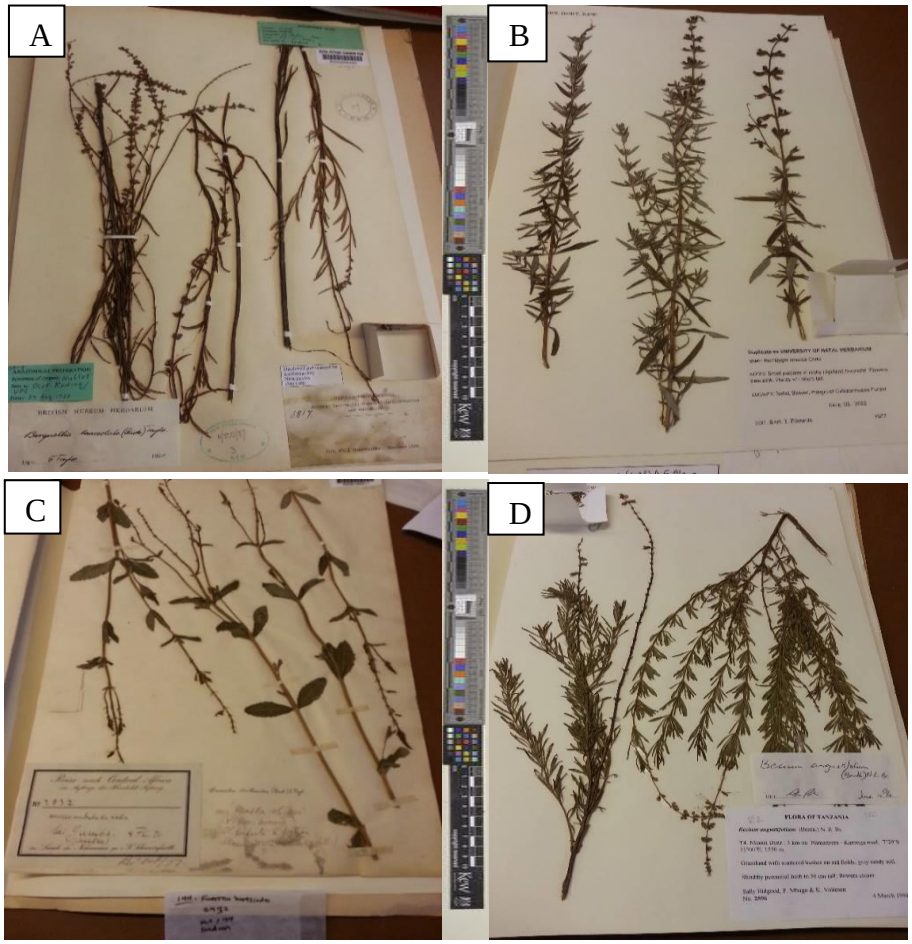


Figure 2.2 Pictures of herbarium specimens representing some samples in Appendix 1. A. *Benguellia lanceolata* (27) B. *Syncolostemon cinereum* (49) C. *Fuerstia bartsioides* (65) D. *Ocimum angustifolium* (81).

Table 2.2 List of accession numbers for plant samples from GenBank/EMBL with voucher information.

Species	Genbank accession	Region	Voucher specimens
***Basilicum polystachyon* (L.) Moench**	AJ505439.1	*trn*L–*trn*F intergenic spacer	Greenway & Kanuri 14501 (K).
***Hoslundia opposita* Vahl**	AJ505551.1	*trn*L–*trn*F intergenic spacer	Edwards & Tewolde-Berhan G-E, 3667 (K).
***Ocimum basilicum* L.**	AJ505351.1	*rps*16 intron	Cult., Suddee at al 894 (BKF, K).
***Ocimum labiatum* (N. E. Br.) A.J.Paton**	AJ505471.1	*trn*L–*trn*F	Balkwill et al 10848 (K).
	AJ505356.1	*rps*16 intron	Balkwill et al 10848 (J, K).
***Ocimum serratum* (Schltr.) A.J.Paton**	AJ505357.1	*rps*16 intron	Balkwill et al 10861 (J, K).
***Orthosiphon aristatus* (Blume) Miq.**	AJ505474.1	*trn*L–*trn*F	Suddee et al 969 (BKF, K, TCD).
	JF357830.1	*mat*K	S Suddee 969(K)
***Plectranthus nitidus* P.I.Forst.**	KU564713.1	*trn*H-*psb*A intergenic spacer	Howard 300201114
***Plectranthus parishii* Hook.f.**	AJ505390.1	*rps*16 intron	Suddee 1144 (BKF, K, TCD).
***Plectranthus laxiflorus* Benth.**	AJ505389.1	*rps*16 intron	Edwards s.n. (K).
***Plectranthus petiolaris* E.Mey.**	AJ505391.1	*rps*16 intron	Cult., K-1996-2729, U of Natal (K).

Table 2.3 Samples of outgroup from the DNA and Tissue Bank at Kew Science (2018).

ID	Taxon	Country	Collector	Collection date	Collector no.	Extraction date
22695	*Plectranthus bracteolatus* A.J.Paton	Tanzania	Bridson, D.	08/09/1984	640	2005-09-29
19766	*Plectranthus hadiensis* (Forssk.) Schweinf. ex Sprenger	Zimbabwe	Chase, N.C.	-	8548	2004-07-20
19755	*Plectranthus masukensis* Baker	Tanzania	Richards, H.M.	-	11159B	2004-07-20
19769	*Plectranthus triangularis* A.J.Paton	Tanzania	Mlangwa, J.A.	-	631, TaxonomicNote: Plectranthus sp., P. sp. aff. seretii (De Wild.) Vollesen	2004-07-20

2.3.2 DNA extraction

DNA was extracted from 0.01-0.02g dry weight of each specimen. Herbarium specimens were extracted using the CTAB method of Doyle & Doyle (1990), without modification and then with modification using different isopropanol precipitation times. Replicated extraction were made for each specimen with isopropanol precipitation time of one week and three weeks. Finally, the extraction of modified (CTAB) method, (Doyle & Doyle, 1990), with isopropanol precipitation of three weeks was cleaned up the DNA using the DNeasy® Plant Mini Kit (Qiagen) (Appendix 2).

The concentration of extracted DNA was determined using the NanoDrop Lite spectrophotometer. The quality of DNA was checked on 0.7% agarose gel stained with ethidium bromide (in 2015) and GelRed (Biotium) (from 2016–2018), during electrophoresis with a field strength of $5VCm^{-1}$. The gel was run for 50 minutes. The size of the DNA fragments was determined using HyperLadder ™ 1kb as a marker.

2.3.3 Polymerase chain reaction (PCR)

2.3.3.1 Primers

Herbarium specimens were amplified using the primers in Table 2.4. Amplification was difficult for some herbarium specimens using *trn*L–*trn*F primers c and f, which were eventually amplified using primer pairs c and d, and e and f, as described by Taberlet et al. (1991).

Table 2.4 Primers used in this study.

Locus	**Primer name**	**Primer sequence (5´– 3´)**	**Author**
***trn*L intron, *trn*L-*trn*F intergenic spacer**	c	CGAAATCGGTAGACGCTACG	Taberlet et al., 1991
	d	GGGGATAGAGGGACTTGAAC	Taberlet et al., 1991
	e	GGTTCAAGTCCCTCTATCCC	Taberlet et al., 1991
	f	ATTTGAACTGGTGACACGAG	Taberlet et al., 1991
***trn*H-*psb*A intergenic spacer**	*trn*H	CGCGCATGGTGGATTCACAATCC	Steven & Subramanyam, 2009
	*psb*A3'f	GTTATGCATGAACGTAATGCTC	Steven & Subramanyam, 2009
***mat*K**	*mat*K472F	CCC RTY CAT CTG GAA ATC TTG GTT C	Yu et al., 2011
	*mat*K1248R	GCT RTR ATA ATG AGA AAG ATT TCT GC	Yu et al., 2011
***rps*16 intron**	*rps*F	GTGGTAGAAAGCAACGTGCGACTT)	Oxelman et al.,1997
	rps2R	TCGGGATCGAACATCAATTGCAAC	Oxelman et al.,1997

2.3.3.2 PCR amplification, conditions, programs, purification and sequencing

PCR reactions were performed in 30µ1 volumes containing final concentrations of lx Bioline Biomix, 0.35µM of each primer, 0.13mg/ml BSA (bovine serum albumin), 2.67% v/v DMSO (dimethyl sulfoxide), 1.5–2.0 mM $MgCl_2$ and 10-50 ng DNA template. The successful cycling conditions for amplifying four regions (*trn*L–*trn*F, *mat*K, *trn*H–*psb*A and *rps*16) of DNA were described in Table 2.5 after modified (Zhong et al., 2010; Pastore et al., 2011). PCR products were separated on 1% agarose gel in 1x TAE buffer stained by ethidium bromide (in 2015) and it was changed to GelRed (Biotium) (from 2016–2018) because it is less toxic. Gels were illuminated with UV light and photographs were taken to record the presence of PCR amplicons. Approximate size and concentration of the PCR amplicons was determined using HyperLadder ™ 1kb (Bioline Reagents Ltd., London, UK). Direct sequencing of PCR products was carried out by–GATC Biotech (Konstanz, Germany) in 2015 and Eurofins (Germany) in 2016-2018.

Table 2.5 The list of optimized PCR programs used to amplify and produce PCR products.

The region	**Initial DNA denaturation**	**Cycles**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
***trn*L–*trn*F**	94 °C for 5 minute	40	94 °C 30 seconds	57 °C 30 seconds	72 °C 1.5 minute	72 °C 8 minute
***trn*H-*psb*Af**	94 °C 4 minutes	40	94 °C 30 seconds	54 °C 1 minute	72 °C 1 minute	72 °C 7 minutes
***mat*K**	94 °C 3 minutes	40	94 °C 30 seconds	50 °C 40 seconds	72 °C 1 minute	72 °C 10 minutes
***rps*16**	94 °C 3 minutes	40	94 °C 30 seconds	56 °C 1 minute	72 °C 1 minute	72 °C 10 minutes

2.3.4 Sequence editing

Sequence trace files were assembled and edited using SeqMan 15.3 (DNAStar, Inc., Madison, Wl, USA.). Sequences were aligned with the MUSCLE algorithm (Edgar, 2004) implemented in Bioedit[3]. The beginning and end of the alignment, where base callings were ambiguous, were excluded from the analysis. *trn*L intron and *trn*L–*trn*F intergenic spacer for some herbarium specimens were concatenated using Mesquite (Maddison & Maddison, 2018).

2.3.5 Phylogenetic analysis

The program MrBayes 3.1.2 (Ronquist et al., 2012) was used to carry out phylogenetic analyses. The optimal nucleotide substitution model was selected for each alignment via the AIC criterion (Akaike, 1974) using PAUP 4.0b10 (Swofford, 2003) and the MrModelblock command (from MrModeltest 2.3 (Nylander, 2004)). GTR+G was selected for *trn*H-*psb*A as the best fit model, for *mat*K the best-fit model selected was GTR+G, for *trn*L-t*rn*F the best-fit model selected was GTR+I+G, and for *rps*16 the best-fit model selected was GTR+G.

For each analysis, four chains were run for 10 million generations, sampling taking place every 10,000 generations. The first 25% of trees were discarded as burn in. Tracer v1. 6.0 was used to assess burn-in and analyse log files (Rambaut et al., 2015), to ensure proper mixing. Combinable component consensus trees were generated in BayesTrees (Pagel, Meade & Barker, 2004) and were used in subsequent investigations because these show the best-supported clades.

2.4 Results

2.4.1 DNA extraction

The DNA was extracted successfully from herbarium specimens after several attempts. The herbarium samples produced no bands using the standard Doyle & Doyle CTAB method (1990): overnight isopropanol precipitation (Figure 2.3, A), however, the DNA yields were good after this method was modified to use a three weeks isopropanol precipitation (Figure 2.3, C), rather than one week of precipitation (Figure 2.3, B). Amplifying the DNA was not successful without clean-up of the CTAB, although the band became weak (Figure 2.3, D). Therefore, the CTAB method (Doyle & Doyle, 1990), with isopropanol precipitation for three weeks was used and subsequently cleaned up with the DNeasy® Plant Mini Kit (Qiagen), which is a successful method for herbarium specimens.

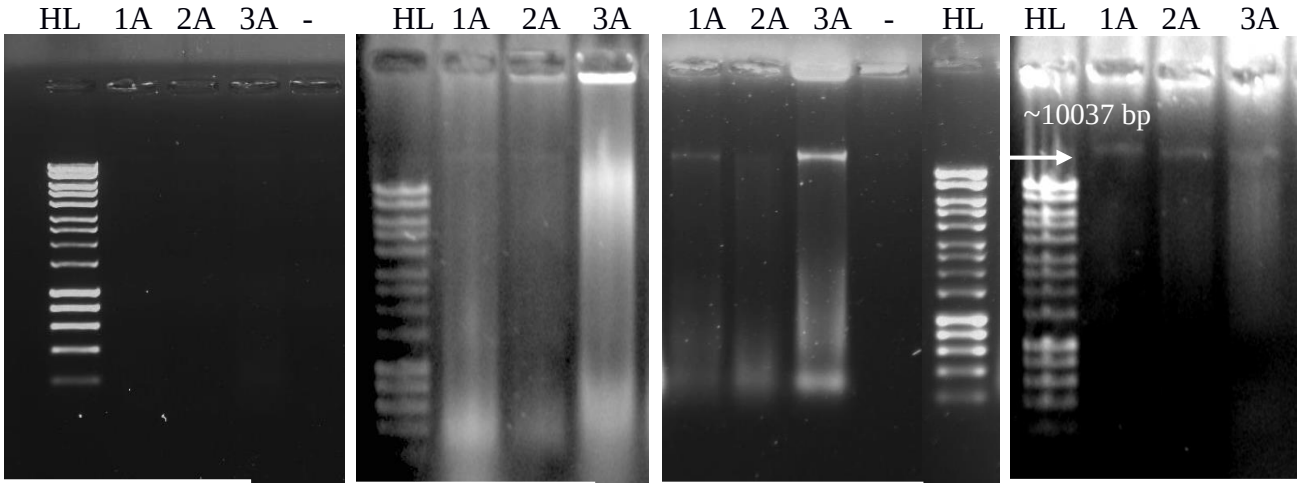


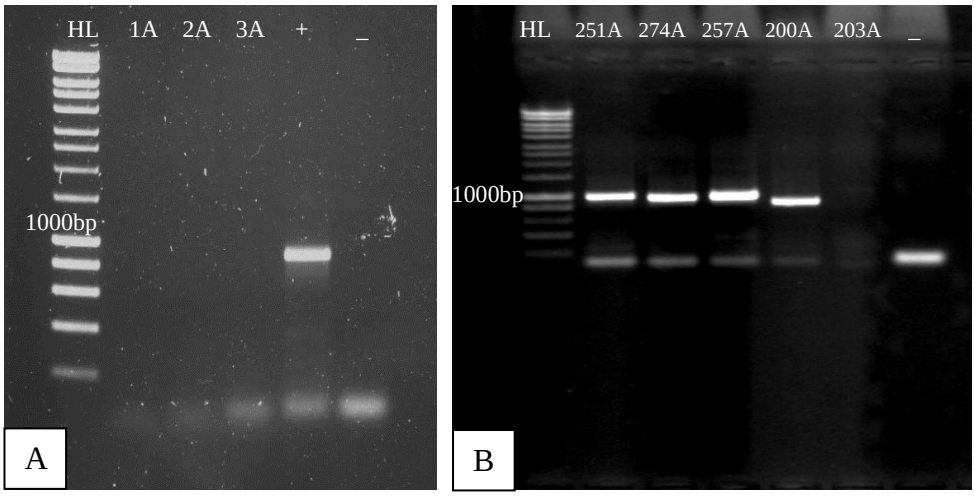
Figure 2.3 A. The herbarium samples produced no bands by Doyle & Doyle CTAB method (1990). B. The herbarium samples produced very weak bands by the Doyle & Doyle CTAB method (1990) modified with isopropanol precipitation of one week. C. The herbarium samples produced bands by the Doyle & Doyle CTAB method (1990 modified with isopropanol precipitation of three weeks. D. The herbarium samples produced bands by the Doyle & Doyle CTAB method (1990) modified with isopropanol precipitation of three weeks and cleaned up using the DNeasy® Plant Mini Kit. 1A. *Hoslundia opposite* Vahl. 2A. *Orthosiphon serratus* Schltr. and 3A. *Orthosiphon pallidus* Royle ex Benth. with HyperLadder™ 1kb and negative control.

2.4.2 PCR amplification and sequencing

PCR amplification was not successful for the herbarium specimens without cleaning up the DNA using the DNeasy® Plant Mini Kit (Figure 2.4, A), but was successful with cleaning up the DNA using the DNeasy® Plant Mini Kit (Figure 2.4, B).

Amplification of the four regions was successful in the 99 samples which produced the bands of greatest intensity (Figure 2.4, B), with the exception one species from *trn*H-*psb*A, nine species from *rps*16 and seven species from *mat*K (Appendix 1), however, they were included in this analysis.

Sequencing was successful in most of the samples. GenBank BLASTn was used to check all other sequences matched with identities and query covers ranging from 80-99% of the submitted sequence. It means that identifications of gene based on BLASTn were possible. The identifications of the samples from GenBank/EMBL checked in the original paper which cited the voucher specimens in Table 2.2.



HL: Hyperladder™ 1 K

Figure 2.4 A. Failed amplification of the herbarium specimens with primers c and f of *trn*L–*trn*F with HyperLadder™ 1kb, positive and negative control. 1A. *Hoslundia opposite* Vahl. 2A. *Orthosiphon serratus* Schltr. and 3A. *Orthosiphon pallidus* Royle ex Benth. B. Amplification of 1000 bp product of the herbarium specimens with primers c and f of *trn*L–*trn*F with HyperLadder™ 1kb and negative control. 251A. *Ocimum africanum,* 274A. *Ocimum ellenbeckii,* 257A. *Ocimum verticillifolium,* 200A. *Orthosiphon hanningtonii* and 203A. *Hoslundia opposita.*

2.4.3 Sequence alignment

Overall alignment of the *trn*L-*trn*F region was 1130 bp long, which was trimmed to 854 bp; *trn*H-*psb*A region of 515 bp, which was trimmed to 401 bp; *rps*16 region of 1050 bp, which was trimmed to 957 bp; and *mat*K region of 880 bp, which was trimmed to 758 bp. This was done in order to make all the alignment of the same length for later analyses.

2.4.4 Phylogenetic analysis for four cpDNA

2.4.4.1. *trn*L–*trn*F region

All species from the subtribe Ociminae were retrieved as a monophyletic group with a posterior probability (PP) of 1.0 (Figure 2.5).

All species from *Endostemon* and *Syncolostemon* formed clades (Figure 2.5, Clade C and D) each has a PP of 1.0. All samples of *Ocimum* (Figure 2.4, Clade A), formed a paraphyletic group with one species from *Orthosiphon* (*Or. fruticosus*) in *Ocimum* subgenus *Nautochilus.* All species from *Orthosiphon*, *Fuerstia* and *Hoslundia* together formed a clade (Figure 2.5, Clade B) with a PP 1.0, in which all species from *Orthosiphon* formed a clade with a PP 0.91 except for one species belonging *Ocimum*, so it is not a monophyletic group. All species from *Fuerstia* and *Hoslundia* together formed a clade with a PP 0.90. The *Fuerstia* and *Hoslundia* clade is a sister to the *Orthosiphon* group. Clade E in Figure 2.5 contains all species from *Catoferia* and formed a clade with a PP 1.0, *Basilicum* clade with a PP 1.0, all samples of *Haumaniastrum* and *Platostoma* formed a clade and they are sister to each other with a PP 1.0.

2.4.4.2 *trn*H–*psb*A region

All species from the subtribe Ociminae were retrieved as a monophyletic group with a PP of 1.0 (Figure 2.6).

All species from *Catoferia* and *Syncolostemon* formed clades (Figure 2.6, Clade A and B) both have PP 1.0. All species from *Orthosiphon*, *Fuerstia* and *Hoslundia* formed a clade (Figure 2.6, Clade C) with a PP 1.0, in which all species from *Orthosiphon* formed a clade with a PP 1.0 except for one species belonging *Ocimum* (*Or. fruticosus*), therefore, it is not a monophyletic group. All species from *Fuerstia* and *Hoslundia* form a clade with a PP 0.87. The *Fuerstia* and *Hoslundia* clade is a sister to the *Orthosiphon* group. All species from *Endostemon* formed a clade (Figure 2.6, Clade D) with a PP 0.98. *Platostoma* clade is a sister to *Haumaniastrum* clade with a PP 1.0 (Figure 2.6, Clade E), in which all species from *Platostoma* formed a clade with a PP 0.74 and all species from *Haumaniastrum* formed a clade with a PP 1.0. *Benguellia, Basilicum* and all

species from *Ocimum* formed a clade with a PP 0.74 (Figure 2.6, Clade F), in which all species from *Ocimum* formed a clade with a PP 0.95, with one species from *Orthosiphon* (*Or. fruticosus*) in *Ocimum* subgenus *Nautochilus.* Thus, *Ocimum* is a paraphyletic group.

2.4.4.3 *rps*16 region

All species from the subtribe Ociminae were retrieved as a monophyletic group with a PP of 1.0 (Figure 2.7).

All species from *Syncolostemon* and *Endostemon* formed clades (Figure 2.7, Clade B and D) each has a PP of 1.0. All species from *Ocimum* formed a clade with a PP of 0.99 (Figure 2.7, Clade E), with one species from *Orthosiphon* (*Or. fruticosus*) in *Ocimum* subgenus *Nautochilus*, so *Ocimum* is a paraphyletic group. *Platostoma* clade is a sister to *Haumaniastrum* clade with a PP 1.0 (Figure 2.7, Clade A), in which all species from *Platostoma* formed a clade with a PP 0.59 and all species from *Haumaniastrum* formed a clade with a PP 1.0. All species from *Orthosiphon*, *Fuerstia* and *Hoslundia* formed a clade (Figure 2.7, Clade C) with a PP 1.0, in which all species from *Orthosiphon* formed a clade with a PP 0.97 except for one species belonging *Ocimum* (*Or. fruticosus*), therefore, it is not a monophyletic group. All species from *Fuerstia* and *Hoslundia* form a clade with a PP 1.0. The *Fuerstia* and *Hoslundia* clade is a sister to the *Orthosiphon* group.

2.4.4. 4 *mat*K region

All species from the subtribe Ociminae were retrieved as a monophyletic group with a PP of 1.0 (Figure 2.8).

All species from *Catoferia* and *Endostemon* formed clades (Figure 2.8, Clade A and E) both with a PP 1.0. *Platostoma* clade is a sister to *Haumaniastrum* clade with a PP 1.0 (Figure 2.8, Clade B), each has a PP 1.0. The *Haumaniastrum* clade is found most closely related to *Platostoma* sect. *Limniboza* subsection *Rotundifolia.* All species from *Syncolostemon* formed a clade with a PP 0.99 (Figure 2.8, Clade D). All species from *Orthosiphon*, *Fuerstia* and *Hoslundia* formed a clade (Figure 2.8, Clade C) with a PP 0.88, in which all species from *Orthosiphon* formed a clade with a PP 0.79 except for one species belonging *Ocimum*, therefore, it is not a monophyletic group. All species from *Fuerstia* and *Hoslundia* formed a clade with a PP 1.0. The *Fuerstia* and *Hoslundia* clade is a sister to the *Orthosiphon* group. All species from *Ocimum* formed a clade with a PP 0.96 (Figure 2.8, Clade F), with one species from *Orthosiphon* (*Or. fruticosus*) in *Ocimum* subgenus *Nautochilus.* Thus, *Ocimum* is a paraphyletic group.

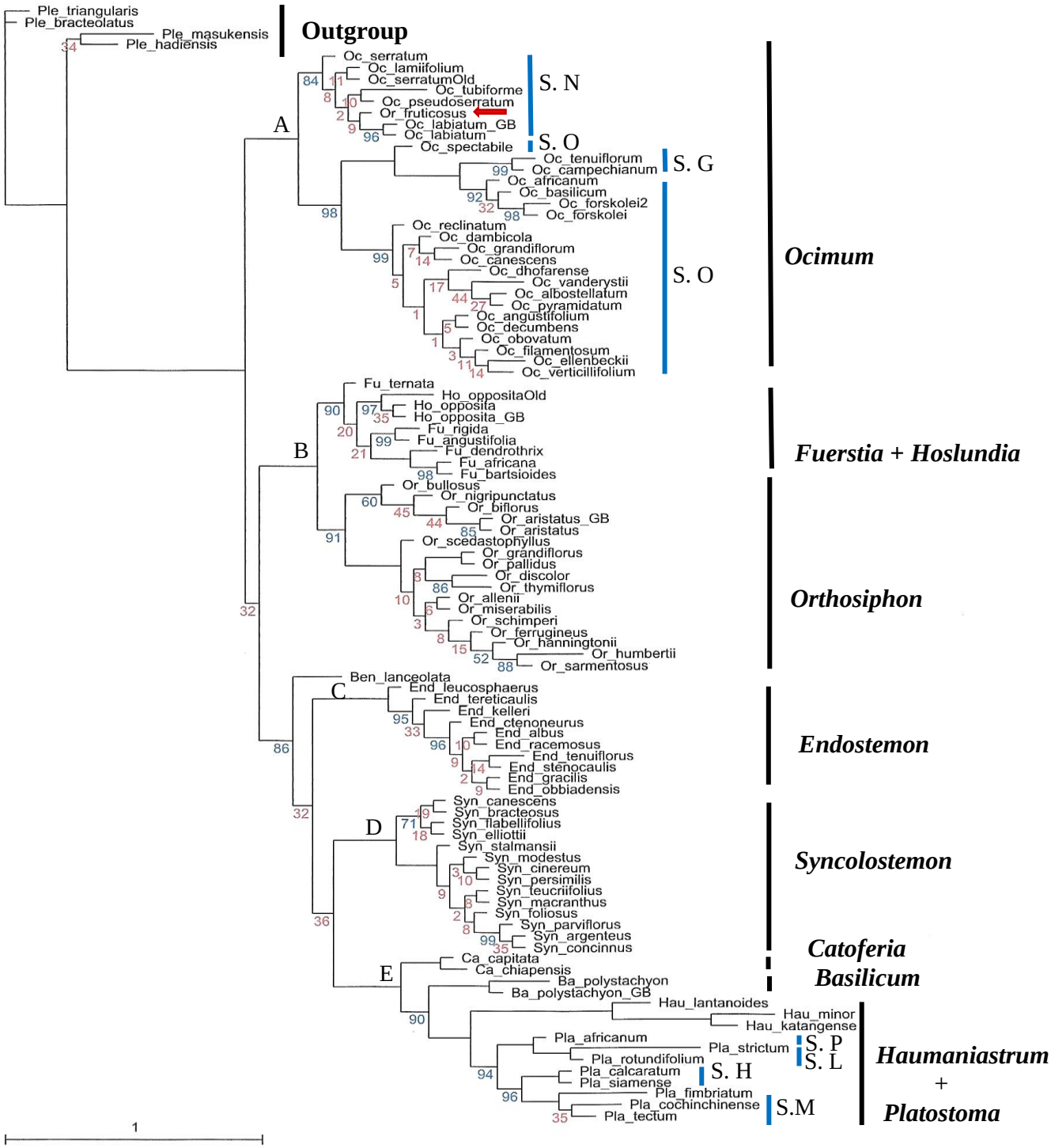


Figure 2.5 Combinable component consensus tree from Bayesian analysis of the cpDNA *trn*L-*trn*F region showing the outgroup, and subtribe Ociminae clades A-E. Posterior probabilities are given below the branches: blue > 0.50 support, red < 0.50, all unlabelled nodes have a posterior probability of 1.0. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum* and Syn = *Syncolostemon*. *Ocimum* subgenera; S. G = Subgenus *Gymnocimum* S. O = Subgenus *Ocimum* and S. N = Subgenus *Nautochilus*. *Platostoma* sections; S. H = Section *Heterodonta*, S. L = Section *Limniboza*, S. M = Section *Mesona* and S. P = Section *Platostoma*. Dark red arrow indicates *Orthosiphon fruticosus* which is in the *Ocimum* group.

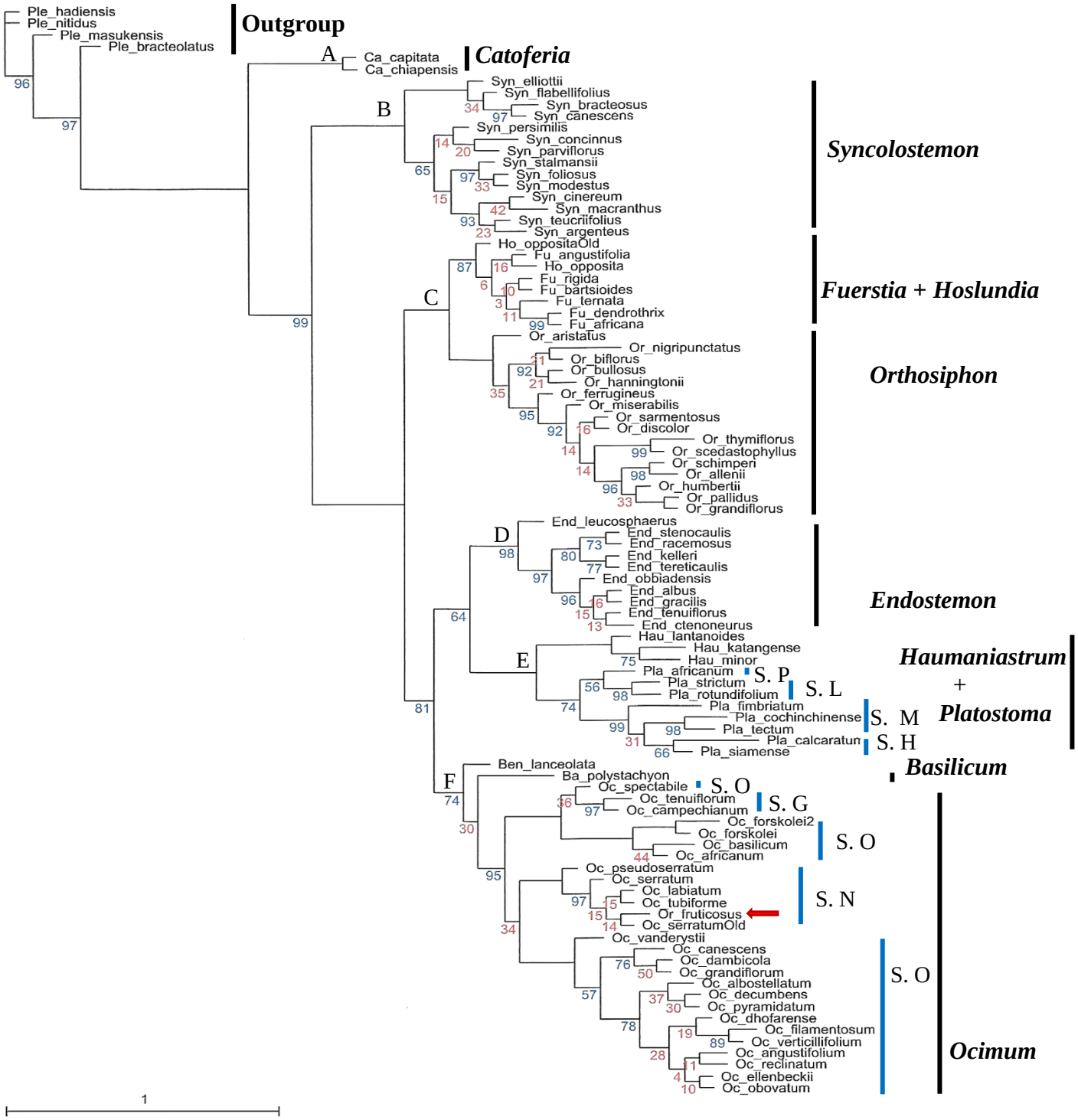


Figure 2.6 Combinable component consensus tree from Bayesian analysis of the cpDNA *trn*H–*psb*A region showing the outgroup, and subtribe Ociminae clades A-F. Posterior probabilities are given below the branches: blue > 0.50 support, red < 0.50, all unlabelled nodes have a posterior probability of 1.0. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum* and Syn = *Syncolostemon*. *Ocimum* subgenera; S. G = Subgenus *Gymnocimum* S. O = Subgenus *Ocimum* and S. N = Subgenus *Nautochilus*. *Platostoma* sections; S. H = Section *Heterodonta*, S. L = Section *Limniboza*, S. M = Section *Mesona* and S. P = Section *Platostoma*. Dark red arrow indicates the *Orthosiphon fruticosus* which is existed in *Ocimum* group.

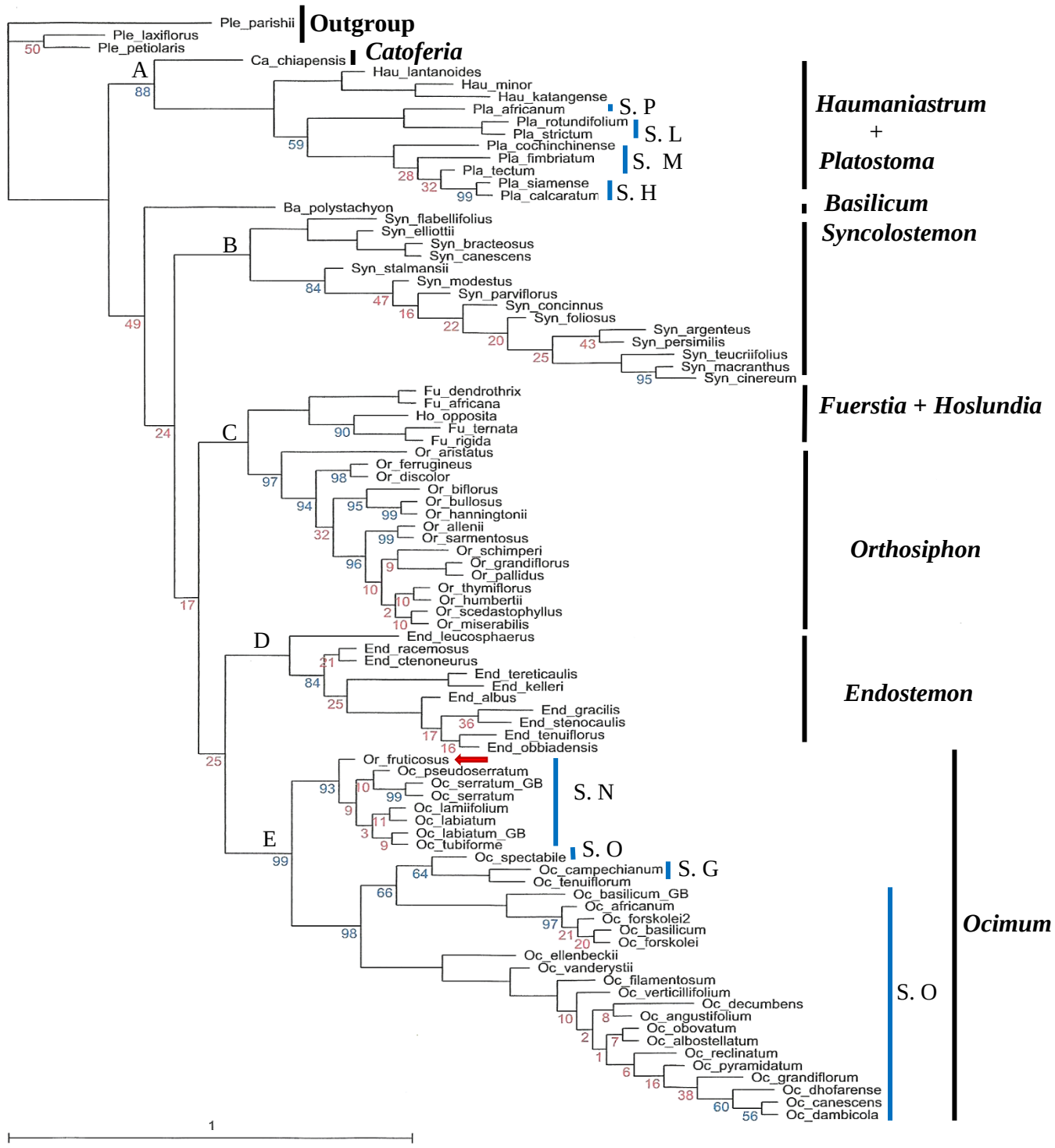


Figure 2.7 Combinable component consensus tree from Bayesian analysis of the cpDNA *rps*16 region showing the outgroup, and subtribe Ociminae clades A-E. Posterior probabilities are given below the branches: blue > 0.50 support, red < 0.50, all unlabelled nodes have a posterior probability of 1.0. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum* and Syn = *Syncolostemon*. *Ocimum* subgenera; S. G = Subgenus *Gymnocimum* S. O = Subgenus *Ocimum* and S. N = Subgenus *Nautochilus. Platostoma* sections; S. H = Section *Heterodonta*, S. L = Section *Limniboza*, S. M = Section *Mesona* and S. P = Section *Platostoma*. Dark red arrow indicates the *Orthosiphon fruticosus* which is existed in *Ocimum* group.

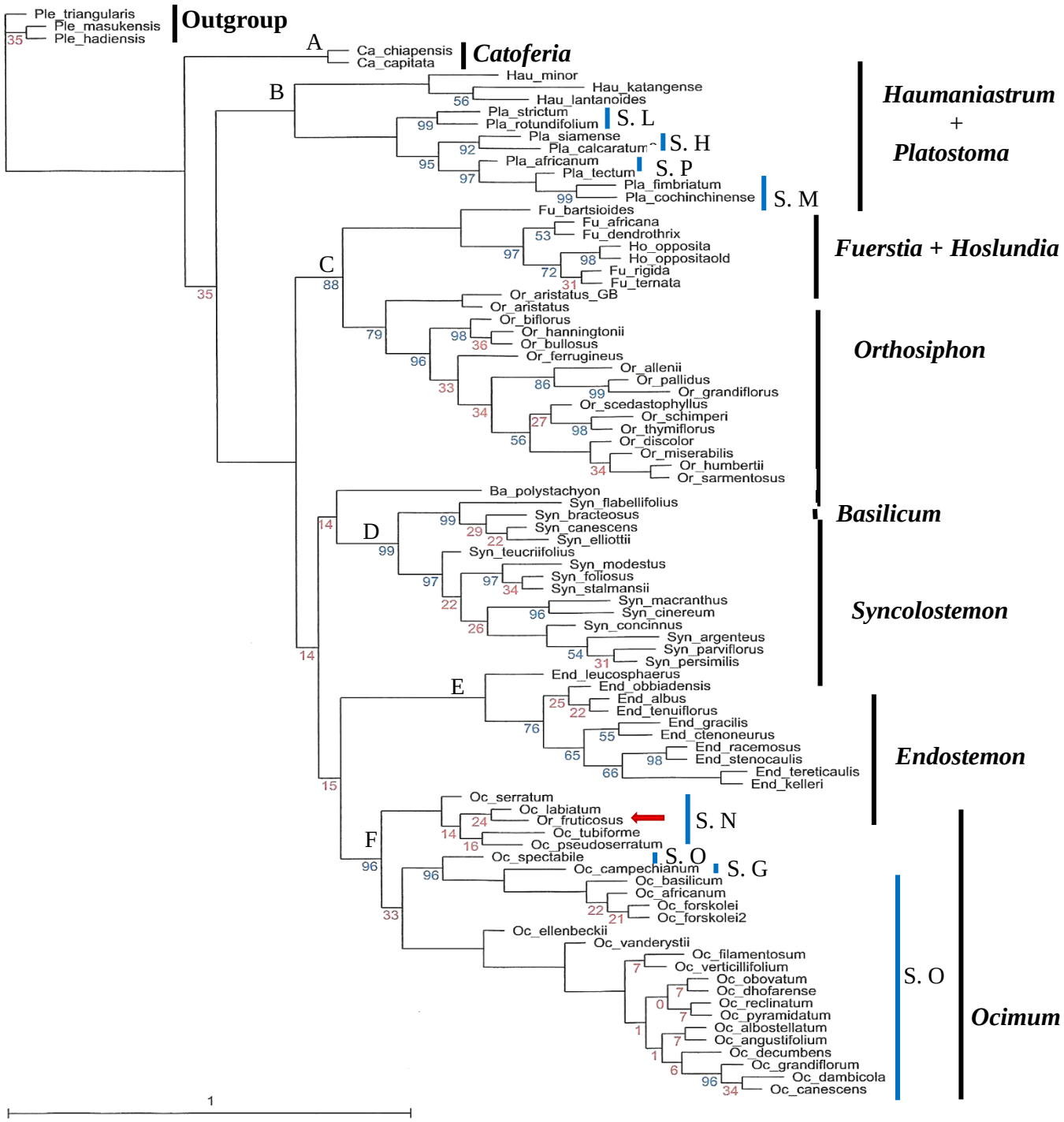


Figure 2.8 Combinable component consensus tree from Bayesian analysis of the cpDNA *mat*K region showing the outgroup, and subtribe Ociminae clades A-F. Posterior probabilities are given below the branches: blue > 0.50 support, red < 0.50, all unlabelled nodes have a posterior probability of 1.0. Ba = *Basilicum,* Ben = *Benguellia,* Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia,* Hau = *Haumaniastrum,* Ho = *Hoslundia,* Or = *Orthosiphon*, Pla = *Platostoma,* Ple = *Plectranthus,* Oc = *Ocimum* and Syn = *Syncolostemon*. *Ocimum* subgenera; S. G = Subgenus *Gymnocimum* S. O = Subgenus *Ocimum* and S. N = Subgenus *Nautochilus. Platostoma* sections; S. H = Section *Heterodonta,* S. L = Section *Limniboza,* S. M = Section *Mesona* and S. P = Section *Platostoma*. Dark red arrow indicates the *Orthosiphon fruticosus* which is existed in *Ocimum* group.

2.5 Discussion

2.5.1 DNA extraction, amplification and sequencing

In this study, problems were encountered when trying to extract DNA from the herbarium specimens which is consistent with observations by Harley & Paton, (2012), who failed at their attempt to extract DNA from the herbarium of *Orthosiphon americanus*. The herbarium materials was very difficult to extract DNA from because the DNA was degraded and the reason for this may be as reported by Erkens et al. (2008) that "the botanists should annotate how specimens were collected and dried because this information is essential for successful DNA extraction", it means the DNA extraction was challenging in this study. As a result, several extraction methods were tested before the best method for obtaining high-quality DNA was determined. The CTAB method (Doyle & Doyle, 1990), modified to include long isopropanol precipitation of three weeks with CTAB clean up by DNeasy® Plant Mini Kit (Qiagen), has been used successfully for herbarium specimens. In this study, the DNA yields were good after three weeks of isopropanol precipitation rather than one week, this result consistent with Fay et al. (1998), who recommended that DNA from herbarium specimens be allowed to precipitate for a minimum of three weeks at −20°C, so, modified with long isopropanol precipitation. After only one week of precipitation, there were very low DNA yields and bands were not visible clearly. Richardson et al. (2000) stated that the reasons for increased yield after lengthy precipitation are uncertain, but it may be because modified secondary compounds produced by degradation during drying make the quick precipitation of DNA difficult. It may also be because the DNA obtained from the herbarium specimens was degraded, which necessitated more time for precipitation. The success of the modification of the CTAB method might be related to removes of secondary compounds, avoiding them overlapping with subsequent PCR amplification. Amplifying the DNA would not be successful without cleaning up using CTAB.

Paton et al.'s (2004) study suggested that one fragment c to f was useful for PCR amplification of the *trn*L-*trn*F region for herbarium specimens, fresh and silica-dried of the tribe Ocimeae, but this study showed that c - d and e - f were useful for some herbarium specimens of Ociminae such as in the sample of *Platostoma calcaratum* which was 119 years old. This indicates that it was not possible to amplify herbarium specimens for the intron and spacer as a single fragment; therefore, internal primers d and e were used in combination with c and f for the samples, and this result seems to be consistent with Pastore et al. (2011). Perhaps the DNA from the herbarium specimens was more degraded than fresh and silica-dried plant materials and therefore this region was not able to be amplified as one fragment.

2.5.2 Phylogenetic analyses

The phylogenetic analysis presented from four chloroplast regions (*trn*L–*trn*F, *trn*H–*psb*A, *mat*K and *rps*16) supports the monophyly of Ociminae with a PP value of 1.0. The *Orthosiphon* clade is monophyletic if *Or. fruticosus* is merged to *Ocimum* subgenus *Nautochilus.* However, Paton et al. (2004) showed that *Orthosiphon* is monophyletic with the inclusion of *Fuerstia* and *Hoslundia*, but with a bootstrap of <50%. *Ocimum* as currently recognised is polyphyletic (Paton, 1997b). By separately recognising the monophyletic subgenus *Nautochilus* and the monophyletic remainder of *Ocimum* subgenus *Ocimum* and subgenus *Gymnocimum* a pair of monophyletic groups (Paton et al, 2004) is produced. In this study, the *Ocimum* clade is monophyletic with the inclusion of *Nautochilus* with a PP value of 1.0, 0.99, 0.96 and 0.95 from *trn*L–*trn*F, *rps*16, *mat*K and *trn*H–*psb*A regions respectively if *Or. fruticosus* is included in *Ocimum* subgenus *Nautochilus*. This study has demonstrated that *Platostoma* clade is monophyletic and also *Haumaniastrum* clade, but Paton et al. (2004) reported that *Platostoma* is monophyletic with the inclusion of *Basilicum* and *Haumaniastrum*. The *Syncolostemon* clade is monophyletic, this finding is consistent with that of Paton et al. (2004) and Otieno et al. (2006). The *Catoferia* clade is monophyletic with a PP = 1.0 from chloroplast regions in this study and *Endostemon* clade is monophyletic with a PP = 1.0 from *trn*L-*trn*F, *trn*H-*psb*A, and *rps*16, and with a PP = 0.99 PP from *mat*K regions.

This study sought to determine the generic delimitation and investigate the relationships among *Orthosiphon*, *Fuerstia*, and *Hoslundia.* Based on *trn*L-*trn*F, *trn*H-*psb*A, *mat*K and *rps*16 region evidence, it was found that *Fuerstia* and the *Hoslundia* group is a sister to *Orthosiphon* clade, with a PP = 1.0 for all these regions except *mat*K, which had a PP = 0.88. Surprisingly, *Hoslundia* was found nested with *Fuerstia* with a PP = 1.0 from *rps*16 and *mat*K, with a PP = 0.90 from *trn*L-*trn*F, and with a PP = 0.87 from *trn*H-*psb*A regions. Because these genera form a monophyletic group, *Hoslundia* and *Fuerstia* will be merged under the earliest name *Hoslundia.* Because *Hoslundia* was published in 1804 and *Fuerstia* were published in 1929 (International Plant Names Index, 2019), therefore, the name of *Fuerstia* should be changed to *Hoslundia*. Therefore, some changes to the taxonomy/nomenclature are required.

According, the relationship between *Ocimum* and subgenus *Nautochilus,* some studies have been unable to demonstrate the relationship between them. Al-Qassabi (2014) recommended placing *Nautochilus* into a new genus because of differences between it and the Asian clade, but the two regions (*trn*L-F and *rps*16) were not sufficient to verify these findings. From this study, *Ocimum* subgenus *Nautochilus* is a sister to the rest of *Ocimum* and *Orthosiphon fruticosus* belongs in this subgenus.

The *Catoferia* clade is a sister group to *Platostoma* and *Haumaniastrum* with a PP = 1.0 in *trn*L–*trn*F and PP = 0.88 in *rps*16, however, this relationship is not supported by *trn*H–*psb*A. This

result was also reported by Paton et al. (2004), who showed that *Catoferia* is more closely related to *Platostoma.*

The *Platostoma* clade is a sister to *Haumaniastrum* clade with a PP= 1.0 based on all the chloroplast regions which were used in this study. The *Haumaniastrum* clade is found to be most closely related to *Platostoma* sect. *Limniboza* subsection *Rotundifolia* by *mat*K. This result is consistent with Paton (1997b), who showed that *Haumaniastrum* more closely related to *Platostoma* sect. *Limniboza* rather than *Acrocephalus.* However, it is close to sect. *Platostoma* according to the rest of the genes in this study.

To sum up, most previous recognised genera (*Catoferia, Endostemon, Syncolostemon, Platostoma* and *Haumaniastrum*) when sampled formed a clade in the molecular analysis. These results offer a framework for making decisions regarding taxonomy in terms of genera as previously mentioned and provide a starting point for greater comprehension of the phylogeny of Ociminae.

2.6 Conclusion

The successful method of DNA extraction with modification was determined for herbarium specimens. The study used DNA sequence data based on cpDNA regions *trn*L–*trn*F, *trn*H–*psb*A, *rps*16, and *mat*K, to determine the generic delimitation and investigate the relationships among Ociminae. *Fuerstia* and *Hoslundia* are the sister group to *Orthosiphon. Hoslundia*, a monotypic genus originally placed in the Ociminae, forms part of a small clade nested within the genus *Fuerstia*. Because these genera form a monophyletic group, *Hoslundia* and *Fuerstia* will be merged under the earliest name later on in the paper, therefore, the name of *Fuerstia* should be changed to *Hoslundia*. *Ocimum* subgenus *Nautochilus* is a sister to the rest of *Ocimum* (subgenus *Ocimum* and subgenus *Gymnocimum*). *Orthosiphon fruticosus* is a member of subgenus *Nautochilus.* Therefore, nomenclatural changes are appropriate.

The monophyly of Ociminae, *Catoferia, Endostemon, Syncolostemon, Platostoma* and *Haumaniastrum,* were confirmed. *Orthosiphon* is monophyletic if *Or. fruticosus* is excluded, *Fuerstia* is monophyletic with inclusion *Hoslundia, Ocimum* is monophyletic with the inclusion subgenus *Nautochilus* after including *Orthosiphon fruticosus.*

Chapter 3 : Molecular phylogeny of nrDNA regions

3.1 Introduction

The problems outlined in chapter 2.

Nuclear ribosomal ITS and ETS regions were previously used for molecular phylogenetics within Lamiaceae successfully: ITS were used by Jamzad et al. (2003) for *Nepeta* L.; by Bramley (2009) for *Callicarpa* L.; by Conn et al. (2009) for *Chloantheae*; by Bräuchler et al. (2010) for Menthinae; by Zhong et al. (2010) for *Isodon*; by Pastore et al. (2011) for subtribe Hyptidinae; by Jenks et al. (2013) for *Salvia* subgenus *Calosphace*; by Yao et al. (2016) for *Pogostemon* sl; and by Li et al. (2017) for Elsholtzieae. ETS was used by Wilson et al. (2012) for *Prostanthera* and by (Li et al., 2017) for Elsholtzieae. It was used together with ITS by Silveira (2010) for *Pogogyne*; by Chen et al. (2016) for *Ombrocharis*; by Li et al. (2017) for *Heterolamium*; by Takano (2017) for Japanese *Salvia;* and by Hu et al. (2018) for *Salvia*. ITS and ETS showed the best results in previous studies and appeared to be significantly informative.

3.2 Aims and objectives

The main aim in this chapter is to determine the generic delimitation the genera of *Orthosiphon, Fuerstia,* and *Hoslundia,* and between *Ocimum* and *Nautochilus* using nrDNA evidence (ITS and ETS) and then investigate the relationships among these genera.

The objectives

1. To test if the subtribe Ociminae is a monophyletic group.
2. To test if *Orthosiphon, Ocimum,* and *Fuerstia* each comprise a monophyletic group.
3. To investigate the relationship among *Orthosiphon, Fuerstia, Hoslundia, Ocimum*, and subgenus *Nautochilus.*

3.3 Materials and methods

3.3.1 Plant materials

Samples were collected as reported in Chapter 2 (Appendix 1). One sequence was taken from NCBI- GenBank which is *Plectranthus thyrsoideus* (Baker) B. Mathew for ETS. The GenBank accession for this is JF304198 and voucher specimen is LCD5638704012.

3.3.2 DNA extraction

Described in detail in section 2.3.2

3.3.3 Polymerase chain reaction (PCR)

3.3.3.1 Primers

Herbarium specimens were amplified using the primers in Table 3.1. Amplification was difficult for ITS some herbarium specimens could not be amplified in a single reaction and so the region was amplified in two distinct sections with the help of primers 17SE from Sun et al. (1994) and ITS2 from White et al., (1990), 26SE from Sun et al. (1994) and ITS3 from White et al., (1990). ETS was amplified in one fragment with primers 18S-IGS from Baldwin & Markos (1998) and ETS-B from Beardsley & Olmstead (2002).

Table 3.1 Primers used in this chapter.

Locus	**Primer name**	**Primer sequence (5´– 3´)**	**Author**
ITS	17SE	ACG AAT TCA TGG TCC GGT G AA GTG TTC	Sun et al., 1994
	26SE	TAG AAT TCC CCG GTT CGC TCG CCG TTA C	Sun et al., 1994
	ITS2	GCTGCGTTCTTCATCGATGC	White et al., 1990
	ITS3	GCATCGATGAAGAACGCAGC	White et al., 1990
ETS	18S-IGS	GAGACAAGCATATGACTACTGGCA GGATCAACCAG	Baldwin & Markos, 1998
	ETS-B	ATAGAGCGCGTGAGTGGTG	Beardsley & Olmstead, 2002

3.3.3.2 PCR amplification, conditions, programs, purification and sequencing

The two regions (ITS and ETS) were successfully amplified independently in PCR thermocyclers described in Table 3.2 after modified (Zhong et al., 2010; Pastore et al., 2011). The conditions, purification, and sequencing were as outlined in 2.3.3.2.

Table 3.2 The list of programs were successful to amplify and produce PCR products.

The region	Initial DNA denaturation	Cycles	Denaturation	Annealing	Extension	Final extension
ITS	94 °C 5 minute	40	94 °C 45 seconds	57 °C 50 seconds	72 °C 1.5 minutes	72 °C 7 minutes
ETS	94 °C 5 minute	40	94 °C 1 minute	58 °C 1 minute	72 °C 1 minute	72 °C 7 minutes

3.3.4 Sequence editing

Sequence editing was outlined in Chapter 2. ITS1 and ITS2 for some herbarium specimens were concatenated using Mesquite (Maddison & Maddison, 2018).

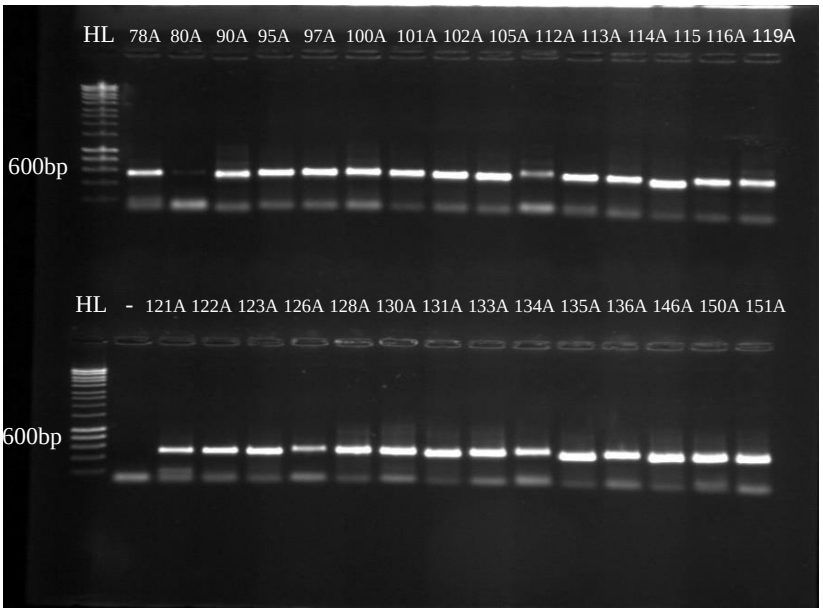
3.3.5 Phylogenetic analysis

Phylogenetic analyses were performed as in section 2.3.5. For the nuclear region (ITS) the best-fit model was selected as GTR+G and for ETS the best-fit model was selected GTR+I+G.

3.4 Results

3.4.1 PCR amplification and sequencing

Amplification of the two regions was successful in the 99 samples. There produced the highest number of bands of greatest intensity (e.g. Figure 3.1), except four species from ITS and four species from ETS, (Appendix 1). Sequencing was successful in most of the samples.



HL: Hyperladder™ 1 K

Figure 3.1 Amplification of 600 bp product of herbarium specimens with primers ITS 17SE and ITS2 with HyperLadder™ 1kb and negative control. 78A. *Haumaniastrum katangense,* 80A. *Haumaniastrum lantanoides,* 90A. *Haumaniastrum minor,* 95A. *Platostoma cochinchinense,* 97A. *Platostoma africanum,* 100A. *Platostoma tectum,* 101A. *Platostoma fimbriatum,* 102A. *Platostoma siamense,* 105A. *Platostoma strictum,* 112A. *Platostoma rotundifolium,* 113A. *Platostoma calcaratum,* 114A. *Syncolostemon elliottii,* 115A. *Syncolostemon canescens,* 116A. *Syncolostemon bracteosus,* 119A. *Syncolostemon flabellifolius,* 121A. *Syncolostemon concinnus,* 122A. *Syncolostemon parviflorus,* 123A. *Syncolostemon argenteus,* 126A. *Syncolostemon macranthus,* 128A. *Syncolostemon cinereum,* 130A. *Syncolostemon teucriifolius,* 131A. *Syncolostemon modestus,* 133A. *Syncolostemon stalmansii,* 134A. *Syncolostemon foliosus,* 135A. *Syncolostemon persimilis,* 136. *Orthosiphon aristatus,* 146A. *Orthosiphon ferrugineus,* 150A.*Orthosiphon grandiflorus* and 151A. *Orthosiphon bullosus.*

3.4.2 Sequence alignment

Alignment resulted in an ITS region of 1010 bp which was trimmed to 885 bp and an ETS region of 539 bp which was trimmed to 513 bp. This was done in order to make all the alignment of the same length for later analyses.

3.4.3 Phylogenetic analysis for two nrDNA regions

3.4.3.1 ITS region

All species from the subtribe Ociminae were retrieved as a monophyletic group with a PP of 1.0 (Figure 3.2).

All species from *Syncolostemon* and *Catoferia* formed clades (Figure 3.2, Clade A and E) both have PP 1.0. *Benguellia* and all species from *Endostemon* formed a clade (Figure 3.2, Clade C) with a PP 1.0, in which all species from *Endostemon* formed a clade with a PP 0.73. *Platostoma* clade is a sister to and *Haumaniastrum* clade with a PP 0.95 (Figure 3.2, Clade D), in which all species from *Platostoma* formed a clade with a PP 0.94 and all species from *Haumaniastrum* formed a clade with a PP 1.0. The *Haumaniastrum* clade is found from most closely related to *Platostoma* sect. *Limniboza* subsection *Rotundifolia.* All species from *Orthosiphon*, *Fuerstia* and *Hoslundia* formed a clade (Figure 3.2, Clade B) with a PP 1.0, in which all species from *Orthosiphon* formed a clade with a PP 0.92 except for one species belonging *Ocimum*, therefore, it is not a monophyletic group. All species from *Fuerstia* and *Hoslundia* formed a clade with a PP 1.0. The *Fuerstia* and *Hoslundia* clade is a sister to the *Orthosiphon* group. *Basilicum* and all species from *Ocimum* formed a clade with a PP 0.99 (Figure 3.2, Clade F), in which all species from *Ocimum* formed a clade with a PP 0.51 with one species from *Orthosiphon* (*Or. fruticosus*) in *Ocimum* subgenus *Nautochilus*, therefore, it is a paraphyletic group.

3.4.3.2 ETS region

All species from the subtribe Ociminae were retrieved as a monophyletic group with a PP of 1.0 (Figure 3.3).

All species from *Syncolostemon, Catoferia* and *Endostemon* formed clades (Figure 3.3, Clade A, B and E) each has a PP 1.0. All species from *Ocimum* formed a clade with a PP 0.93 with one species from *Orthosiphon* (*Or. fruticosus*) in *Ocimum* subgenus *Nautochilus*, therefore, it is a paraphyletic group (Figure 3.3, Clade C). The *Platostoma* clade is a sister to the *Haumaniastrum* clade with a PP 1.0 (Figure 3.3, Clade D), in which all species from *Platostoma* formed a clade with a PP 0.93 and all species from *Haumaniastrum* formed a clade with a PP 1.0. *Haumaniastrum* clade is found most closely related to *Platostoma* sect. *Limniboza* subsection *Rotundifolia. Basilicum,* all species from *Orthosiphon*, *Fuerstia* and *Hoslundia* formed a clade (Figure 3.3,

Clade F) with a PP 0.98, in which *Basilicum* formed a clade with a PP 1.0, all species from *Orthosiphon* formed a clade with a PP 0.96 except for one species belonging *Ocimum*, therefore, it is not a monophyletic group. All species from *Fuerstia* and *Hoslundia* formed a clade with a PP 0.99. The *Fuerstia* and *Hoslundia* clade is a sister to the *Orthosiphon* group.

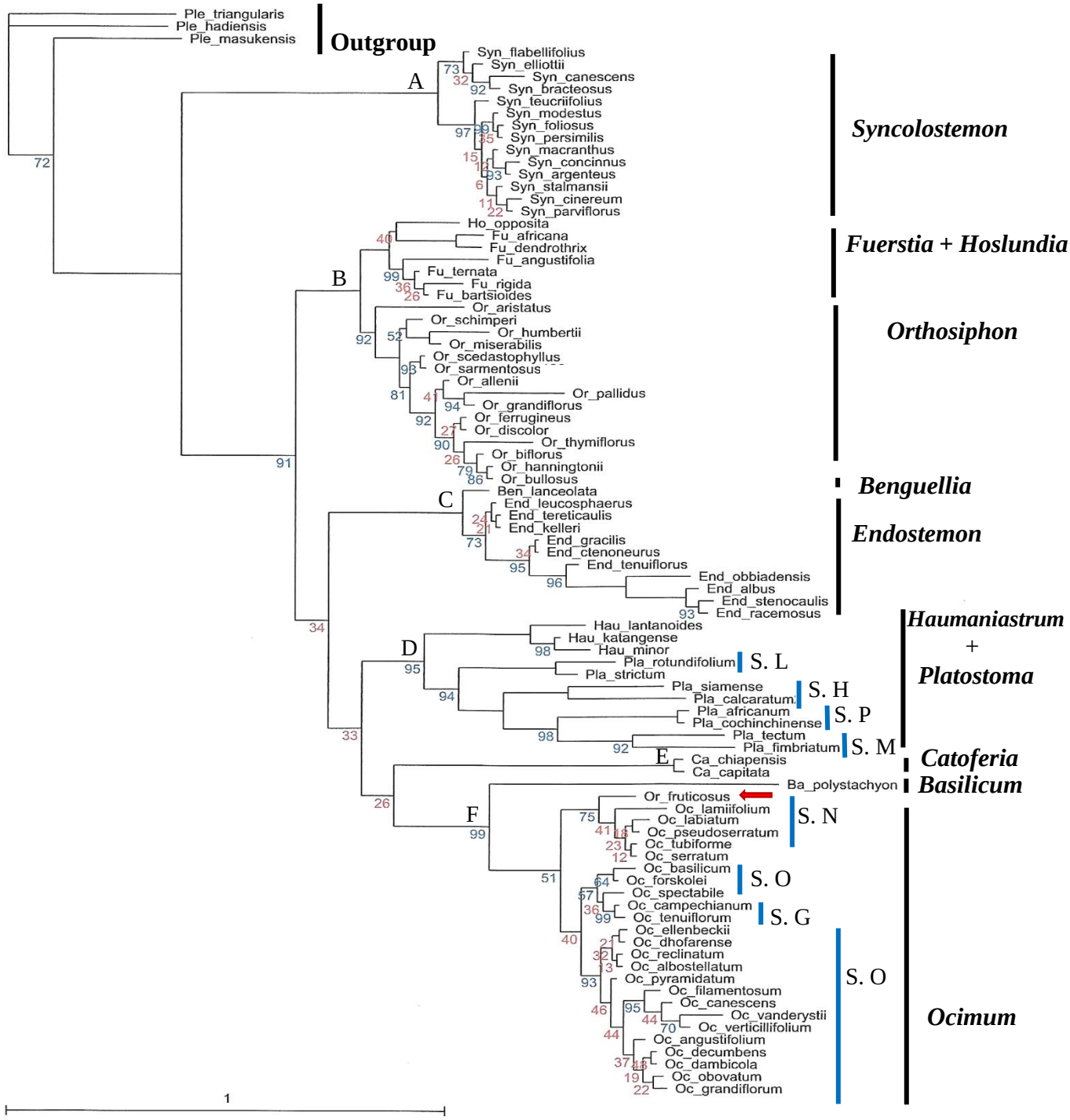


Figure 3.2 Combinable component consensus tree from Bayesian analysis of the nrDNA ITS region showing the outgroup, and subtribe Ociminae clades A-F. Posterior probabilities are given below the branches: blue > 0.50 support, red < 0.50, all unlabelled nodes have a posterior probability of 1.0. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum* and Syn = *Syncolostemon*. *Ocimum* subgenera; S. G = Subgenus *Gymnocimum* S. O = Subgenus *Ocimum* and S. N = Subgenus *Nautochilus*. *Platostoma* sections; S. H = Section *Heterodonta*, S. L = Section *Limniboza*, S. M = Section *Mesona* and S. P = Section *Platostoma*. Dark red arrow indicates the *Orthosiphon fruticosus* which is existed in *Ocimum* group.

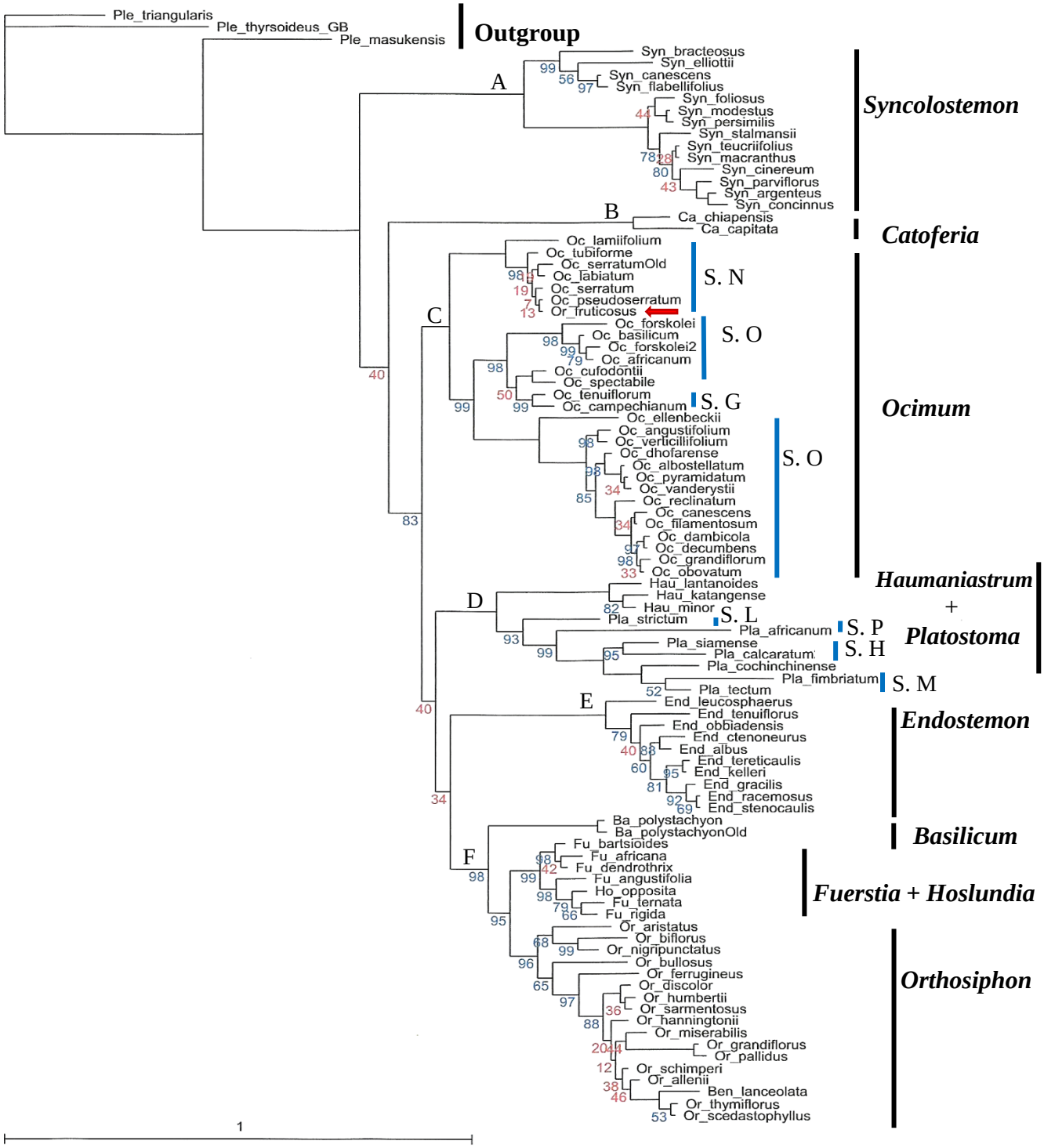


Figure 3.3 Combinable component consensus tree from Bayesian analysis of the nrDNA ETS region showing the outgroup, and subtribe Ociminae clades A-F. Posterior probabilities are given below the branches: blue > 0.50 support, red < 0.50, all unlabelled nodes have a posterior probability of 1.0. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum* and Syn = *Syncolostemon*. *Ocimum* subgenera; S. G = Subgenus *Gymnocimum* S. O = Subgenus *Ocimum* and S. N = Subgenus *Nautochilus. Platostoma* sections; S. H = Section *Heterodonta*, S. L = Section *Limniboza*, S. M = Section *Mesona* and S. P = Section *Platostoma*. Dark red arrow indicates the *Orthosiphon fruticosus* which is existed in *Ocimum* group.

3.5 Discussion

3.5.1 Amplification and Sequencing

Interestingly, like Pastore et al.'s (2011) findings, the extracts resulted in successful amplification of ITS in one fragment, using primers 17SE and 26SE for most herbarium specimens and, in two separate fragments, using primers 17SE and its2, and 26SE and its3 for some herbarium specimens. It is possible that the degradation of the DNA made it impossible to amplify this region in one fragment. There was not a link between the age of the herbarium specimens and the success in amplification, for example, *Platostoma calcaratum* collected in 1900, is 119 years old but *Syncolostemon teucriifolius* collected in 1994, is 25 years old but both not amplified ITS in one fragment but they amplified ITS in two fragments (Appendix 1). Erkens et al. (2008) showed that "the age and greenness of leaves are unreliable indicators of extraction and amplification success and the botanists should annotate how specimens were collected and dried because this information is essential for successful DNA extraction".

3.5.2 Phylogenetic analyses

The phylogenetic analysis by two nuclear regions (ITS and ETS) supports the monophyly of Ociminae with a PP value of 1.0.

The *Orthosiphon* clade is monophyletic if *Or. fruticosus* is transferred to *Ocimum* subgenus *Nautochilus.* However, Paton et al. (2004) showed that *Orthosiphon* is monophyletic with the inclusion of *Fuerstia* and *Hoslundia*, but with a bootstrap of <50%. This differs from the findings presented here, which show a PP =1.0 from ITS and a PP = 0.98 from ETS. *Ocimum* as currently recognised is polyphyletic (Paton, 1997b). By separately recognising the monophyletic subgenus *Nautochilus* and the monophyletic remainder of subgenus *Ocimum* and subgenus *Gymnocimum* produces a pair of monophyletic groups (Paton et al, 2004). In this study, *Ocimum* clade is monophyletic with the inclusion of *Nautochilus*, with a PP 1.0 from ETS and with a PP 0.51 from ITS, if the taxonomic change was done to include *Or. fruticosus* in *Ocimum* subgenus *Nautochilus*. This study has revealed that *Platostoma* clade is monophyletic and also *Haumaniastrum* clade, but Paton et al. (2004) reported that *Platostoma* is monophyletic with the inclusion of *Basilicum* and *Haumaniastrum*. The *Syncolostemon* clade is monophyletic, this finding is consistent with that of Paton et al. (2004) and Otieno et al. (2006). *Catoferia* and *Endostemon* clades are monophyletic for each genus with a PP value of 1.0 from nrDNA regions in this study.

In this study I aimed to determine the generic delimitation and investigate the relationships among the genera of *Orthosiphon*, *Fuerstia*, and *Hoslundia.* Based on ITS and ETS region

evidence, it was found that the *Fuerstia* and the *Hoslundia* group is a sister to *Orthosiphon* clade, with a PP value of 1.0 by ITS and with a PP value of 0.95 by ETS. Surprisingly, *Hoslundia* was found nested with *Fuerstia* with a PP 1.0 from ITS and with a PP 0.99 from ETS. Because these genera form a monophyletic group, *Hoslundia* and *Fuerstia* will be merged under the earliest name *Hoslundia*, therefore, the name of *Fuerstia* should be changed to *Hoslundia*. Therefore, some changes to the taxonomy/nomenclature are required.

According, the relationship between *Ocimum* and subgenus *Nautochilus,* some studies have been unable to demonstrate the relationship between them. Al-Qassabi (2014) recommended placing *Nautochilus* into a new genus because of differences between it and the Asian clade, but two regions were not sufficient to verify these findings. From this study, *Ocimum* subgenus *Nautochilus* is a sister to the rest of *Ocimum* and *Orthosiphon fruticosus* belongs in this subgenus.

The *Catoferia* clade is close to *Ocimum* when using ETS and close to *Ocimum* and *Basilicum* when using ITS. This outcome is contrary to that of Paton et al. (2004), who found that *Catoferia* is more closely related to *Platostoma.*

The *Platostoma* clade is a sister to *Haumaniastrum* clade with a PP value of 1.0 from ETS and a PP = 0.95 from ITS. The *Haumaniastrum* clade is more closely related to the subgenus *Platostoma,* section *Limniboza,* subsection *Rotundifolia.* This result is consistent with Paton's (1997b) finding which showed that *Haumaniastrum* is more closely related to *Platostoma* sect. *Limniboza* rather than *Acrocephalus.* Overall, this clade supports the classification determined by Paton (1997b).

Most previous recognised genera (*Catoferia*, *Endostemon, Syncolostemon, Platostoma*, and *Haumaniastrum*) when sampled formed a clade in the molecular analysis by ITS and ETS regions. These results offer a framework for making decisions regarding taxonomy in terms of genera as, previously mentioned, and provide a starting point for greater comprehension of the phylogeny of Ociminae.

3.6 Conclusion

This chapter, based on nuclear ribosomal DNA regions, determine the generic delimitation and investigate the relationships among Ociminae. *Fuerstia* and *Hoslundia* are the sister group to *Orthosiphon. Hoslundia*, a monotypic genus originally placed in the Ociminae, forms part of a small clade nested within the genus *Fuerstia*. Because these genera form a monophyletic group, *Hoslundia* and *Fuerstia* will be merged under the earliest name *Hoslundia* later on in the paper, therefore, the name of *Fuerstia* should be changed to *Hoslundia*. *Ocimum* subgenus *Nautochilus* is a sister to the rest of *Ocimum* (subgenus *Ocimum* and subgenus *Gymnocimum*). *Orthosiphon fruticosus* is a member of *Ocimum* subgenus *Nautochilus.* Therefore, some nomenclatural changes

are necessary if generic delimitation and nomenclature are to reflect phylogeny. *Platostoma* is a sister to *Haumaniastrum.*

The monophyly of Ociminae, *Catoferia*, *Endostemon*, *Syncolostemon, Platostoma*, and *Haumaniastrum*, were confirmed. *Orthosiphon* is monophyletic if *Or. fruticosus* is excluded, *Fuerstia* is monophyletic with inclusion *Hoslundia, Ocimum* is monophyletic with the inclusion subgenus *Nautochilus* after including *Orthosiphon fruticosus.*

Chapter 4 : Combined analyses

4.1 Introduction

Phylogenetic incongruence can be caused by gene duplication and loss, incomplete lineage sorting, and horizontal gene transfer (Mallo & Posada, 2016) or by lack of signal against random mutation noise. Combining regions aims to overcome these problems can result in the greater precision of species resolution, in order to reflect the ideal species tree more accurately than when using single gene phylogenetic trees (Mallo & Posada, 2016). It has been shown that many loci may be necessary for the resolution of certain clades (Leaché & Rannala, 2010; Liu & Yu, 2011), particularly when DNA sequences may not have diverged sufficiently to resolve a phylogeny using a single locus (Beltrán et al., 2002). Concatenated regions are likely to have been a positive influence on phylogenetic resolution unless they have an incongruent phylogenetic signal.

4.2 Aims and objectives

The main aim in this chapter is to discover where the data set between different genes congruent, and if so, then where better resolution than from nuclear or plastid genes alone can be gained to determine the generic delimitation the genera of *Orthosiphon, Fuerstia*, and *Hoslundia*; and between *Ocimum* and *Nautochilus* based on combined cpDNA and nrDNA regions.

The objectives

1. Test if the subtribe Ociminae remains a monophyletic group.
2. Test if *Orthosiphon, Ocimum,* and *Fuerstia* remain monophyletic groups.

4.3 Materials and methods

Plant samples were listed in Chapter 2 (Appendix 1), DNA extraction is detailed in section 2.3.2. Data generated in Chapters 2–3 were used in this chapter.

4.3.1 Sequence concatenation

Sequence alignments of *trn*L–*trn*F, *trn*H–*psb*A, *rps*16, *mat*K, ITS and ETS, were concatenated using Mesquite (Maddison & Maddison, 2018). Concatenated alignments were then automatically edited then manually so that gaps without data did not remain. In order to check for incongruencies between gene trees, it was compared strict consensus trees from preliminary parsimony phylogenetic analyses of the six genetic regions.

4.3.2 Phylogenetic analyses

Phylogenetic analyses were performed as in section 2.3.5. Separate models (partitioned models) were specified for each dataset within concatenated alignments and were as follows: GTR+G for *trn*H-*psb*A, GTR+G for *mat*K, GTR+G for *rps*16, GTR+I+G for *trn*L-t*rn*F, GTR+G for ITS and GTR+I+G for ETS. Analyses of combined regions were run for 10,000,000 generations.

4.4 Results

4.4.1 Phylogenetic analysis for combined cpDNA regions

All species from the subtribe Ociminae were retrieved as a monophyletic group with a PP of 1.0 (Figure 4.1).

All species from *Catoferia*, *Syncolostemon* and *Endostemon* formed clades with a PP 1.0 (Figure 4.1, Clade A, C and E). The *Platostoma* clade is a sister to the *Haumaniastrum* clade with a PP 1.0 (Figure 4.1, Clade B), in which all species from *Platostoma* formed a clade with a PP 1.0 and all species from *Haumaniastrum* formed a clade with a PP 1.0. All species from *Orthosiphon*, *Fuerstia* and *Hoslundia* formed a clade (Figure 4.1, Clade D) with a PP 1.0, in which all species from *Orthosiphon* formed a clade with a PP 1.0 except for one species belonging *Ocimum*, therefore, it is not a monophyletic group. All species from *Fuerstia* and *Hoslundia* form a clade with a PP 1.0. The *Fuerstia* and *Hoslundia* clade is a sister to the *Orthosiphon* group. *Basilicum*, *Benguellia* and all species from *Ocimum* formed a clade with a PP 0.92 (Figure 4.1, Clade F), in which all species from *Ocimum* formed a clade with a PP 1.0, with one species from *Orthosiphon* (*Or. fruticosus*) in *Ocimum* subgenus *Nautochilus*, therefore, *Ocimum* is a paraphyletic group.

4.4.2 Phylogenetic analysis for combined nrDNA regions

All species from the subtribe Ociminae were retrieved as a monophyletic group with a PP of 1.0 (Figure 4.2).

All species from *Syncolostemon* and *Catoferia* formed clades with a PP 1.0 (Figure 4.2, Clade A and B). The *Platostoma* clade is a sister to *Haumaniastrum* with a PP 1.0 (Figure 4.2, Clade C), in which all species from *Platostoma* formed a clade with a PP 1.0 and all species from *Haumaniastrum* formed a clade with a PP 1.0. The *Haumaniastrum* clade was found to be most closely related to *Platostoma* sect. *Limniboza* subsection *Rotundifolia. Basilicum* and all species from *Ocimum* formed a clade with a PP 0.69 (Figure 4.2, Clade D), in which all species from *Ocimum* formed a clade with a PP 1.0, with one species from *Orthosiphon* (*Or. fruticosus*) in *Ocimum* subgenus *Nautochilus*, so *Ocimum* is a paraphyletic group. *Benguellia* and all species from *Endostemon* formed a clade with a PP 1.0 (Figure 4.2, Clade E), in which all species from *Endostemon* formed a clade with a PP 1.0. All species from *Orthosiphon*, *Fuerstia* and *Hoslundia* formed a clade (Figure 4.2, Clade F) with a PP 1.0, in which all species from *Orthosiphon* formed a clade with a PP 0.97 except for one species belonging *Ocimum*, therefore, it is not a monophyletic group. All species from *Fuerstia* and *Hoslundia* formed a clade with a PP 1.0. The *Fuerstia* and *Hoslundia* is a sister to the *Orthosiphon* group.

4.4.3 Phylogenetic analysis for combined cpDNA and nrDNA regions

All species from the subtribe Ociminae were retrieved as a monophyletic group with a PP of 1.0 (Figure 4.3).

All species from *Catoferia* and *Syncolostemon* formed clades with a PP 1.0 (Figure 4.3, Clade A and B). The *Platostoma* clade is a sister to *Haumaniastrum* clade with a PP 1.0 (Figure 4.3, Clade C), in which all species from *Platostoma* formed a clade with a PP 1.0 and all species from *Haumaniastrum* formed a clade with a PP 1.0. The *Haumaniastrum* clade was found to be most closely related to *Platostoma* sect. *Limniboza* subsection *Rotundifolia*. All species from *Orthosiphon*, *Fuerstia* and *Hoslundia* formed a clade (Figure 4.3, Clade D) with a PP 1.0, in which all species from *Orthosiphon* formed a clade with a PP 1.0 except for one species belonging *Ocimum*, therefore, it is not a monophyletic group. All species from *Fuerstia* and *Hoslundia* form a clade with a PP 1.0. The *Fuerstia* and *Hoslundia* clade is a sister to the *Orthosiphon* group. *Benguellia* and all species from *Endostemon* formed a clade with a PP 1.0 (Figure 4.3, Clade E), in which all species from *Endostemon* formed a clade with a PP 1.0. *Basilicum* and all species from *Ocimum* formed a clade with a PP 0.91 (Figure 4.3, Clade F), in which all species from *Ocimum* forms a clade with a PP 1.0 with one species from *Orthosiphon* (*Or. fruticosus*) in *Ocimum* subgenus *Nautochilus*, therefore, it is a paraphyletic group.

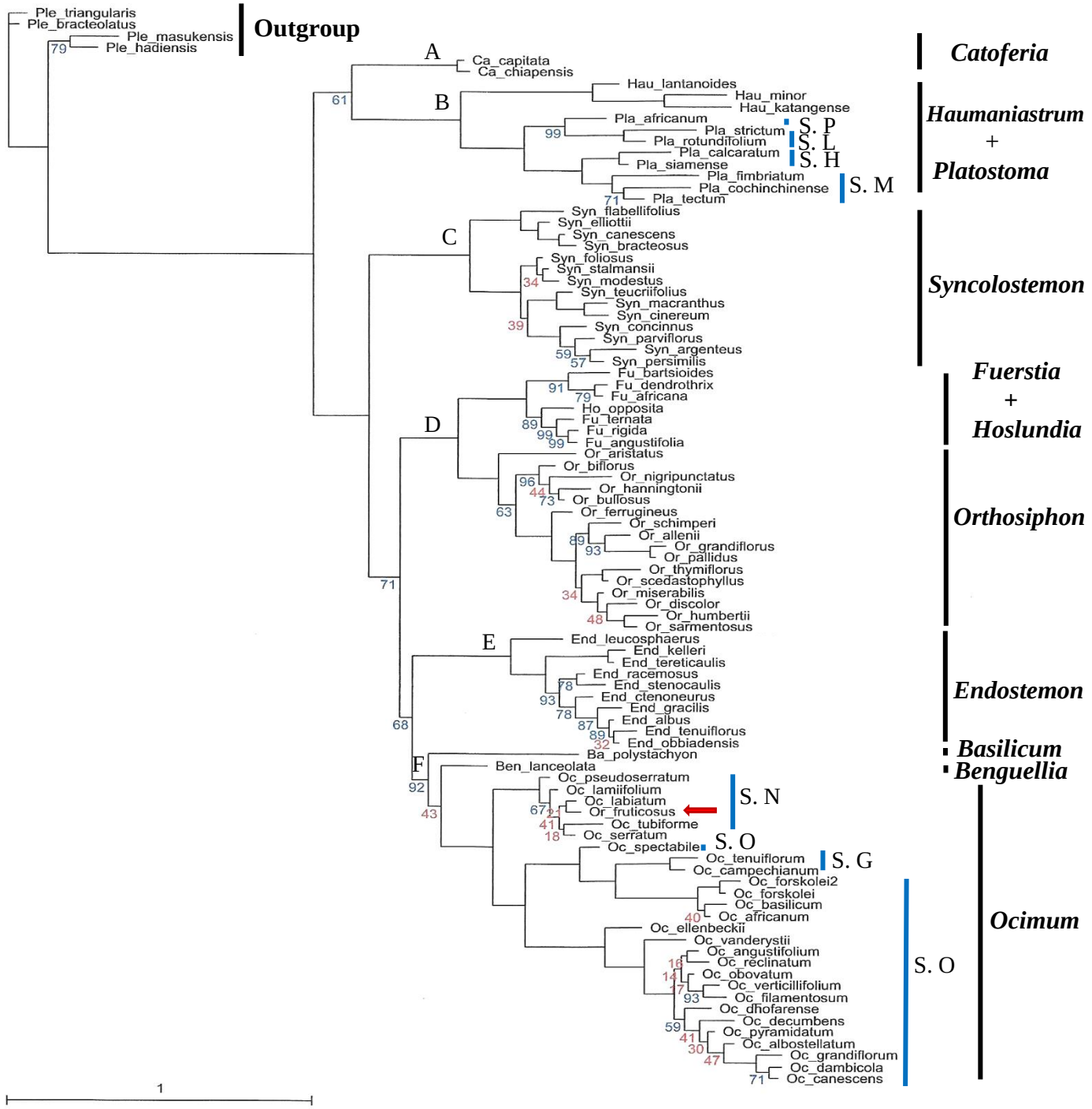


Figure 4.1 Combinable component consensus tree from Bayesian analysis of combined cpDNA regions showing the outgroup, and subtribe Ociminae clades A-F. Posterior probabilities are given below the branches: blue > 0.50 support, red < 0.50, all unlabelled nodes have a posterior probability of 1.0. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum* and Syn = *Syncolostemon*. *Ocimum* subgenera; S. G = Subgenus *Gymnocimum* S. O = Subgenus *Ocimum* and S. N = Subgenus *Nautochilus. Platostoma* sections; S. H = Section *Heterodonta*, S. L = Section *Limniboza*, S. M = Section *Mesona* and S. P = Section *Platostoma*. Dark red arrow indicates the *Orthosiphon fruticosus* which is existed in *Ocimum* group.

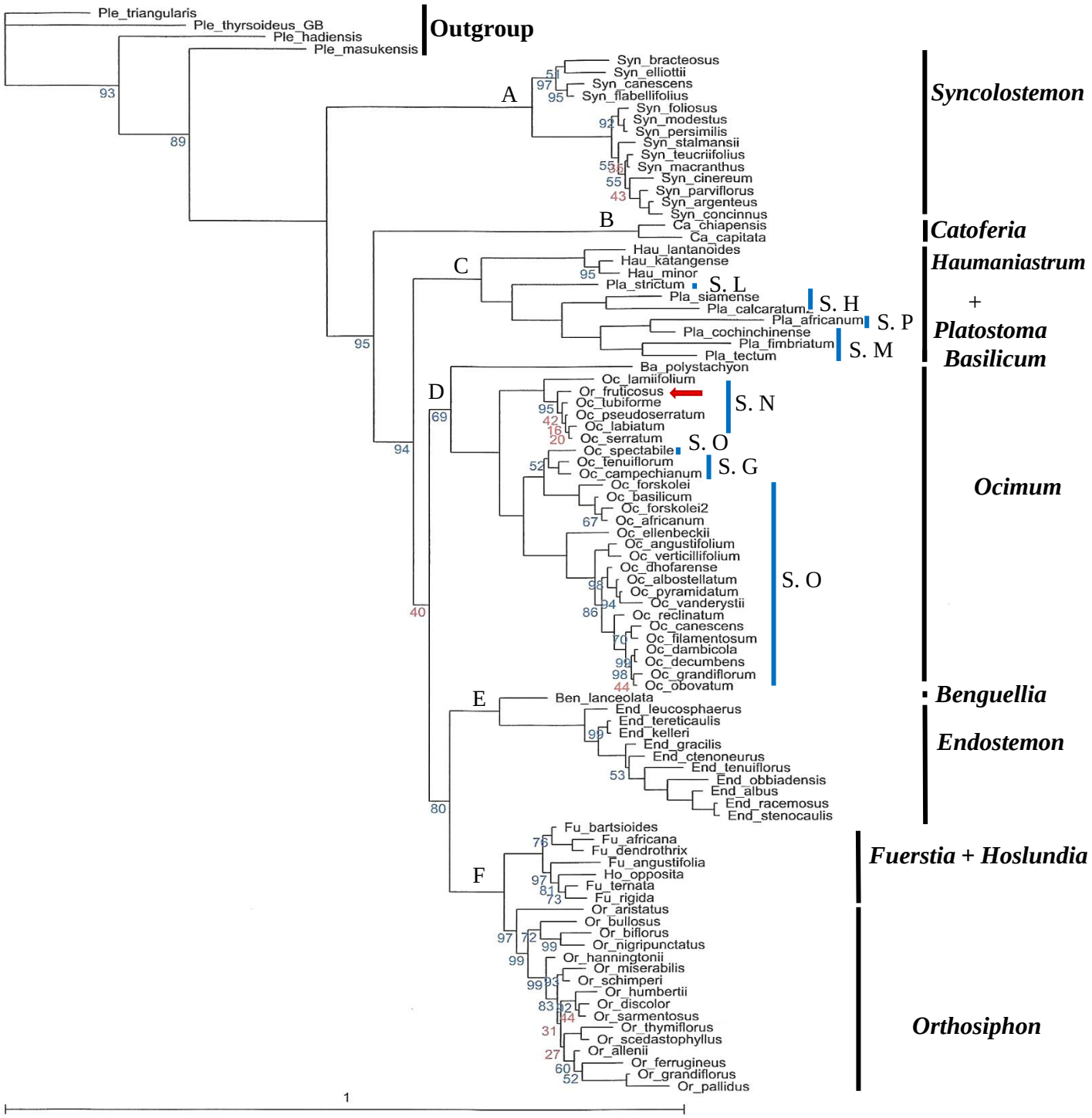


Figure 4.2 Combinable component consensus tree from Bayesian analysis of combined nrDNA regions showing the outgroup, and subtribe Ociminae clades A-F. Posterior probabilities are given below the branches: blue > 0.50 support, red < 0.50, all unlabelled nodes have a posterior probability of 1.0. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum* and Syn = *Syncolostemon*. *Ocimum* subgenera; S. G = Subgenus *Gymnocimum* S. O = Subgenus *Ocimum* and S. N = Subgenus *Nautochilus. Platostoma* sections; S. H = Section *Heterodonta*, S. L = Section *Limniboza*, S. M = Section *Mesona* and S. P = Section *Platostoma*. Dark red arrow indicates the *Orthosiphon fruticosus* which is existed in *Ocimum* group.

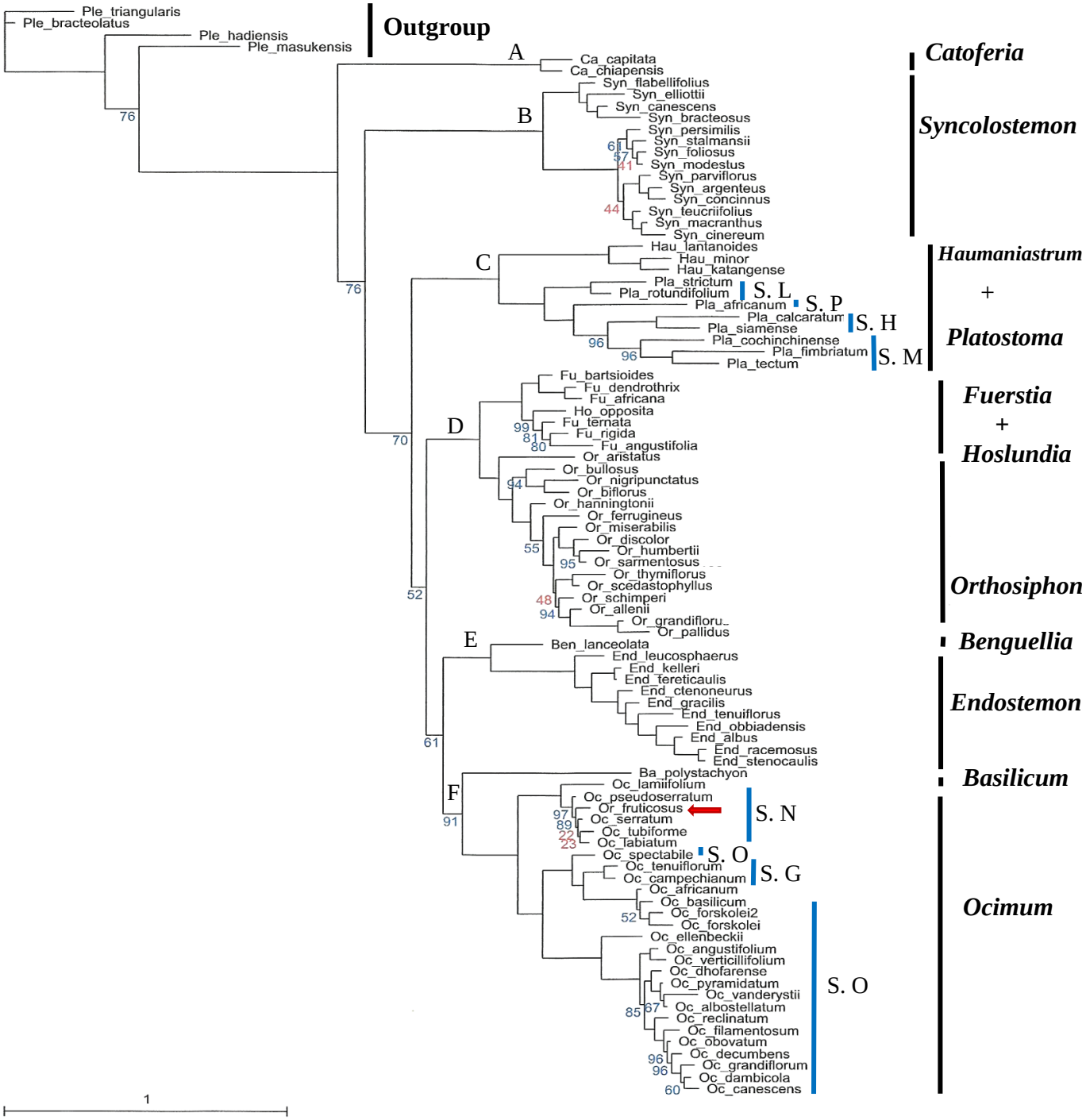


Figure 4.3 Combinable component consensus tree from Bayesian analysis of combined cpDNA and nrDNA regions showing the outgroup, and subtribe Ociminae clades A-F. Posterior probabilities are given below the branches: blue > 0.50 support, red < 0.50, all unlabelled nodes have a posterior probability of 1.0. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum* and Syn = *Syncolostemon*. *Ocimum* subgenera; S. G = Subgenus *Gymnocimum* S. O = Subgenus *Ocimum* and S. N = Subgenus *Nautochilus*. *Platostoma* sections; S. H = Section *Heterodonta*, S. L = Section *Limniboza*, S. M = Section *Mesona* and S. P = Section *Platostoma*. Dark red arrow indicates the *Orthosiphon fruticosus* which is existed in *Ocimum* group.

4.5 Discussion

The combined phylogenetic analysis presented from four chloroplast regions *trn*L–*trn*F, *trn*H–*psb*A, *mat*K, and *rps*16, and two nuclear regions, ITS and ETS, supports the monophyly of Ociminae with a PP value of 1.0. The *Orthosiphon* clade is monophyletic, with a PP= 1.0 from all combined regions of the chloroplast, nuclear, and chloroplast with nuclear, after the taxonomic change to transfer *Or. fruticosus* in *Ocimum* subgenus *Nautochilus*, but the study by Paton et al. (2004) suggested that *Orthosiphon* is monophyletic with the inclusion of *Fuerstia* and *Hoslundia* with a bootstrap of <50%. *Ocimum* as currently recognised is polyphyletic (Paton, 1997b). By separately recognising the monophyletic subgenus *Nautochilus* and the monophyletic remainder of subgenus *Ocimum* and subgenus *Gymnocimum* there by producing a pair of monophyletic groups (Paton et al, 2004). In this study, *Ocimum* clade is monophyletic with the inclusion of *Nautochilus* with a PP 1.0 from all combined regions chloroplast, nuclear and chloroplast with nuclear if the taxonomic change was done to include *Or. fruticosus* in *Ocimum* subgenus *Nautochilus*. This study has revealed that the *Platostoma* clade is monophyletic as is the *Haumaniastrum* clade, but Paton et al. (2004) reported that *Platostoma* is monophyletic with the inclusion of *Basilicum* and *Haumaniastrum*, although *Basilicum* was placed in the ‘‘*Orthosiphon* Group’’ group by Ryding (1993). *Basilicum* is more closely related to *Ocimum* in the combined chloroplast, combined nuclear regions and chloroplast combined with nuclear regions with a PP 0.92, 0.69 and 0.91 respectively. The *Syncolostemon* clade is monophyletic, this finding is consistent with that of Paton et al. (2004) and Otieno et al. (2006). *Catoferia* and *Endostemon* clades are monophyletic for each genus with a PP value of 1.0 from all combined regions: chloroplast, nuclear, and chloroplast combined with nuclear.

Some clades appear in all analyses: the *Syncolostemon* clade, *Haumaniastrum* and *Platostoma* clade, *Fuerstia*, *Hoslundia* and *Orthosiphon* clade, *Endostemon* clade, *Basilicum*, *Ocimum* and *Ocimum* subgenus *Nautochilus* with *Or. fruticosus* clade. Some genera move around; eg. *Benguellia* forms a clade with *Basilicum* and *Ocimum* in combined chloroplast analysis and with *Endostemon* in the combined nuclear analysis and chloroplast with nuclear analysis. *Catoferia* forms a clade with *Haumaniastrum* and *Platostoma* in the combined chloroplast analysis, but it is a single branch in combined nuclear analysis and chloroplast with nuclear analysis. Although there are differences in generic level relationships, all the analyses show that the genera currently recognised are monophyletic with the exception of *Fuerstia*, due to the inclusion of *Hoslundia, Orthosiphon*, due to the exclusion *Or. fruticosus*, and *Ocimum*, due to the inclusion of *Or. fruticosus* (Table 4.1). The incongruence between the regions does not alter any discussion of the genera as monophyletic units.

Table 4.1 Monophyletic Ociminae genera from combined the cpDNA regions analysis, nrDNA regions analysis, and combined cpDNA and nrDNA regions analysis.

The genus	**In combined cpDNA regions**	**In combined nrDNA regions**	**In combined cpDNA and nrDNA regions**
Catoferia	Monophyletic	Monophyletic	Monophyletic
Syncolostemon	Monophyletic	Monophyletic	Monophyletic
Platostoma	Monophyletic	Monophyletic	Monophyletic
Haumaniastrum	Monophyletic	Monophyletic	Monophyletic
Orthosiphon	Monophyletic after the taxonomic change to transfer *Or. fruticosus* in *Ocimum* subgenus *Nautochilus*	Monophyletic after the taxonomic change to transfer *Or. fruticosus* in *Ocimum* subgenus *Nautochilus*	Monophyletic after the taxonomic change to transfer *Or. fruticosus* in *Ocimum* subgenus *Nautochilus*
Fuerstia* + *Hoslundia	Monophyletic with the inclusion of *Hoslundia*	Monophyletic with the inclusion of *Hoslundia*	Monophyletic with the inclusion of *Hoslundia*
Hoslundia	Monotypic	Monotypic	Monotypic
Benguellia	Monotypic	Monotypic	Monotypic
Endostemon	Monophyletic	Monophyletic	Monophyletic
Basilicum	Monotypic	Monotypic	Monotypic
Ocimum* + *Ocimum* subgenus *Nautochilus	Monophyletic after include *Or. fruticosus* in *Ocimum* subgenus *Nautochilus*	Monophyletic after include *Or. fruticosus* in *Ocimum* subgenus *Nautochilus*	Monophyletic after include *Or. fruticosus* in *Ocimum* subgenus *Nautochilus*

One of the principal aims of this project was to identify the relationships among the genera of *Orthosiphon*, *Fuerstia*, and *Hoslundia.* The *Fuerstia* and *Hoslundia* group is a sister to the *Orthosiphon* group, with a PP value of 1.0 through cpDNA regions, nrDNA regions, and combined cpDNA and nrDNA regions. What is surprising in the current study is that *Hoslundia* was found nested with *Fuerstia* with a PP 1.0 from all combined regions. These findings suggest that *Fuerstia* is monophyletic with the inclusion of *Hoslundia.* It can thus be suggested that because these genera form a monophyletic group, *Hoslundia* and *Fuerstia* will be merged under the earliest name *Hoslundia*, therefore, the name of *Fuerstia* should be changed to *Hoslundia.* Nomenclatural changes will be necessary if the taxonomy and nomenclature are to reflect this phylogeny.

According, the relationship between *Ocimum* and subgenus *Nautochilus,* some studies have been unable to demonstrate the relationship between them. Al-Qassabi (2014) recommended placing *Nautochilus* into a new genus because of differences between it and the Asian clade, but two regions were not sufficient to verify these findings. From this study, *Ocimum* subgenus *Nautochilus* is a sister to the rest of *Ocimum* and *Orthosiphon fruticosus* belongs in this subgenus.

The *Platostoma* clade is a sister to the *Haumaniastrum* clade with a PP = 1.0 from all combined regions of the chloroplast, nuclear, and chloroplast with nuclear. The *Haumaniastrum*

clade is more closely related to the subgenus *Platostoma,* section *Limniboza,* subsection *Rotundifolia* based on the combined nuclear regions and combined chloroplast and nuclear regions. This result is consistent with Paton (1997b), who found that *Haumaniastrum* is more closely related to *Platostoma* sect. *Limniboza* rather than *Acrocephalus.* However, from combined chloroplast regions, it can be seen that it is closely related to *Platostoma* sect. *Platostoma.*

Because *Benguellia* and *Catoferia* show different relationships in different analyses I cannot really conclude much about their relationships.

The results obtained from the phylogeny of the subtribe Ociminae are based on combined regions showing most previous recognised genera (*Catoferia*, *Endostemon, Syncolostemon, Platostoma* and *Haumaniastrum*) when sampled formed a clade in the molecular analysis. Concatenation of regions in the current study has been shown to improve Ociminae phylogenetic resolution generic delimitation and increases the support values for clades. This result, regarding the benefit of combined regions, was also reported by Bena et al. (1998), Leaché and Rannala (2010), and Liu and Yu (2011). Strong trees and better resolution resulted from a combined analysis of the datasets; therefore, this method shows great promise.

4.6 Conclusion

This chapter, based on combined chloroplast, nuclear and combined chloroplast and nuclear regions, determine the generic delimitation and investigate the relationships among the subtribe Ociminae. *Fuerstia* and *Hoslundia* are the sister group to *Orthosiphon. Hoslundia*, a monotypic genus originally placed in the Ociminae, forms part of a small clade nested within the genus *Fuerstia*. Because these genera form a monophyletic group, *Hoslundia* and *Fuerstia* will be merged under the earliest name *Hoslundia* later on in the paper, therefore, the name of *Fuerstia* should be changed to *Hoslundia*. *Ocimum* subgenus *Nautochilus* is a sister to the rest of *Ocimum* (subgenus *Ocimum* and subgenus *Gymnocimum*). *Orthosiphon fruticosus* is a member of *Ocimum* subgenus *Nautochilus.* Therefore, nomenclature changes are necessary.

Based on combined chloroplast, nuclear and combined chloroplast and nuclear regions, it was identified that the monophyly of the subtribe Ociminae is well-supported, *Catoferia*, *Endostemon, Syncolostemon, Platostoma* and *Haumaniastrum,* were confirmed. *Orthosiphon* is monophyletic if *Or. fruticosus* is excluded, *Fuerstia* is monophyletic with inclusion *Hoslundia*, and *Ocimum* is monophyletic with the inclusion subgenus *Nautochilus* after including *Orthosiphon fruticosus.* Results show clear improvements in resolution from combined regions and outperform individual regions.

Chapter 5 : Evolution of the subtribe Ociminae

5.1 Introduction

5.1.1 Mapping morphological characters

Morphological character evolution is problematic in terms of examination as it does not often occur at a swift enough pace for direct observation; however, evolutionary processes can often be observed by characters are distributed in living organisms (Zhang et al., 2008). Thus for any specific phylogenetic tree, the evolutionary history for individual characters may be reconstructed (Zhang et al., 2008). To recreate individual character states and observe morphological character evolution, mapping morphological characters onto phylogenetic trees is effective (Zhang et al., 2008), as is observing the way states are distributed through the internal nodes (Pasqualin et al., 2017). Thus character mapping with phylogenies offers the historical framework that is necessary to comprehend the ways in which morphological traits have evolved and been distributed.

Wiens (2004) reported that "Until we reach the stage where all molecular phylogenies are reconstructed without error, it is still important to have rigorous, morphology-based phylogenies as a "reality check" for molecular results". It has been argued (Pasqualin et al., 2017; Wiens, 2004) that although molecular data undoubtedly has many virtues, it remains essential to continue with the collection of extra morphological data to use in phylogenetic analysis and to persist with improving methodologies in terms of morphology-based phylogenetics.

There are several studies on mapping morphological characters on phylogenetic trees to investigated staminal evolution in Lamiaceae. For example, traditionally the genus *Salvia* has encompassed all members of the tribe Mentheae (Lamiaceae) that have only two stamens where each stamen expresses an elongate connective (Walker & Sytsma, 2006). Schematic sketches have been offered for the staminal morphology of all major lineages of *Salvia* and related genera (Walker & Sytsma, 2006). This analysis indicated that the staminal elongate connective had independent origins on at least three points in the history of the tribe Mentheae, with a unique morphology on each occasion (Walker & Sytsma, 2006); it is presumed that such features of the stamens are unique to flowering plants and increase the presumably of pollination being successful (Claßen-Bockhoff et al., 2004; Walker et al., 2004). The researchers' findings demonstrate that the "*Salvia* clade" results from shared traits where two stamens had been aborted, showing that "staminal morphology is the major defining character in *Salvia*, as well as being integral to the current subgeneric organization of the genus " (Walker et al., 2004; Walker & Sytsma, 2006).

Within the Mentheae tribe, there have been a minimum of four transitions between four and two stamens, this occurs a minimum of two times within the subtribe Menthinae, and once each in the subtribes Salviinae and Lycopinae, when using BayesTraits for the inference of the evolution of stamina within the tribe **(**Drew & Sytsma, 2012). Stamen morphology in Mentheae has been

significantly influenced by parallel evolution. However, Will and Claßen-Bockhoff (2014) do not agree that stamen morphology is paramount in lineage delimitation.

Moreover, the morphological data were applied to the inferred phylogenies of *Pogogyne* in order to reconstruct the ancestral morphological states and how they evolved on the tree. The common ancestor of *Pogogyne* clearly had four fertile stamens, based on both parsimony optimization and likelihood analysis. The analyses suggest that the change from a conspicuous corolla to an inconspicuous corolla and the change from four fertile stamens to two, happened on the same internal branch; these changes are therefore clear apomorphies for the subgenus *Hedeomoides*. Both changes might have occurred because there was possibly a shift from outcrossing to self-pollination along that same branch (Silveira, 2010). Therefore, it is useful to study how various morphological characters evolved within the genus, using the inferred phylogenies acquired.

Molecular divergence is not necessarily commensurate with morphological change (Donoghue & Sanderson, 1992), In Lamiaceae, incongruence between molecular phylogenetic trees and infrageneric classifications based mainly on morphology is common, as seen, for example, in *Clerodendrum* L. (Steane et al., 1999), *Hemigenia* R. Br. (Guerin, 2008), *Mentha* L. (Bunsawat et al., 2004), *Minthostachys* (Benth.) Spach (Schmidt-Lebuhn, 2008), and *Isodon* (Schrad. ex Benth.) Spach (Zhong et al., 2010). However, Xiang et al.'s (2013) analysis displayed strong congruence between the consensus tree based on DNA analysis and the morphology-based infrageneric classification of Wu and Li (1965, 1977). Wu and Li (1965, 1977) recognised two morphological characters, habit and calyx shape, as being the most useful for determining relationships within *Chelonopsis*, especially at the subgeneric level. The molecular results by Xiang et al. (2013) showed that "*Chelonopsis* comprises two clades, one encompassing the taxa of *Chelonopsis* subg. *Chelonopsis* and the genus *Bostrychanthera* and the other consisting of *Chelonopsis* subg. *Aequidens*. This split is supported by several morphological characters". It is clear that sometimes morphological variation seems to be correlated with the molecular phylogeny.

No previous work has mapped morphological characters onto a molecular phylogeny in the Ociminae. In the Ociminae the number of fertile stamens is known to vary between two and four, such as in *Fuerstia*, *Hoslundia* and *Ocimum circinatum* have two while four in the rest of the Ociminae.

There is nevertheless a close relationship between *Orthosiphon* and *Fuerstia* as they both have identically shaped calyx and corolla. Within *Orthosiphon*, *Fuerstia* shows the greatest similarity to the subgenus *Orthosiphon* that also exhibits a clavate style with small, rounded lobes, a four-lobed disk having the anterior lobe largest and posterior stamens which are attached to the corolla above the mid-point of the tube. Paton (1993) created intense doubt on the generic

delimitation of *Fuerstia* from *Orthosiphon* based on morphology. This complex may also include *Hoslundia*. There are identical nutlets in this species (Ryding, 1992), as well as corolla, inflorescence structure, and sterile posterior stamens when compared to *Fuerstia*. However, there are variations in the sense that it has a fleshy, mature calyx. The phylogenetic significance of these morphological characters could not be fully explored because a molecular phylogeny of this subtribe was lacking. This work can clarify the relationships of these taxa and explore the significance of morphological variation within the complex.

There are several morphological characters used to distinguish related genera. *Orthosiphon* is placed in the subtribe Ociminae, characterised by ebracteolate cymes and stamens adnate to the corolla at separate points, usually with the anterior stamens adnate to the corolla at the throat and the posterior stamens adnate to the corolla midway down the tube or nearer the base (Harley et al., 2004). *Fuerstia* is closely related to *Orthosiphon*, having a similar shaped calyx and corolla; however, it is excluded from *Orthosiphon* because it has two fertile stamens (Fries, 1929). *Hoslundia opposita* may have similar nutlets (Ryding, 1992), corolla, inflorescence structure, and sterile posterior stamens to *Fuerstia* (Paton, 1993). However, it differs in having a fleshy, mature calyx. It is clear that the character of sterile posterior stamens is important in distinguishing between *Orthosiphon* on one hand and *Fuerstia* and *Hoslundia* on the other.

Paton et al. (1994) reported that "*Endostemon* can be distinguished from related genera by its short, villous anthers which are attached to the corolla just below the throat, a small shield-like swelling near the base of the style just above the nutlets". *Benguellia* closely resembles *Orthosiphon* in gross morphology and several floral characters, but the nutlets suggest an affinity with the *Platostoma* group (Paton, 1997b). *Catoferia* can be differentiated from *Orthosiphon* by having sessile flowers and a distinct capitate style apex (Harley & Paton, 2012).

Orthosiphon is related to *Ocimum* and *Syncolostemon*; however, *Orthosiphon* is different from both these genera because it has a clavate style with rounded erect lobes rather than the bifid style with subulate, spreading lobes (Paton, 1992). In *Ocimum*, the posterior pair attach near the corolla base, but in *Orthosiphon* subgenus *Orthosiphon*, the posterior pair attach to the corolla at the mid-point of the tube (Paton et al., 1999). *Ocimum* is different from the *Syncolostemon* because it has an equally lobed disk rather than and unequally lobed disk and in having free anterior filaments rather than fused anterior filaments (Paton et al., 1999).

Orthosiphon and the extremely similar *Basilicum* also show very close affinities to *Ocimum*. In these two genera, the fruiting calyx throat is open and glabrous and the corolla is tubular with parallel sides. This combination of characters is also seen in *Ocimum* sect. *Hierocymum* (Paton, 1992). *Orthosiphon* and *Basilicum* can usually be distinguished from *Ocimum* by the clavate rather than the bifid style apex and by their inappendiculate posterior stamens which are adnate to the

corolla at or above the mid-point of the corolla tube, rather than the basally adnate, appendiculate or basally ciliate posterior stamens of *Ocimum* (Paton, 1992).

Platostoma can be recognised from other members of subtribe Ociminae by a combination of coloured bracts and basally swollen and often pubescent posterior staminal filaments (Paton, 1997b). *Haumaniastrum* differs from *Platostoma* by having erect bracts which obscure the calyces, three-flowered cymes in condensed heads, a flattened calyx which curls upwards and can easily be split into its anterior and posterior lips, and with the lateral lobe of the calyx in the same plane as the posterior lobe (Paton, 1997a).

5.1.2 Mapping geographical distribution

Reconstruction of the biogeographical history of plants, through the use of molecular phylogenies the evolutionary history, is aimed at understanding species distribution (Roncal et al., 2012).

Tribe Ocimeae Dumort. is a predominantly Tropical group. There are main centres of diversity in Tropical Africa and Madagascar, China and Indochina, and in South America (Cantino et al., 1992). Many genera in the subtribe Ociminae include a species which is found in India and Africa, e.g. *Basilicum*, *Platostoma*, and *Orthosiphon* (Paton et al., 1994). *Orthosiphon* has one species in Colombia, South America (Harley & Paton, 2012). The genus *Orthosiphon* has been recorded from the Old World in Tropical and Southern Africa, Madagascar, and Tropical or Subtropical Asia (Harley et al., 2004). *Catoferia* has three native species in Mexico and Central America and one species in Colombia (Harley et al., 2004). This genus possibly evolved from an African ancestor (Paton et al., 2004). *Ocimum* has four endemic species from 65 in the New World (Paton et al. 1999). *Ocimum* is more diverse in Africa (Paton et al., 1994). *Endostemon* is widespread in Tropical Africa and there is one species in India, there are two centres of diversity for this genus, Angola and the Somalia-Masai region (Paton et al., 1994). *Platostoma* is found in Tropical Africa, Madagascar, Tropical Asia, and North Queensland (Paton, 1997b). *Haumaniastrum* is recognised with only one species extending out of Africa to Madagascar (Paton, 1997a). *Syncolostemon* is mostly found in Southern Africa (Paton et al., 1999); however, there is one species of *Syncolostemon* in Madagascar and one in India (Paton et al., 1999; Otieno et al., 2006). *Benguellia* found in Angola (Paton, 1997b). *Hoslundia* is widespread in Tropical Africa, Southern Africa, and Madagascar, and *Fuerstia* is widespread in Tropical Africa (*WCSP*, 2018).

Mapping geographic distribution supported an Asiatic origin for Ocimeae (Paton et al., 2004). It is not obvious what the migration mechanism was, though there are possibilities of routes from either Africa or East Asia (Paton et al., 2004). Otieno et al. (2006) suggested that the Afroalpine and Afromontane archipelago-like regional centre of Endemism may be the ancestral

area for *Syncolostemon*. Plectranthinae migration occurred from Africa into Madagascar and Asia; evidence of a return migration to Africa does not exist (Paton et al., 2018).

There are some species that are closely related to others found in the same region or other regions. For example, in *Endostemon*, the only Indian species is closely related to an African one (Paton et al., 1994). The mainly African genus *Haumaniastrum* is found to be more closely related to the African *Platostoma* sect. *Limniboza* rather than the Asiatic sect. *Acrocephalus* (Paton, 1997b). The Indian species of *Ocimum* either is similar to African species or such as *Oc. tenuiflorum* L., which is very close to some South American ones (Paton, 1998). The fewer Indian and South-East Asian members of *Orthosiphon* are either very similar to African species (e.g., *Or. thymiflorum*) or very different by having long exserted stamens (e.g., *Or. aristatus*) (Paton, 1998). Mapping geographical distribution can be used to investigate the distribution of the subtribe Ociminae and the relationships between species based on their regions.

5.2 Aim and Objectives

In this chapter, the first attempt to use the phylogeny to investigate the morphological and geographical distribution of the clades of subtribe Ociminae to further our understanding of the relationships between species and genera was made.

The objectives

1. To examine how morphological characters and geographical occurrence is distributed within the subtribe Ociminae.
2. To re-evaluate the taxonomic status of *Orthosiphon*, *Fuerstia* and *Hoslundia* using both molecular and morphological data.
3. To examine the degree of matching of the molecular data from this study with the existing morphological classifications based on the vegetative (leaf) and floral characters and propose changes to the classification of the group if not so.
4. To assess the taxonomic value of selected characters such as the number of stamens.
5. Mapping geographical continental and regional distribution onto phylogenetic trees of the subtribe Ociminae to investigate geographical distribution in the context of phylogeny.

5.3 Materials and methods

5.3.1. Plant materials

Samples were collected (see Chapter 2).

5.3.2 DNA extraction

Treated in 2.3.2.

5.3.3 PCR amplification, conditions, programs, purification and sequencing

Data generated in Chapters 2-3 were used in this chapter.

5.3.4 Sequence editing

Treated in 4.3.4.

5.3.5 Phylogenetic analysis

See section 4.3.5.

5.3.6 Mapping of morphological characters

Morphological characters used in this study were scored from the literature review (Codd, 1964; Codd, 1976b; Ramamoorthy, 1986; Paton, 1993; Paton et al., 1994; Paton, 1995; Paton, 1997a; Paton, 1997b; Paton et al., 1999; Ryding et al., 2003; Suddee et al., 2005; Otieno et al., 2006; Thulin, 2006; Paton et al., 2009) and examined herbarium material by the researcher where possible from herbarium material at Kew and Reading. From the outset, all character variation was considered potentially informative of relationships. Sixteen characters were identified and are presented below. Eight characters were coded as binary and eight as multistate characters. Fifteen of the characters are associated with inflorescence and flowers while the other ones are derived from the leaves. The morphological data matrix is provided in Appendix 3.

Mesquite v3.51. (Maddison & Maddison, 2018) was used to map morphological characters on the combinable component consensus tree from the Bayesian analysis of the combined molecular datasets. Individual characteristics were traced on the tree to identify morphological synapomorphies.

5.3.6.1 Leaves

5.3.6.1.1Ternate (0): grouped in threes; e.g. an arrangement of leaves in whorls of three (Stevens, 2017); ternately inserted leaves at each node.

Figure 5.1 Photo shows ternate leaves in *Fuerstia ternata* from Paton (1993).

5.3.6.1.2 Opposite (1): leaves borne on the same level yet on opposite sides of the stem node (Stevens, 2017); oppositely inserted leaves at each node.

Figure 5.2 Photo shows opposite leaves in *Platostoma coloratum* from Suddee et al. (2005).

5.3.6.2 Fertile bracts (the bract is often a more or less modified leaf, particularly a smaller one which is associated with a flower or an inflorescence part (PlantNET, 2019)).

5.3.6.2.1 Uniformly green (0): which is the same in all its parts, and is coloured green.

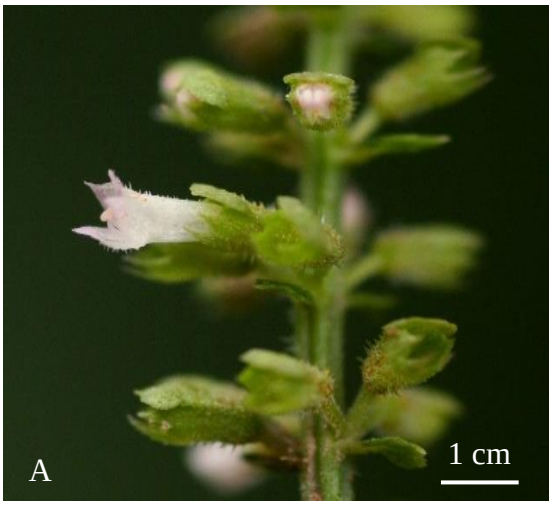


Figure 5.3 Photo shows uniformly green bracts in *Basilicum polystachyon* from Bingham et al. (2019).

5.3.6.2.2 Basally coloured (1): the bract is not green but coloured at its base.

Figure 5.4 Photos show basally coloured bracts. A in *Haumaniastrum caeruleum* and B in *Platostoma rotundifolium* from Bingham et al. (2019).

5.3.6.3 No. of flowers in cyme

5.3.6.3.1 One flower (0): cymes reduced to one flower.

Figure 5.5 Photos show one flower in the cyme. A in *Fuerstia angustifolia* from the Herbarium Catalogue (2019) and B in *Fuerstia dendrothrix* from Paton (1993).

5.3.6.3.2 One to three flowers and variable (1): cymes with a variable number of flowers: one, two and three flowers.

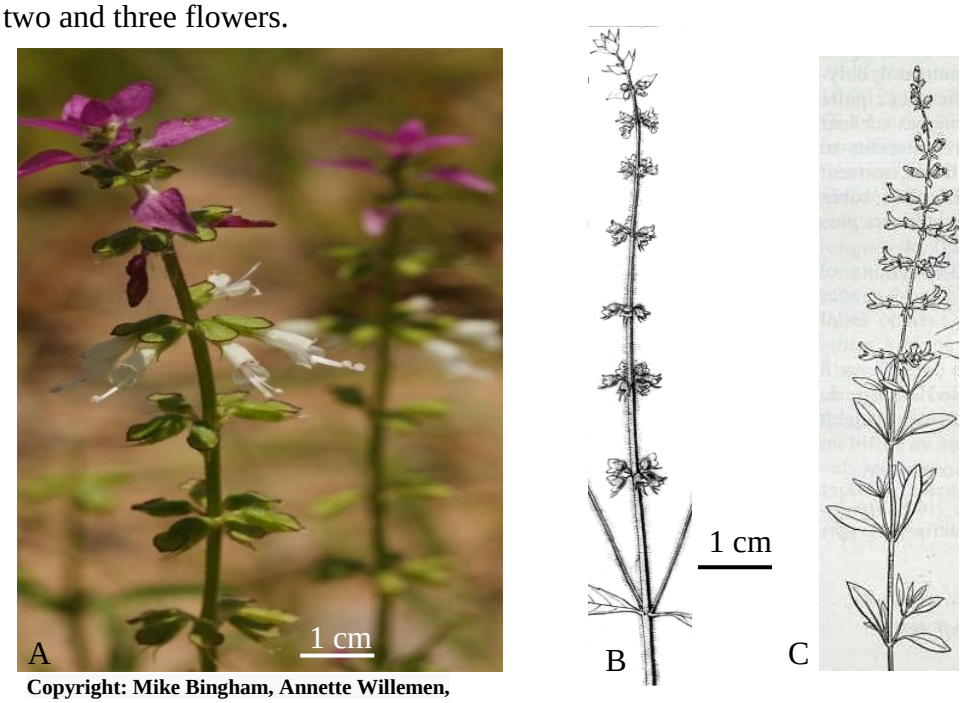


Figure 5.6 Photos show 1-3 variable flowers in the cyme. A in *Syncolostemon bracteosus* from Bingham et al. (2019), B in *Syncolostemon bracteosus* from Watson (1992) and C in *Syncolostemon madagascariensis* from Hedge et al. (1998).

5.3.6.3.3 Three flowers (2): cymes consistently 3-flowered.

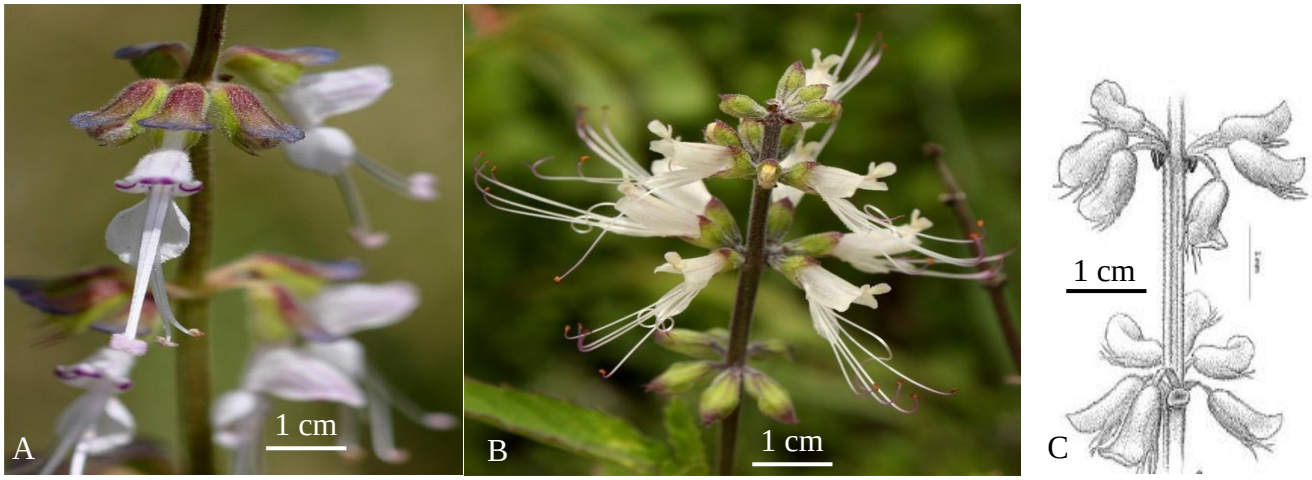


Figure 5.7 Photos show three flowers in the cyme. A in *Ocimum angustifolium* and B in *Ocimum filamentosum* from Bingham et al. (2019) and C in *Ocimum nudicaule* from O'Leary (2017).

5.3.6.3.4 One to many flowers (3): one to many flowers in the cyme, the maximum being more than 3.

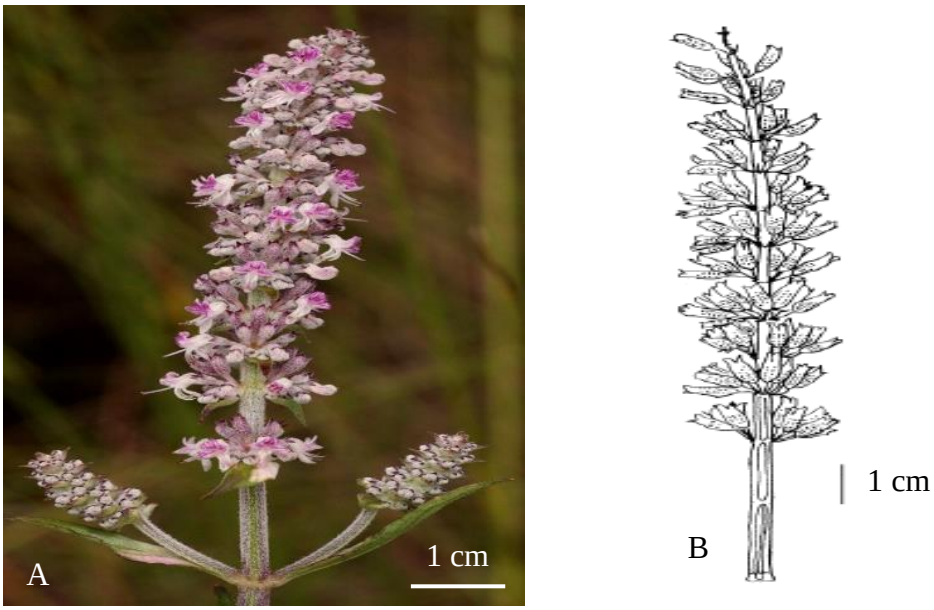


Figure 5.8 Photos show 1-many flowers in the cyme. A in *Platostoma strictum* from Bingham et al. (2019) and B in *Platostoma coloratum* from Suddee et al. (2005).

5.3.6.4 Calyx

5.3.6.4.1 Fleshy mature (0): the calyx is fleshy and coloured brightly (Harley et al., 2004).

Figure 5.9 Photo shows fleshy mature calyx in *Hoslundia opposita* from Bingham et al. (2019).

5.3.6.4.2 Not fleshy mature (1): the calyx does not become fleshy and brightly coloured.

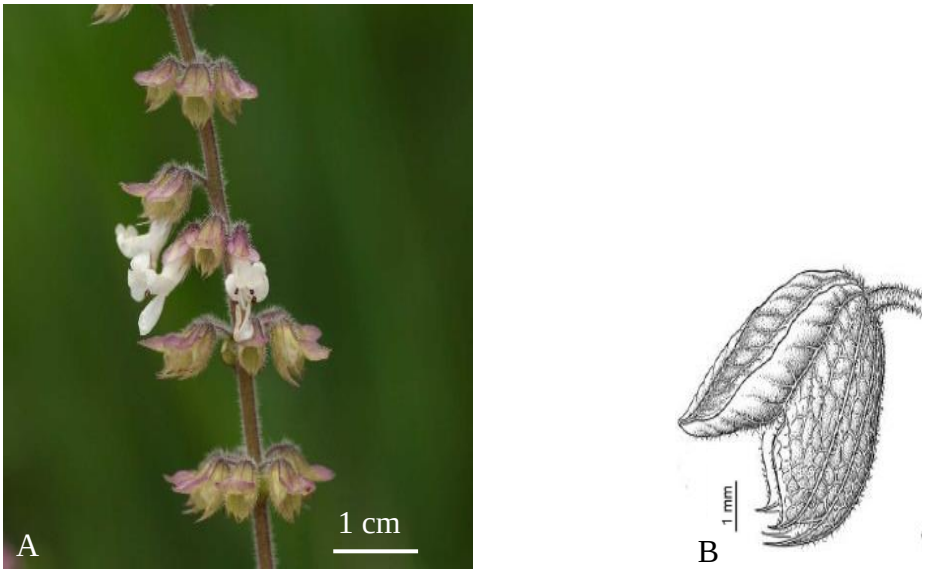


Figure 5.10 Photos show not fleshy mature calyx. A in *Orthosiphon schimperi* from Bingham et al. (2019) and B in *Ocimum ovatum* from O'Leary (2017).

5.3.6.5 Position of lateral calyx lobe

5.3.6.5.1 Median, between posterior and anterior (0): the lateral calyx lobes are usually midway between the median lobes of the anterior lip and posterior lip (Paton et al., 1999).

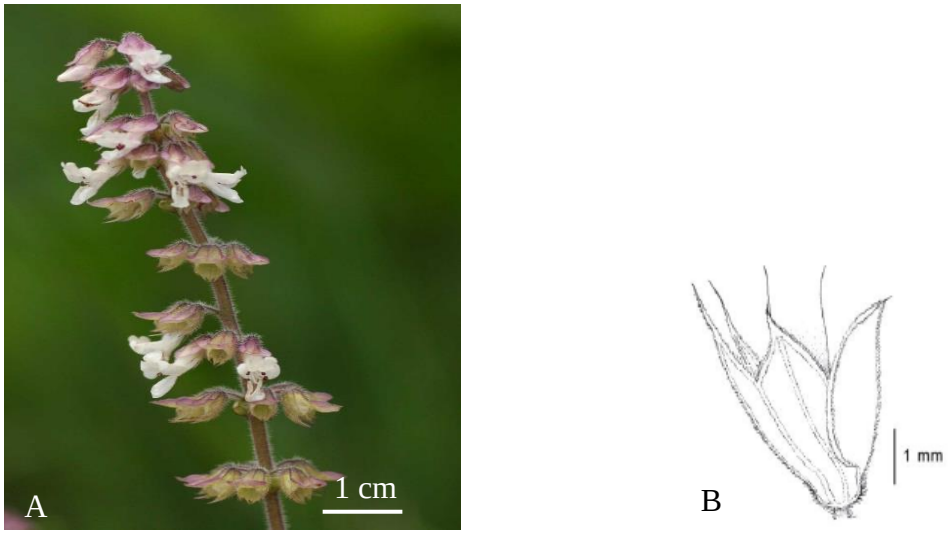


Figure 5.11 Photos show the position of lateral calyx lobe median, between posterior lobe and anterior median lobes. A in *Orthosiphon schimperi* from Bingham et al. (2019) and B in *Ocimum basilicum* from Martínez-Gordillo et al. (2013).

5.3.6.5.2 Close to posterior, not in same plane (1): the lateral calyx lobes are usually located nearer the posterior lobe than to the median lobes of the anterior lip. This provides the impression of a 2-lobed anterior and a 3-lobed posterior lip (Paton et al., 1997b).

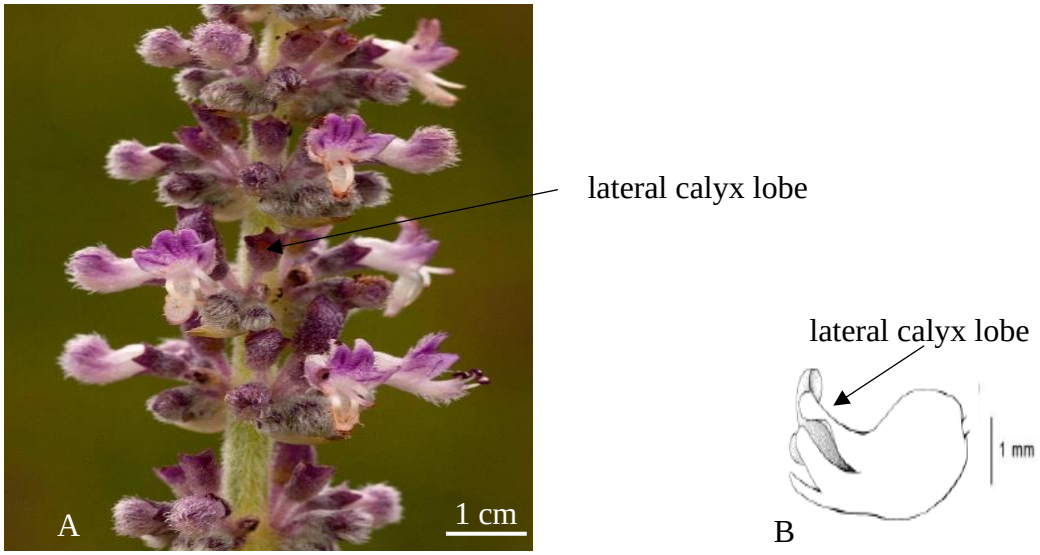


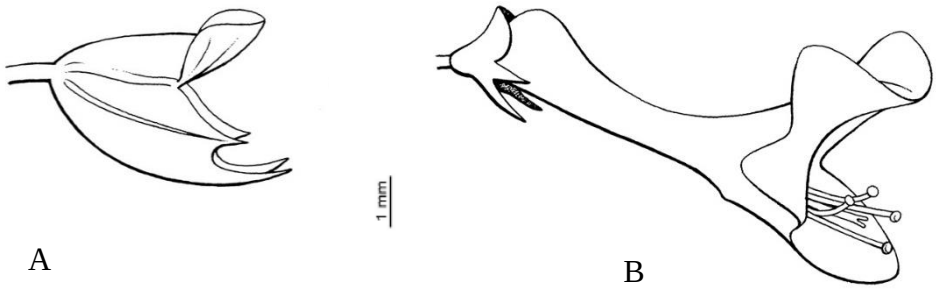
Figure 5.12 Photos show the position of lateral calyx lobe close to posterior, not in same plane. A *Platostoma strictum* from Bingham et al. (2019) and B in *Platostoma coloratum* from Paton (1997b).

5.3.6.5.3 Close to posterior, in same plane (2): the lateral lobes are completely fused to the posterior lobe (Paton et al., 1997b).

A B

Figure 5.13 Photos show the position of lateral calyx lobe close to posterior, in same plane. A in *Haumaniastrum lantanoides* and B in *Haumaniastrum paniculatum* from Paton et al. (1997a).

5.3.6.5.4 Nearer the anterior lobes than posterior lobe (3): the lateral lobes are nearer to the median lobes of the anterior lip than the posterior lip (Paton et al., 1999).



A B

Figure 5.14 Photos show the position of lateral calyx lobe nearer the anterior lobes than posterior lobe. A in *Plectranthus alboviolaceus* and B in *Plectranthus strangulatus* from Paton et al. (2019).

5.3.6.6 Shape of anterior calyx lobes

5.3.6.6.1 Lanceolate (0): the anterior calyx lobes are lance-shaped (lanceolate); 3-6 times as long as broad, being broadest below the middle and tapering to the apex (PlantNET, 2019).

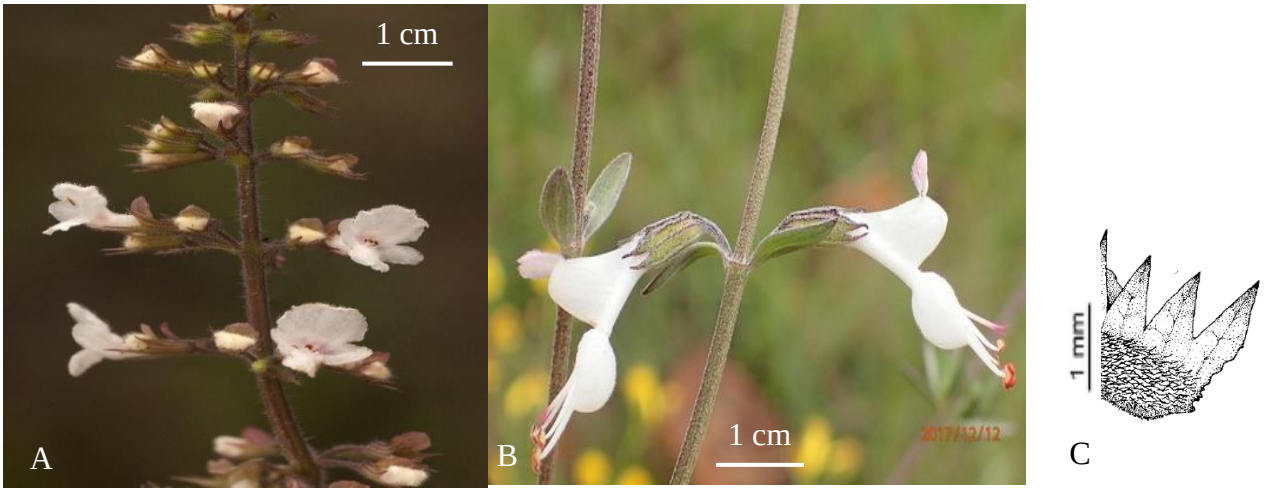


Figure 5.15 Photos show lanceolate of shape of anterior calyx lobes. A in *Endostemon obtusifolius* from Bingham et al. (2019), B in *Syncolostemon parviflorus* var. *parviflorus* from Warren (2017) and C in *Ocimum circinatum* from Paton (1992).

5.3.6.6.2 Subulate (1): the anterior calyx lobes are subulate, meaning they are narrow and taper gradually to a fine point (Stevens, 2017; PlantNET, 2019).

Figure 5.16 Photos show subulate of shape of anterior calyx lobes. A in *Basilicum polystachyon* from Bingham et al. (2019) and B in *Orthosiphon americanus* from (Harley & Paton, 2012).

5.3.6.6.3 Emarginate (2): the anterior calyx lobes are emarginate, being broad and with a shallow notch at the apex (PlantNET, 2019).

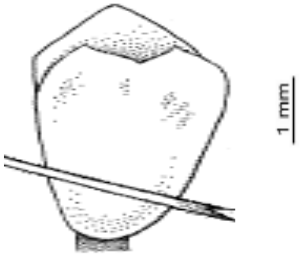
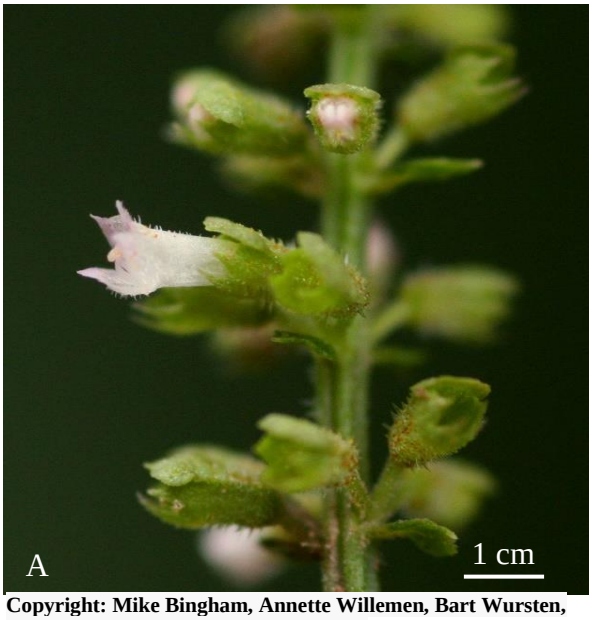
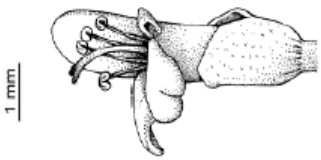


Figure 5.17 Photo shows emarginate of shape of anterior calyx lobes in *Haumaniastrum chartaceum* from Paton (1997a).

5.3.6.7 Corolla tube shape

5.3.6.7.1 Straight (0): the corolla tube is straight not curved or bent.

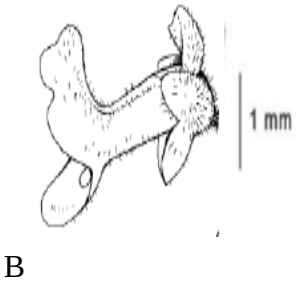




B

Figure 5.18 Photos show straight corolla tube shape. A in *Basilicum polystachyon* from Bingham et al. (2019) and B in *Haumaniastrum chartaceum* from Paton (1997a).

5.3.6.7.2 Curved downward (1): the corolla tube is bent towards a lower place.



B

Figure 5.19 Photos show curved downward corolla tube shape. A in *Syncolostemon bracteosus* from Bingham et al. (2019) and B in *Platostoma intermedium*; the corolla tube is bent downward within calyx from Paton (1997b).

5.3.6.7.3 Curved upward (2): the corolla tube is bent towards a higher place.

C

Figure 5.20 Photos show curved upward corolla tube shape. A and B in *Syncolostemon canescens* from Flora of Southern Africa (2017) and C in *Syncolostemon petiolata* from Otieno et al. (2006).

5.3.6.8 Shape of lower lip corolla

5.3.6.8.1 Flat and the stamens extend over it (0): lower lip corolla is flat not concave and the stamens usually extend over it.

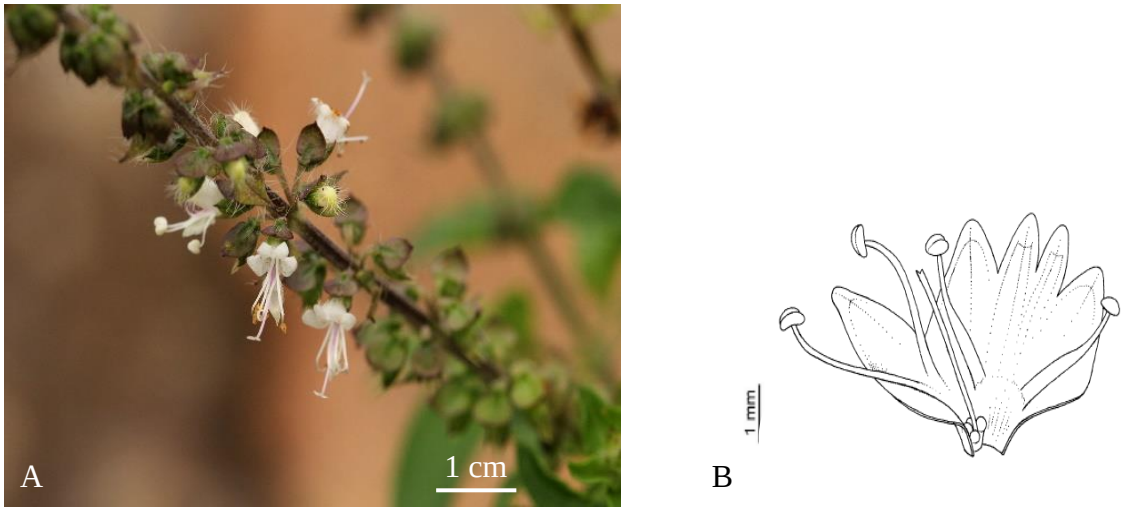


Figure 5.21 Photos show flat and the stamens extend over it in the shape of lower lip corolla. A in *Ocimum americanum* from Bingham et al. (2019) and B in *Platostoma gabonense* from Paton (1997b).

5.3.6.8.2 Boat-shaped and surrounds the stamens (1): lower lip boat-shaped and surrounds the stamens.

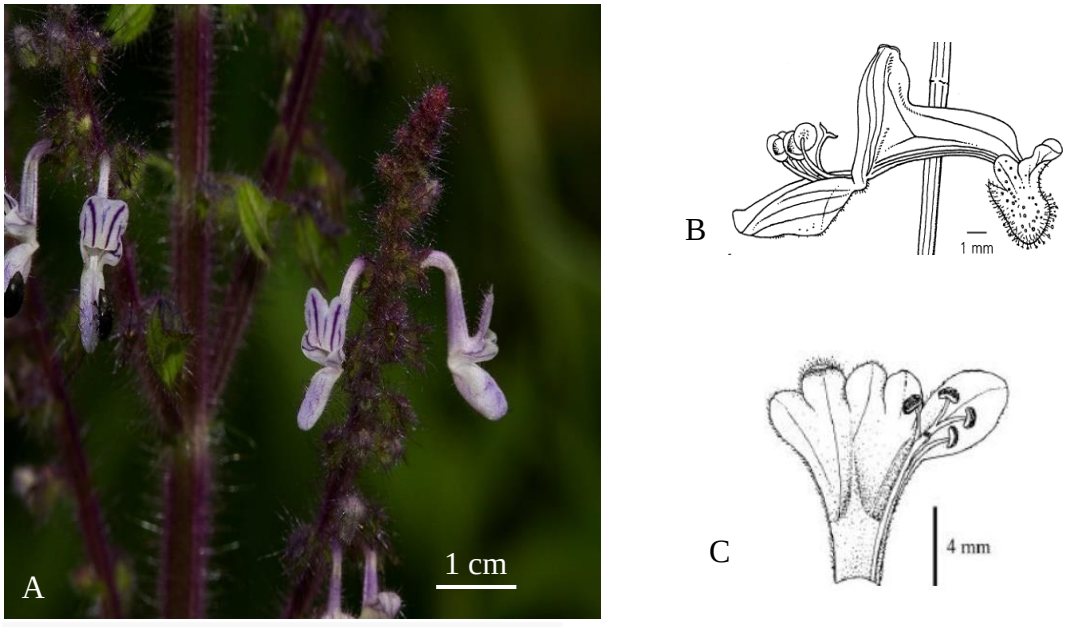


Figure 5.22 Photos show boat-shaped lip that surrounds the stamens. A in *Plectranthus masukensis* var. *masukensis* from Bingham et al. (2019) B in *Plectranthus albicalyx* from Suddee et al. (2005) and C in *Plectranthus phulangkaensis* from Suddee et al. (2014).

5.3.6.9 No. of fertile stamens

5.3.6.9.1 Two (0): two stamens are fertile and two stamens are sterile.

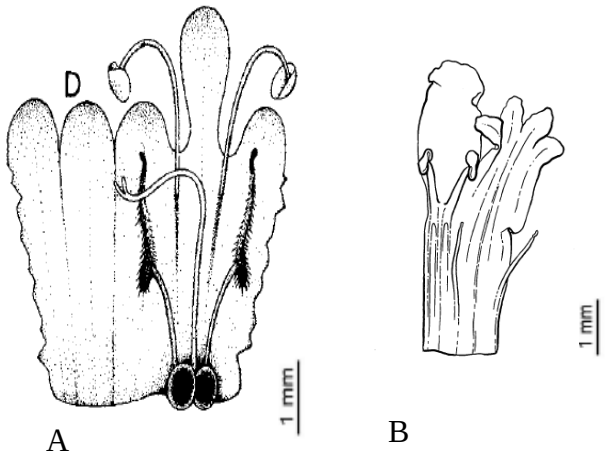


Figure 5.23 Photos show two stamens are fertile. A in *Ocimum circinatum* from Paton (1992) and B in *Fuerstia ternata* from Paton (1993).

5.3.6.9.2 Four (1): four stamens are fertile.

Figure 5.24 Photos show four stamens are fertile. A in *Ocimum filamentosum*, B in *Ocimum obovatum* from Bingham et al. (2019) and C in *Orthosiphon nigripunctatus* from Paton (1993).

5.3.6.10 Stamen

5.3.6.10.1 Included (0): enclosed, do not protrude; stamens held within the corolla tube (Stevens, 2017).

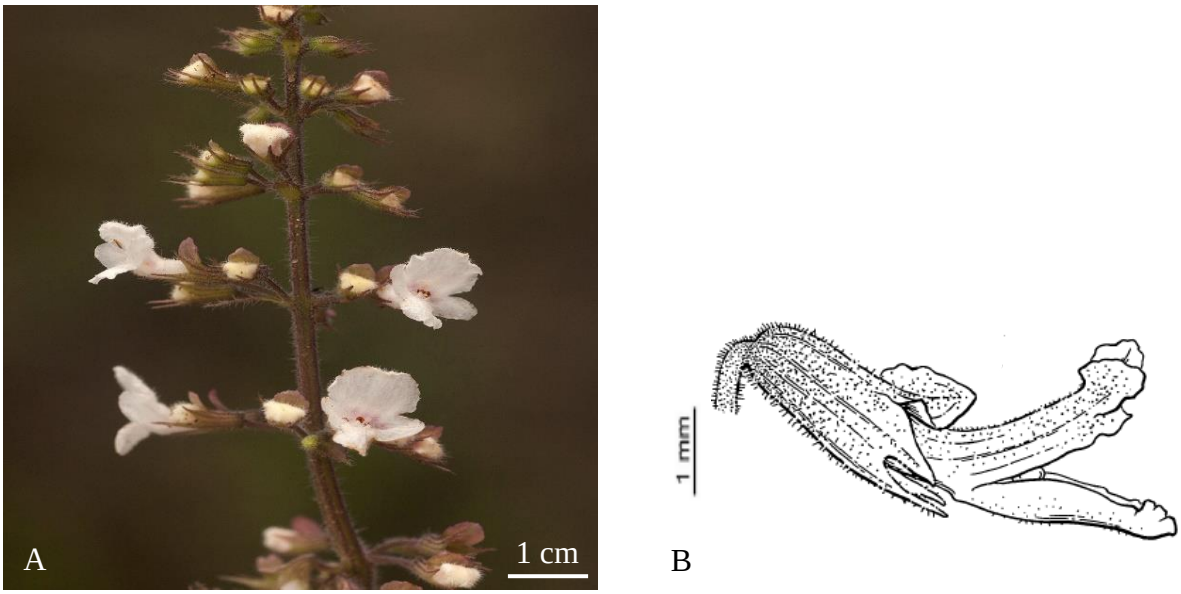


Figure 5.25 Photos show included stamens. A in *Endostemon obtusifolius* from Bingham et al. (2019) and B in *Fuerstia ternata* from Paton (1993).

5.3.6.10.2 Exserted (1): "protruding, e.g. of anthers held outside of the mouth of a corolla tube" (Stevens, 2017).

Figure 5.26 Photos show exserted stamens. A in *Ocimum americanum*, B in *Ocimum filamentosum* from Bingham et al. (2019) and C in *Platostoma tridechii* from Suddee (2010).

5.3.6.11 Fusion of stamens

5.3.6.11.1 No staminal fusion (0): the stamens not fused (not joined together).

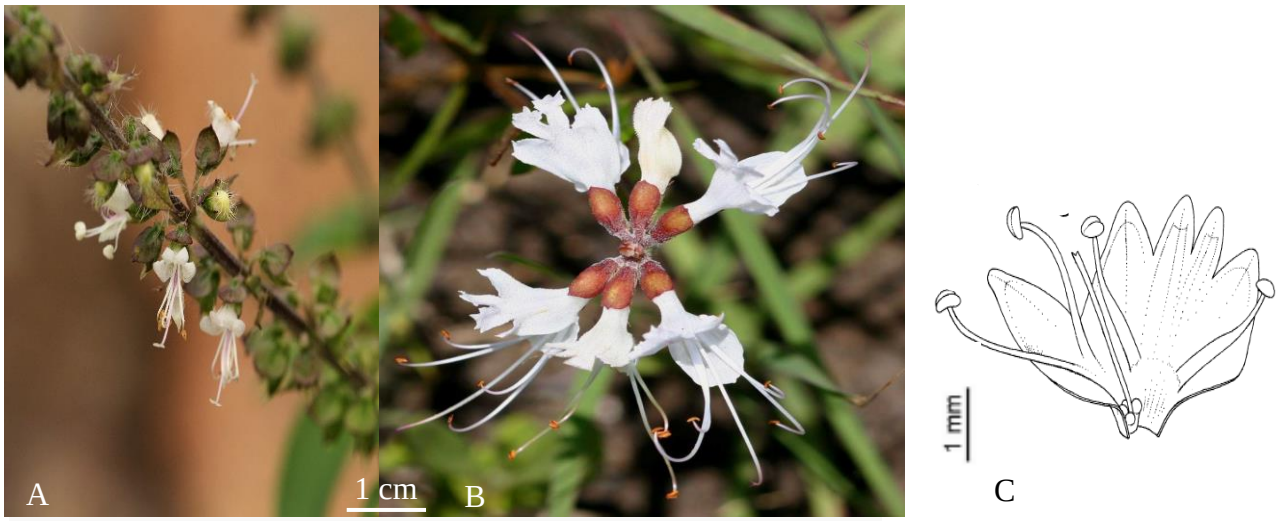


Figure 5.27 Photos show no staminal fusion. A in *Ocimum americanum* and B in *Ocimum obovatum* from Bingham et al. (2019) and C in *Platostoma gabonense* from Paton (1997b).

5.3.6.11.2 All stamens fused, with the two anterior stamens fused together and to the adjacent posterior stamen (1): every stamen is joined, the posterior pair is more shortly connate than the anterior pair (Ryding, 1999).

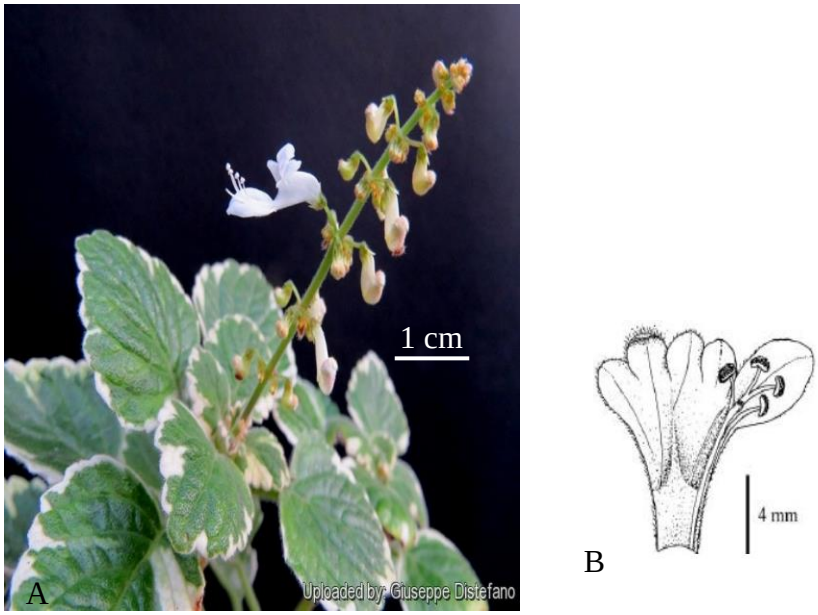


Figure 5.28 Photos show all stamens fused, with the two anterior stamens fused together and to the adjacent posterior stamen. A in *Plectranthus coleoides* from Distefano (2005) and B in *Plectranthus phulangkaensis* from Suddee et al. (2014).

5.3.6.11.3 Anterior stamens only fused, posterior free (2): anterior stamens are joined together, but posterior stamens separate.

Figure 5.29 Photos show anterior stamens only fused, posterior free. A and B in *Syncolostemon parviflorus* var. *parviflorus* from Warren (2017), C in *Syncolostemon densiflorusfrom* from the Indigenous Gardener (2018) and D in *Syncolostemon madagascariensis* from Hedge et al. (1998).

5.3.6.12 Attachment of posterior stamens

5.3.6.12.1 At the throat (0): the posterior stamens attach at the mouth of a corolla.

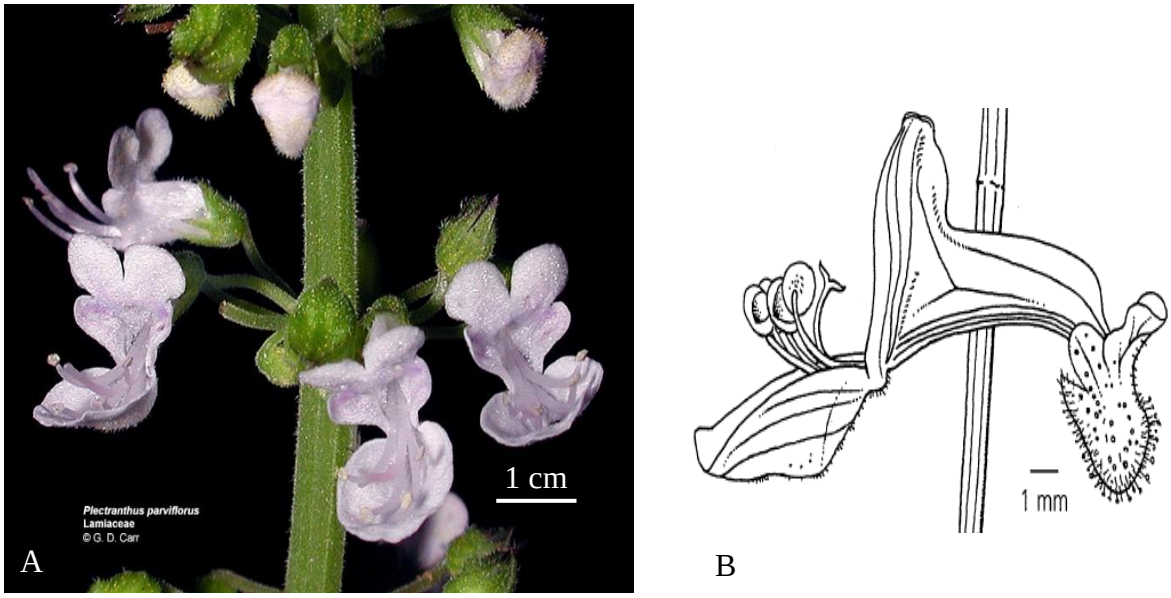


Figure 5.30 Photos show attachment of posterior stamens at the throat. A in *Plectranthus parviflorus* from Carr (2006b) and B in *Plectranthus albicalyx* from Suddee et al. (2005).

5.3.6.12.2 At the base of the tube (1): the posterior stamens attach at the base of the tube.

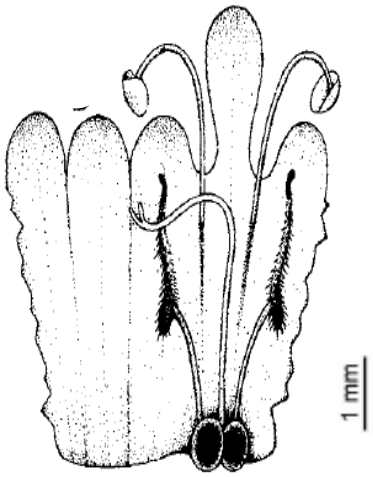


Figure 5.31 Photo shows attachment of posterior stamens at the base of the tube in *Ocimum circinatum* from Paton (1992).

5.3.6.12.3 Around the mid-point of the tube (2): the posterior stamens attach at the middle of the tube.

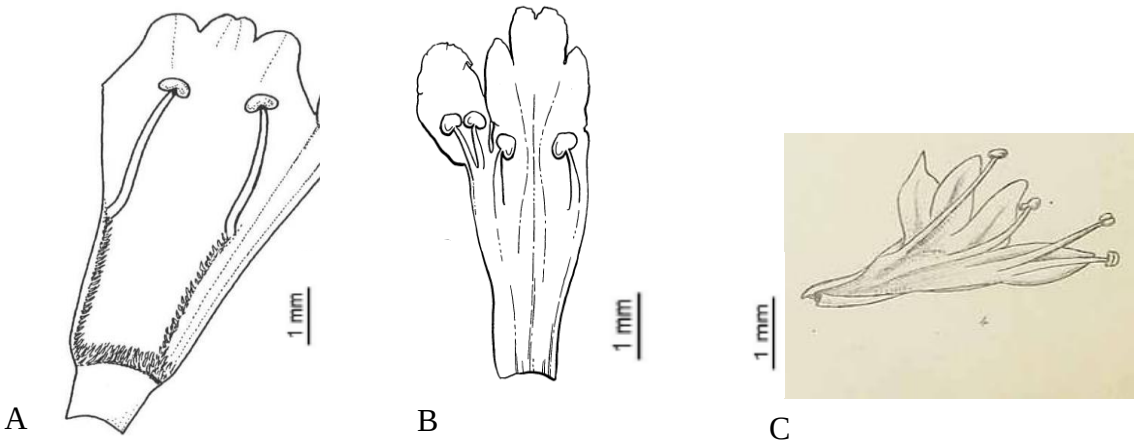
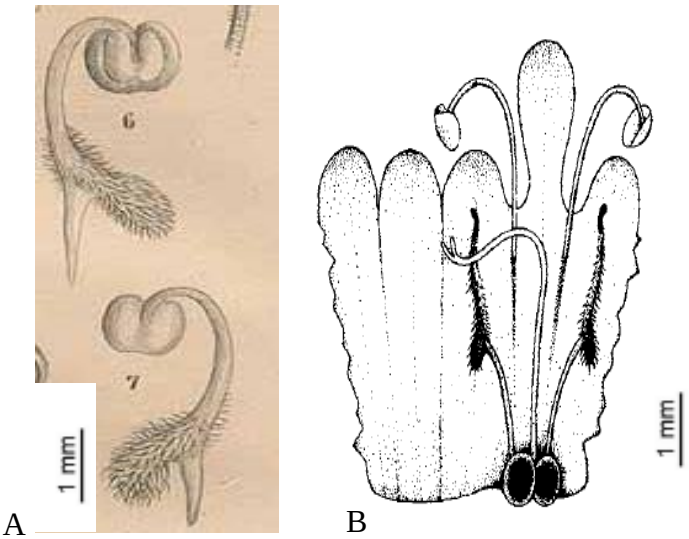


Figure 5.32 Photos show attachment of posterior stamens at the mid-point of the tube. A in *Orthosiphon americanus* from Harley & Paton (2012), B in *Orthosiphon nigripunctatus* from Paton (1993) and C in *Haumaniastrum callianthum* from Pole Evans (1942).

5.3.6.13 Posterior filament form

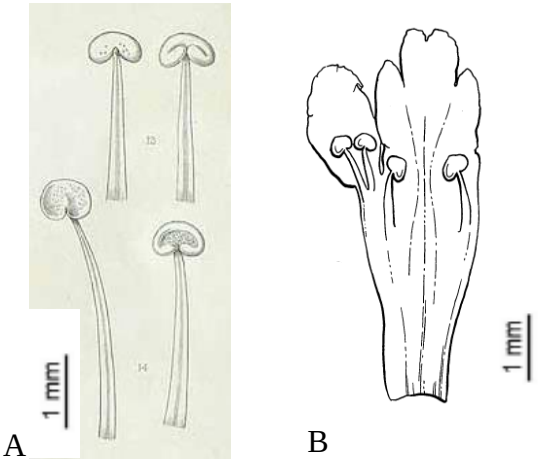
5.3.6.13.1 Appendiculate (0): the posterior filament is formed with a small projection or appendage (PlantNET, 2019). The appendage is an attachment which is developed on and projecting from an organ's surface (PlantNET, 2019).



A B

Figure 5.33 Photos show appendiculate of posterior filament form. A in *Ocimum basilicum* from Spenner et al. (1843) and B in *Ocimum circinatum* from Paton (1992).

5.3.6.13.2 Inappendiculate (1): posterior filament form without a small appendage or projection.



A B

Figure 5.34 Photos show inappendiculate of posterior filament form. A in *Orthosiphon thymiflorus* from De Wildeman & Durand (1899) and B in *Orthosiphon nigripunctatus* from Paton (1993).

5.3.6.13.3 Basally swollen (2): posterior filament swollen at base, tapering apically.

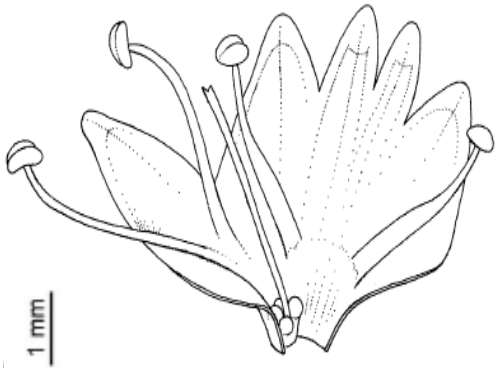


Figure 5.35 Photo shows basally swollen posterior filament in *Platostoma gabonense* form Paton (1997b).

5.3.6.14 Style

5.3.6.14.1 Bifid (0): **bifid (2-fid)**: the style divided into two linear lobes.

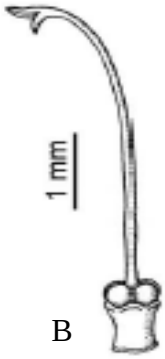


Figure 5.36 Photos show bifid style. A in *Ocimum americanum* from Bingham et al. (2019) and B in *Ocimum selloi* from O'Leary (2017).

5.3.6.14.2 Clavate (1): clavate style; club-shaped with lobes rounded and adpressed (PlantNET, 2019).

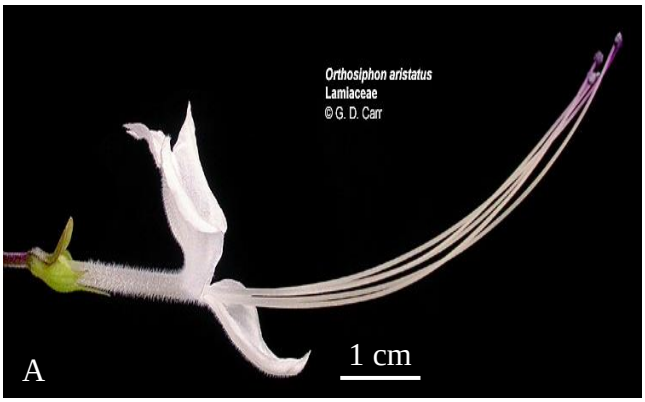


Figure 5.37 Photos show clavate style. A in *Orthosiphon aristatus* from Carr (2006a) and B in *Orthosiphon argenteus* from Hedge et al. (1998).

5.3.6.14.3 Capitate (2): head-shaped style; in a head-like cluster (PlantNET, 2019).

Figure 5.38 Photos show capitate style. A in *Catoferia chiapensis* from Living Collection Virtual Herbarium (2014) and B in *Catoferia capitata* from Martínez-Gordillo et al. (2013).

5.3.6.15 Style shield

5.3.6.15.1 Absent (0): nothing around the style which is between the stigma and ovary.

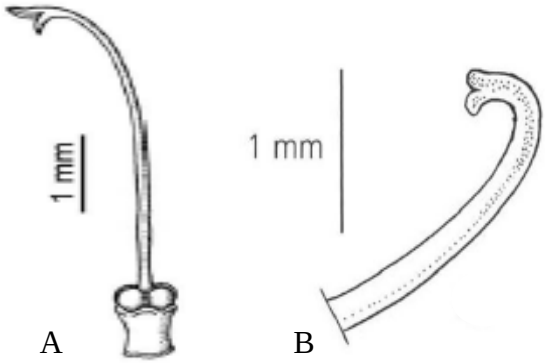


Figure 5.39 Photos show a style lacking the shield. A in *Ocimum selloi* from O'Leary (2017) and B in *Orthosiphon americanus* from Harley & Paton (2012).

5.3.6.15.2 Present (1): there is a shield around the style close to the ovary.

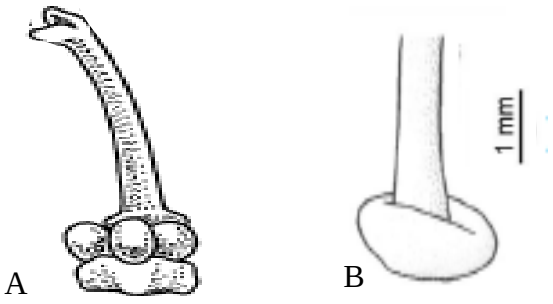


Figure 5.40 Photos show style shield. A in *Endostemon viscosus* from Emanuelsson & Klackenberg (2001) and B in *Endostemon racemosus* from Ryding et al. (2003).

5.3.6.16 Disk lobing

5.3.6.16.1 Equally lobed (0): the disk is divided into equally-sized lobes.

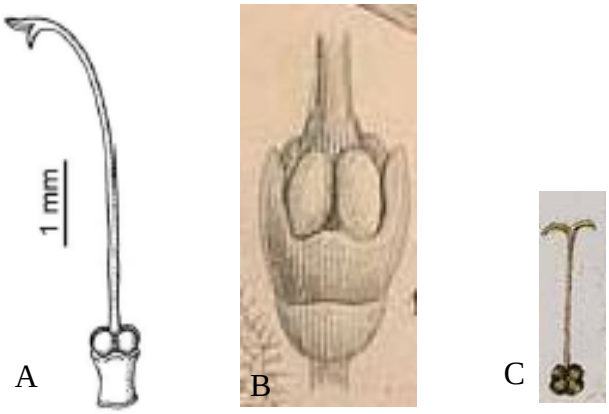


Figure 5.41 Photos show equally lobed disk lobing. A in *Ocimum selloi* from O'Leary (2017), B in *Ocimum basilicum* from Spenner et al. (1843) and C in *Ocimum basilicum* from Zorn (1781).

5.3.6.16.2 Anterior larger (1): the disk is divided into lobes with the anterior lobe larger than the other lobes.

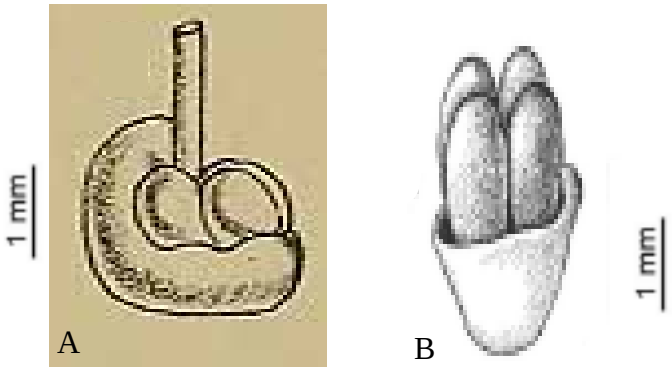


Figure 5.42 Photos show disk with larger antherior lobe. A in *Orthosiphon aristatus* from Kirtikar & Basu (1918) and B in *Orthosiphon americanus* from Harley & Paton (2012).

5.3.7 Mapping geographical distribution

Geographical mapping on the combinable component consensus tree from the Bayesian analysis of the combined dataset using Mesquite v3.51. (Maddison & Maddison, 2018) was conducted. Geographical distribution of continents used in this study was scored from the literature (*WCSP*, 2018). Eight geographical continents (Brummitt et al., 2001) (*WCSP*, 2018) (Figure 5.43), were recognised: 1. Europe= 0, 2. Africa= 1, 3. Western Indian Ocean Madagascar=2 4. Asia-Temperate= 3, 5. Asia-Tropical= 4, 6. Australasia= 5, 7. Pacific= 6, 8. Northern America=7, 9. Southern, America= 8. The geographical data matrix for continental is provided in Appendix 4.

Geographical distribution of regions used in this study was also scored from the literature (*WCSP*, 2018) and also reconstructed using Mesquite v3.51. Thirty-four geographical regions belonging to these continents (Brummitt et al., 2001) (*WCSP*, 2018) (Figure 5.43), were recognised: Eastern Europe= 0; Northern Africa= 1; Macaronesia= 2; West Tropical Africa= 3; West-Central Tropical Africa= 4; Northeast Tropical Africa= 5; East Tropical Africa= 6; South Tropical Africa= 7; Southern Africa= 8; Madagascar= 9; Russian Far East= A; Middle Asia= B; Western Asia= C; Arabian Peninsula= D; China= E; Mongolia= F; Eastern Asia= G; Indian Subcontinent= H; Indo-China= J; Malesia= K; Papuasia= M; Australia= N; South-Western Pacific= P; South-Central Pacific= Q; North-Central Pacific= R; North-Central U.S.A.= S; South-Eastern U.S.A.= T; Mexico= U; Central America= V; Caribbean= W; Northern South America= X; Western South America= Y; Brazil= Z and Southern South America= a. The geographical data matrix for these regions is provided in Appendix 5.

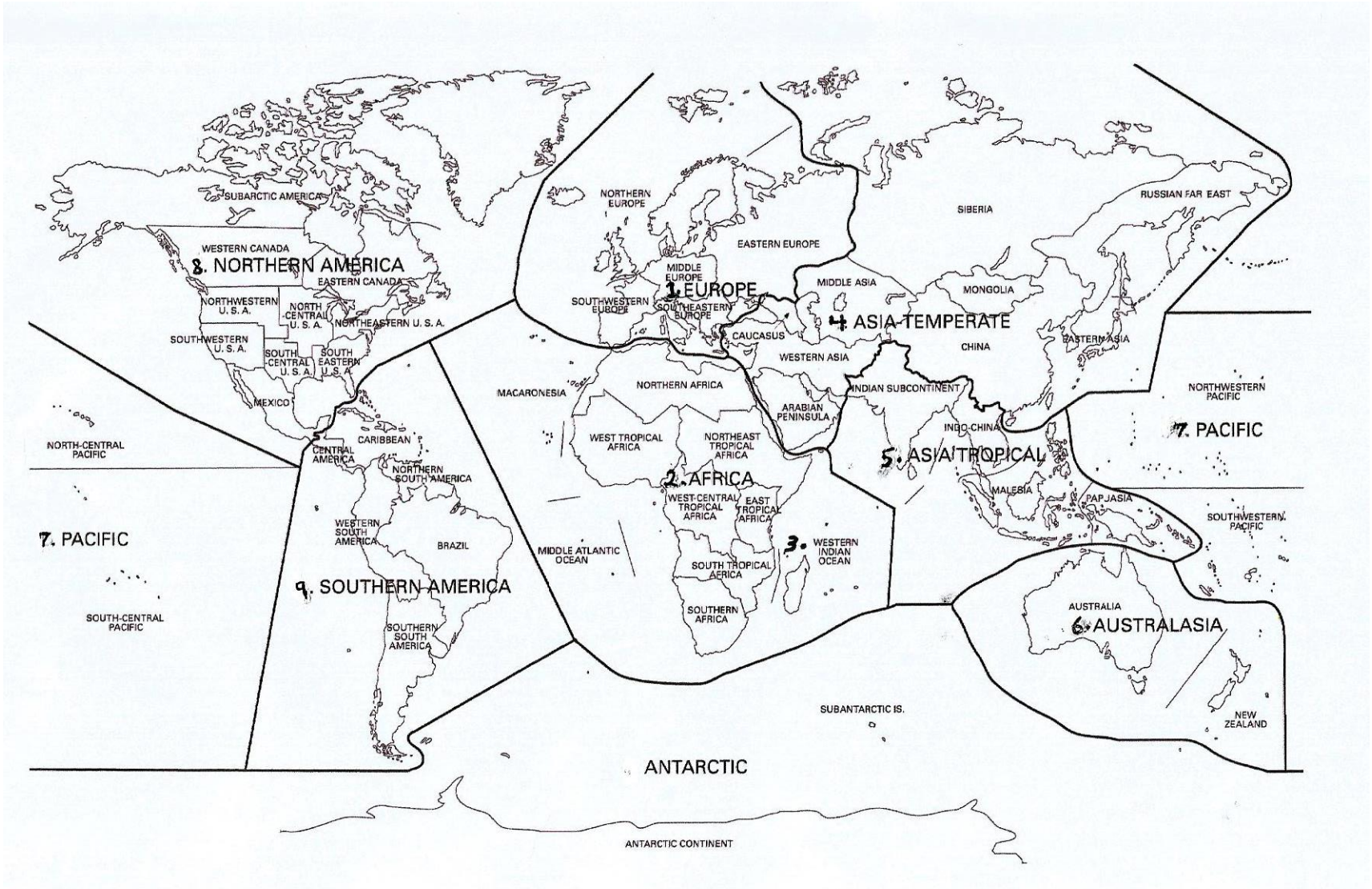


Figure 5.43 A map of geographic regions following Brummitt et al. (2001) used for scoring the presence or absence of taxa.

5.4 Results

5.4.1 Morphological character mapping analyses

The selected morphological characteristics were mapped onto the combinable component consensus tree of the subtribe Ociminae from the combined cpDNA and nrDNA regions dataset (Figures 5.44–5.59).

Leaves are first characteristics mapped onto the combinable component consensus tree of the subtribe Ociminae. *Plectranthus* (as an outgroup) and all genera of the subtribe Ociminae have opposite leaves except for the *Orthosiphon, Fuerstia,* and *Hoslundia* clade. *Ho. opposita* and *Fu. ternata* have opposite and ternate leaves, but *Fu. rigida, Fu. angustifolia* and *Or. nigripunctatus* have ternate leaves (Figure 5.44).

Basally coloured fertile bracts character are found in *Haumaniastrum, Platostoma,* and *Catoferia* but the rest of the genera subtribe Ociminae and *Plectranthus* is an outgroup have uniformly green bracts (Figure 5.45).

Regarding the number of flowers in cyme in this study, *Plectranthus* species have one to many flowers while two species of *Catoferia* have three flowers. *Syncolostemon* have 1–3 variable flowers for most of the species but one flower is in a clade of *Syn. parviflorus, Syn. concinnus*, and *Syn. argenteus,* and *Syn. foliosus,* and *Syn. modestus* are in another clade. The *Haumaniastrum* clade contains *Hau. katangense, Hau. minor,* and *Hau. lantanoides*, which have three flowers. The *Platostoma* species in this study have one to many flowers. All species within *Fuerstia* and *Hoslundia* have one flower, but the *Orthosiphon* clade has three flowers. *Benguellia* also has three flowers. The *Endostemon* clade has one to many flowers for all species except *Endostemon leucosphaerus,* which has only one flower. Clade includes *Basilicum polystachyon* and *Ocimum* group; *Ba. polystachyon* and *Ocimum* species have three flowers, but the subgen *Nautochilus* (*Ocimum lamiifolium, Oc. pseudoserratum Or. fruticosus, Oc. serratum, Oc. labiatum*) has one to many flowers (Figure 5.46).

The fleshy, mature calyx is found only in *Hoslundia* and not in the rest of the genera of the subtribe Ociminae and *Plectranthus* as an outgroup (Figure 5.47).

The position of the lateral calyx lobe character in *Catoferia* is nearer the anterior lobes than posterior lobe (Figure 5.14). In the *Platostoma* clade, *Haumaniastrum* species have the lateral calyx lobe close to posterior, in the same plane (Figure 5.13). *Platostoma* species have the lateral calyx lobe close to posterior, not in the same plane (Figure 5.12), except for *Pla. calcaratum* and *Pla. siamense* where the lateral calyx lobe is in the median position, between the posterior and

anterior (Figure 5.11); this is also similar to the rest of the genera and *Plectranthus* species (Figure 5.48).

The shape of the anterior calyx lobes in the outgroup of *Plectranthus, Catoferia, Syncolostemon, Benguellia, Endostemon* and *Ocimum* excluding subgenus *Nautochilus* have a lanceolate shape. In *Platostoma* and *Haumaniastrum* clade have emarginate shape. In most species of *Orthosiphon*, *Fuerstia, Hoslundia, Basilicum* and *Ocimum* subgenus *Nautochilus*, which includes *Orthosiphon fruticosus* have subulate shape (Figure 5.49).

The corolla tube shape, in the outgroup and *Catoferia* is straight to slightly curved downward. In *Syncolostemon*, all the species have a corolla tube that is curved downward, except *Sy. canescens,* which is curved upward and *Sy. persimilis,* which is straight to slightly curved downward. *Haumaniastrum* species have a straight corolla tube shape. *Platostoma* species have a downward curved corolla tube shape. A straight corolla tube shape is found in the rest of the genera of the subtribe Ociminae (Figure 5.50).

The lower lip corolla is boat-shaped and surrounds the stamens in *Plectranthus*, which is outgroup in this study. However, in the genera of the subtribe Ociminae, the shape of the lower lip corolla is flat and the stamens extend over it (Figure 5.51).

There are two fertile stamens in *Fuerstia* and *Hoslundia,* but four fertile stamens in the rest of the genera of the subtribe Ociminae (Figure 5.52).

The stamen is exserted or included in *Plectranthus* as an outgroup. *Catoferia, Syncolostemon, Platostoma Haumaniastrum,* and the *Ocimum* clade contains *Basilicum* and *Ocimum* subgenus *Nautochilus* with *Orthosiphon fruticosus;* all have exserted stamens. The clade containing *Fuerstia* and *Hoslundia* have included stamens. Moreover, in the clade *Orthosiphon*, all species have included stamens except for *Or. aristatus* and *Or. hanningtonii.* In the *Endostemon* clade, stamens are included except for *Benguellia,* which has exserted stamens (Figure 5.53).

There is no staminal fusion in all genera of the subtribe Ociminae except *Syncolostemon*, which has fused anterior stamens, free posterior stamens, and in the outgroup of *Plectranthus*, either no staminal fusion or all stamens fused, with the two anterior stamens fused together and to the adjacent posterior stamen (Figure 5.54).

There is the attachment of the posterior stamen around the mid-point in *Catoferia, Syncolostemon, Haumaniastrum, Orthosiphon, Fuerstia, Hoslundia, Benguellia, Basilicum* and *Platostoma* except in one species from this genus (*Platostoma calcaratum*). However, attachment is at the throat in *Endostemon* and *Plectranthus*. At the base in *Ocimum* includes the *Ocimum* subgenus *Nautochilus* with *Orthosiphon fruticosus* (Figure 5.55).

The posterior filament form of the stamen in this study is inappendiculate in *Plectranthus, Catoferia, Syncolostemon, Orthosiphon, Fuerstia, Hoslundia, Benguellia, Endostemon, Basilicum*

and *Ocimum* subgen *Nautochilus* including *Orthosiphon fruticosus.* It is appendiculate in *Ocimum* species except for *Oc. tenuiflorum,* which formed a clade with *Oc. campechianum;* both have inappendiculate filaments. A basally swollen filament of the posterior stamen occurs in *Haumaniastrum* and *Platostoma* clade (Figure 5.56).

The style is bifid in the outgroup of *Plectranthus, Syncolostemon, Platostoma, Haumaniastrum,* and *Ocimum* including subgen *Nautochilus* with *Or. fruticosus.* However, it is clavate in *Orthosiphon, Fuerstia, Hoslundia, Benguellia, Endostemon,* and *Basilicum.* The style is capitate in *Catoferia* (Figure 5.57).

A style of shield is present in *Endostemon,* but absent in the rest of genera of the subtribe Ociminae examined in this study (Figure 5.58).

The disk is equally lobed in *Endostemon, Basilicum,* and *Ocimum* except for *Orthosiphon fruticosus,* which forms a clade with *Ocimum* subgen *Nautochilus.* However, the anterior lobes are larger in the rest of the genera subtribe Ociminae and outgroup *Plectranthus* (Figure 5.59).

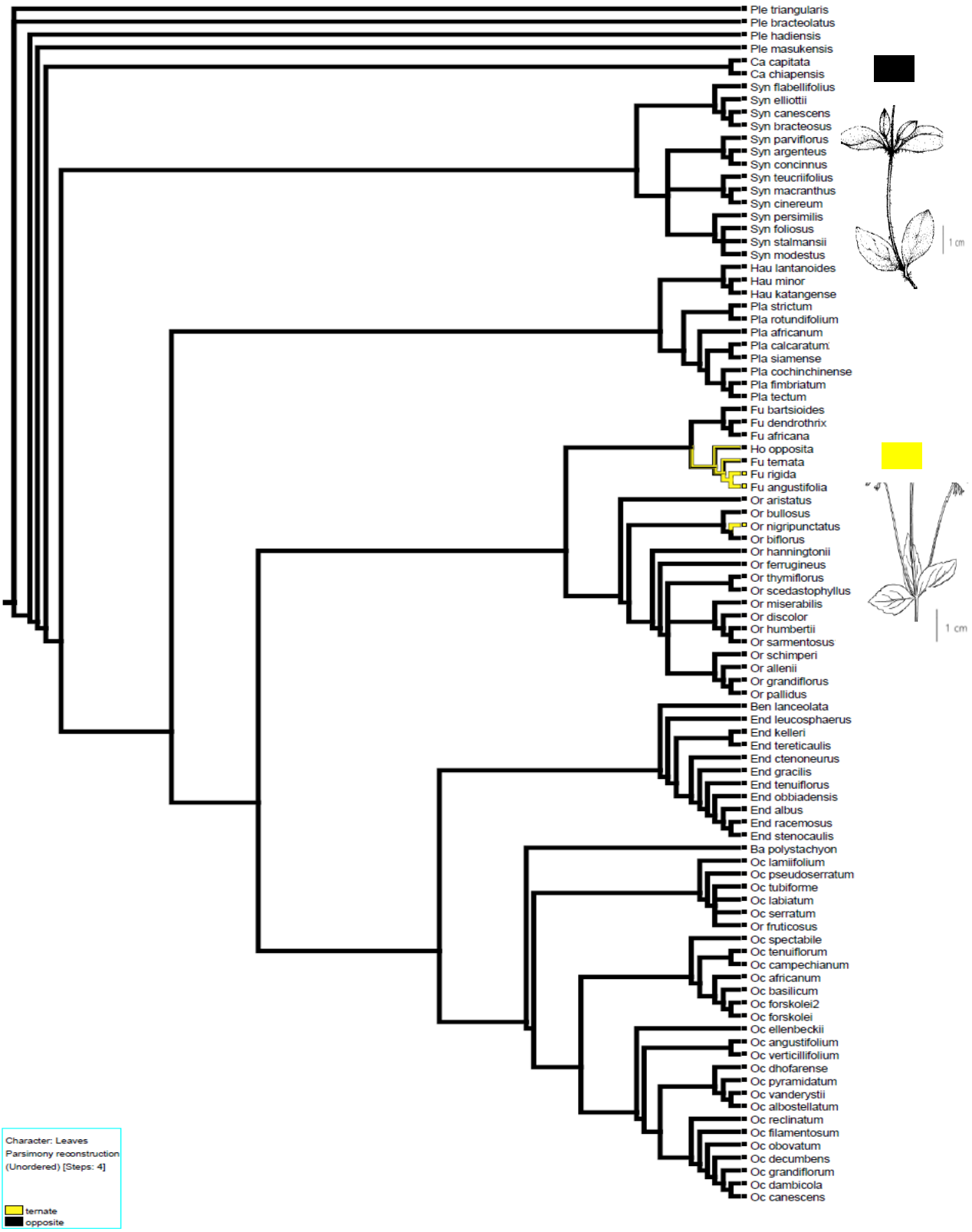


Figure 5.44 History of morphological character 1 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show ternate leaves status 0 yellow (Paton, 1993) and opposite leaves status 1 black (Paton, 1992). Ba = *Basilicum,* Ben = *Benguellia,* Ca = *Catoferia,* End = *Endostemon*, Fu = *Fuerstia,* Hau = *Haumaniastrum*, Ho = *Hoslundia,* Or = *Orthosiphon,* Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

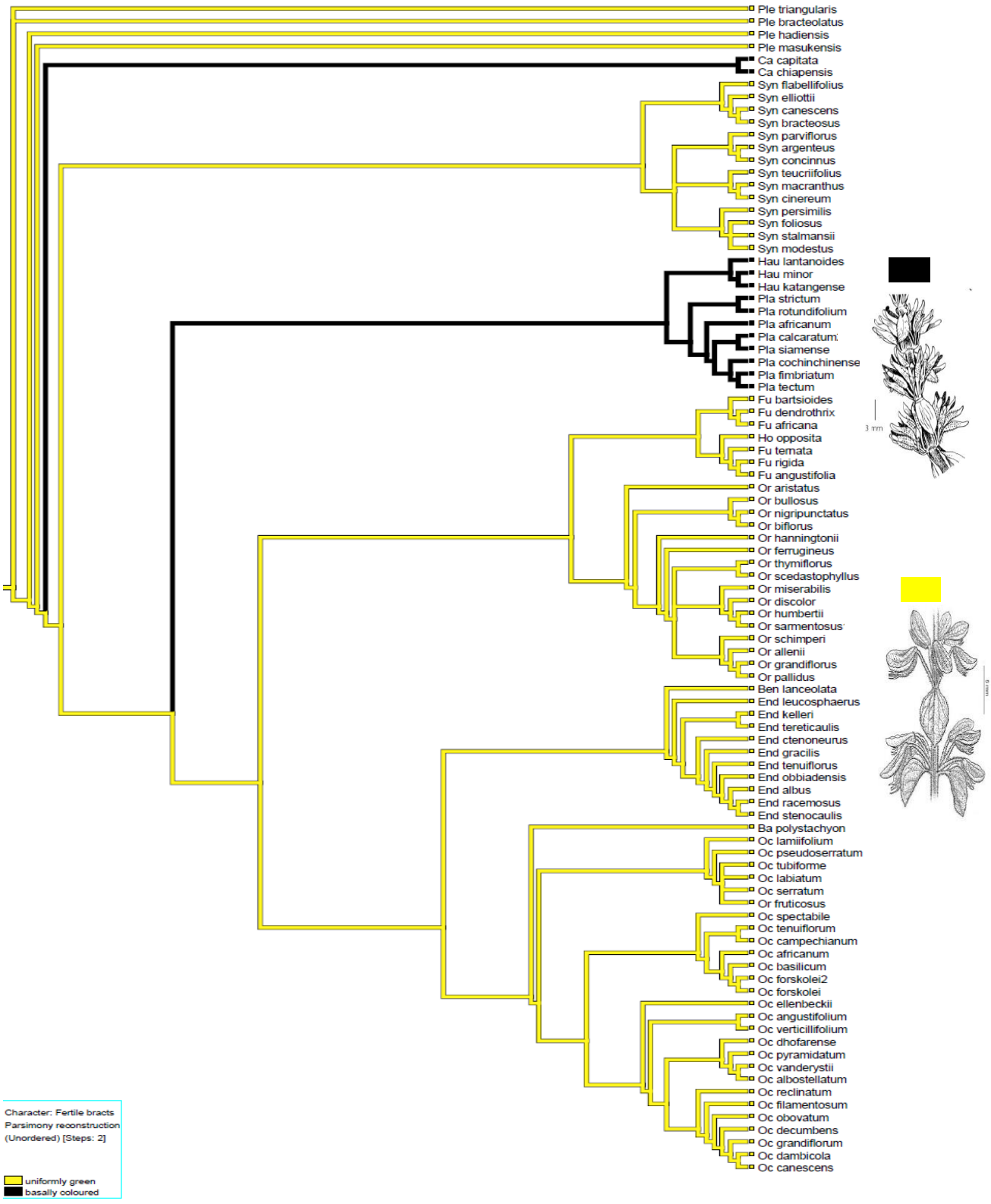


Figure 5.45 History of morphological character 2 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show uniformly green bracts status 0 yellow (O'Leary, 2017) and basally coloured bracts status 1 black (Suddee et al., 2005). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

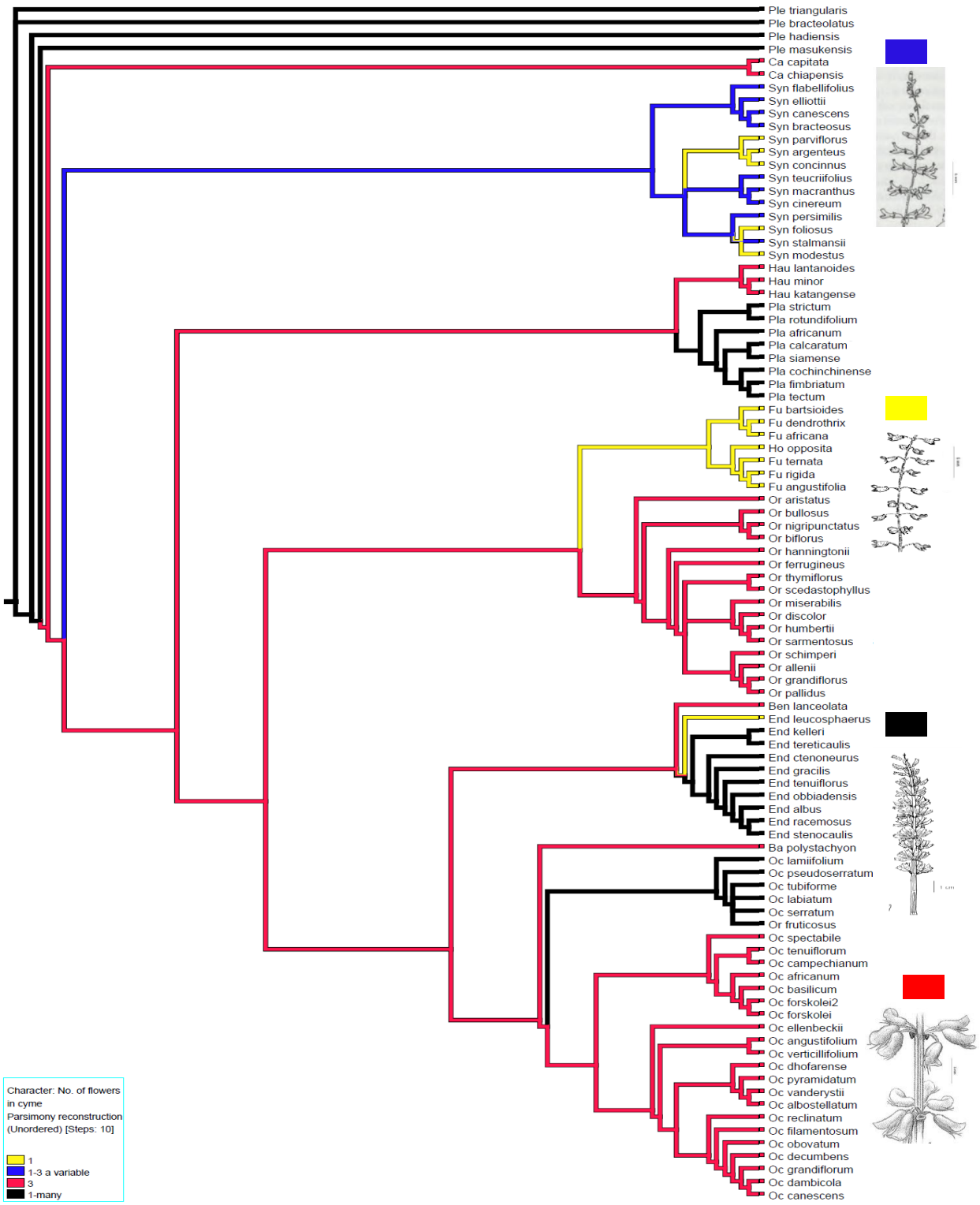


Figure 5.46 History of morphological character 3 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show 1 flower status 0 yellow (Paton, 1993), 1-3 variable flowers status 1 blue (Hedge et al., 1998), 3 flowers status 2 pink (O'Leary, 2017) and 1-many flowers status 3 black (Suddee et al., 2005). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

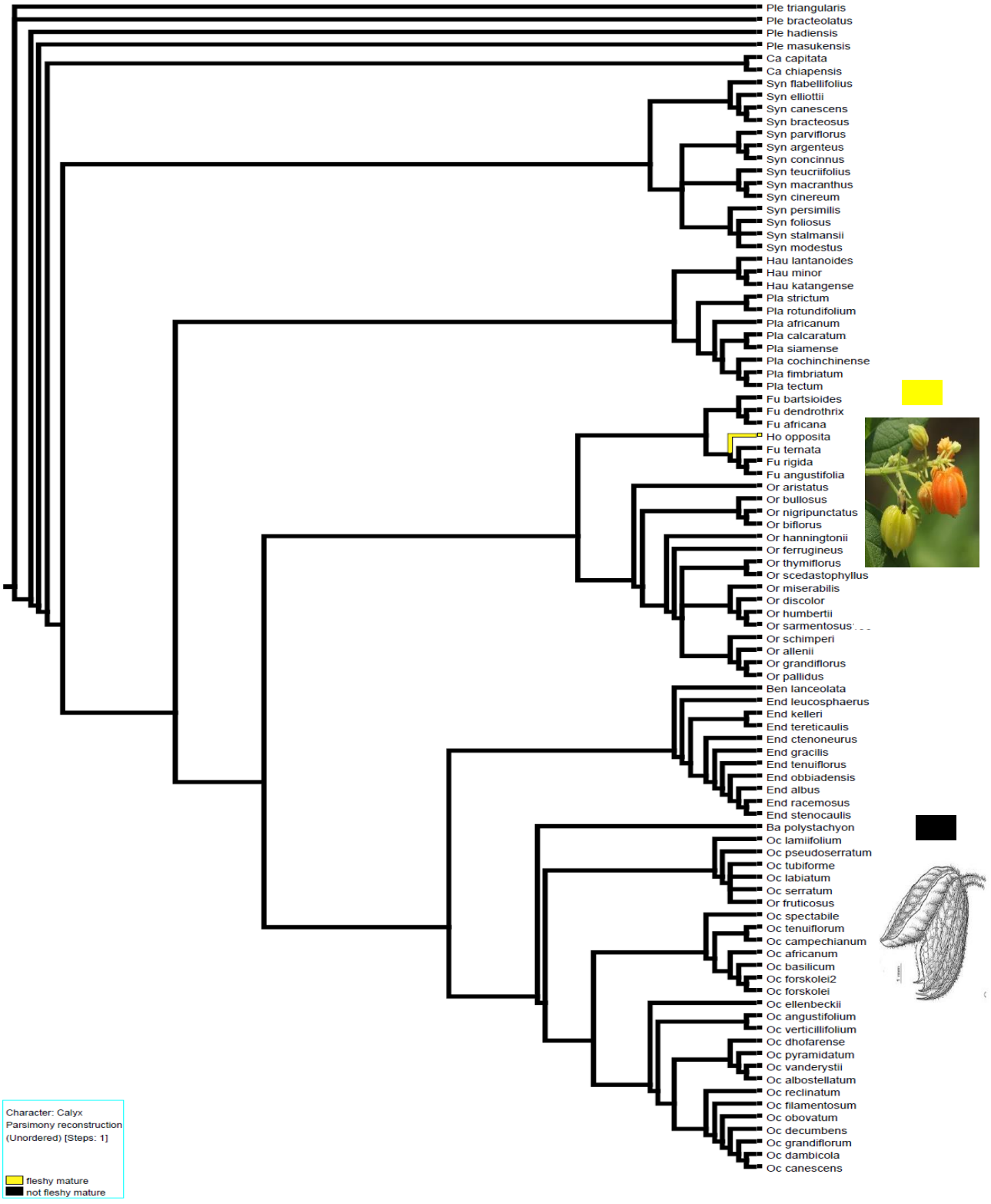


Figure 5.47 History of morphological character 4 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show fleshy mature calyx status 0 is yellow (Hyde et al., 2019**)** and not fleshy mature calyx status 1 is black (O'Leary, 2017). Ba = *Basilicum,* Ben = *Benguellia,* Ca = *Catoferia,* End = *Endostemon,* Fu = *Fuerstia,* Hau = *Haumaniastrum,* Ho = *Hoslundia,* Or = *Orthosiphon,* Pla = *Platostoma,* Ple = *Plectranthus,* Oc= *Ocimum* and Syn = *Syncolostemon*.

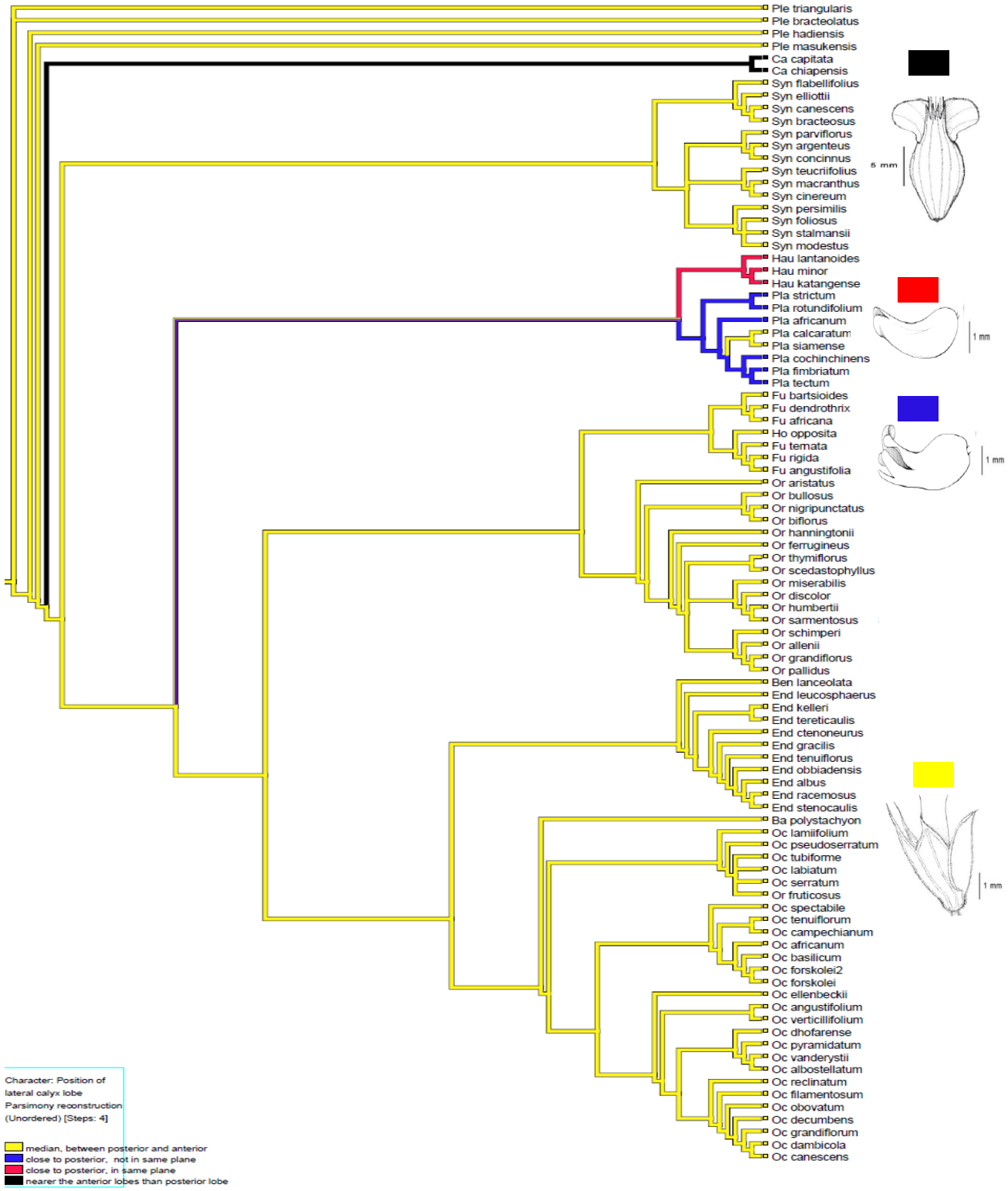


Figure 5.48 History of morphological character 5 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show median, between posterior and anterior status 0 is yellow (Martínez-Gordillo et al., 2013), close to posterior, not in same plane status 1 is blue, close to posterior, in same plane and 2 is pink (Paton, 1997b) and nearer the anterior lobes than posterior lobe status 3 is black (Martínez-Gordillo et al., 2013). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

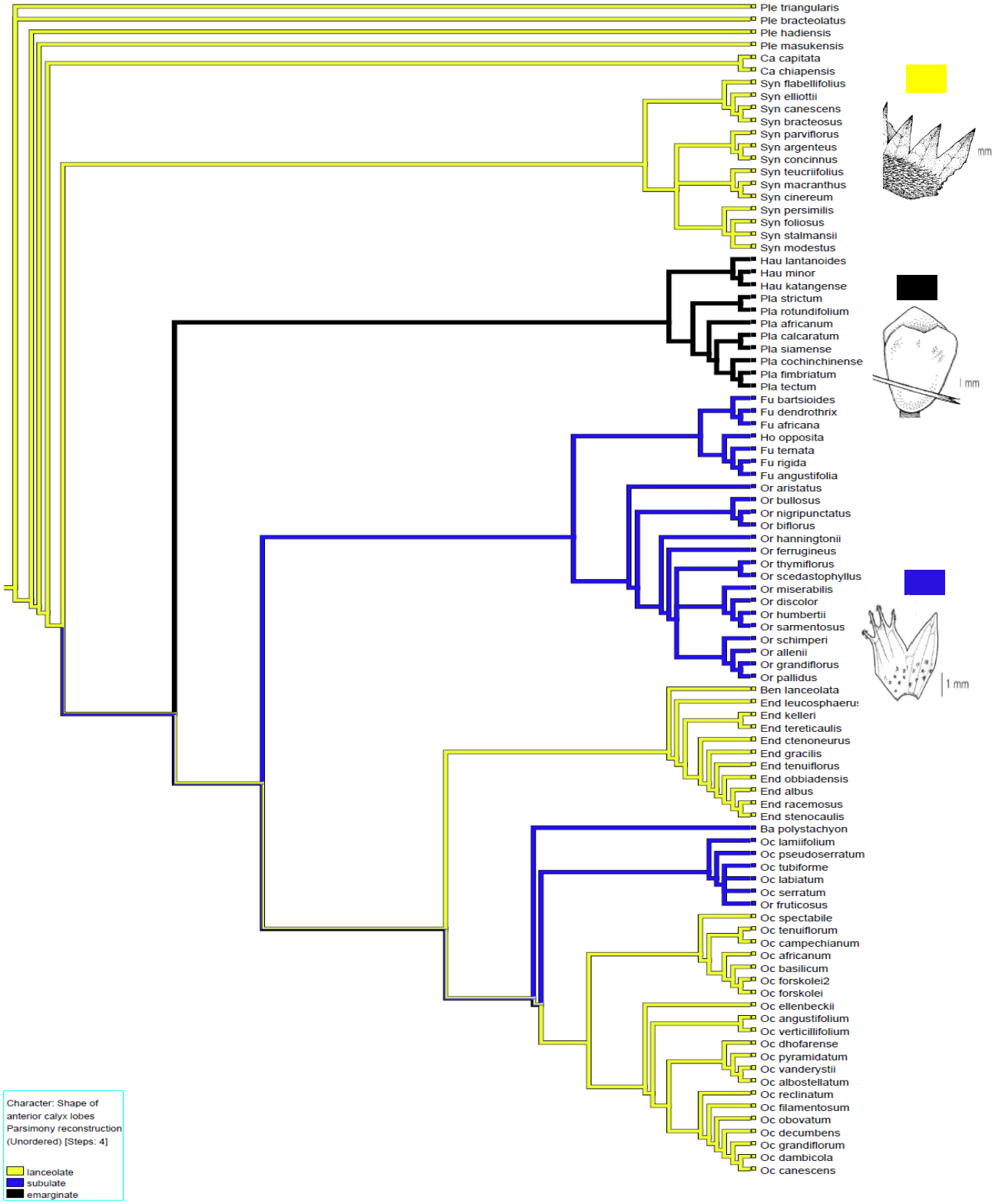


Figure 5.49 History of morphological character 6 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show lanceolate status 0 is yellow Paton (1992), subulate status 1 is blue (Harley & Paton, 2012) and emarginate status 2 is black (Paton, 1997a). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

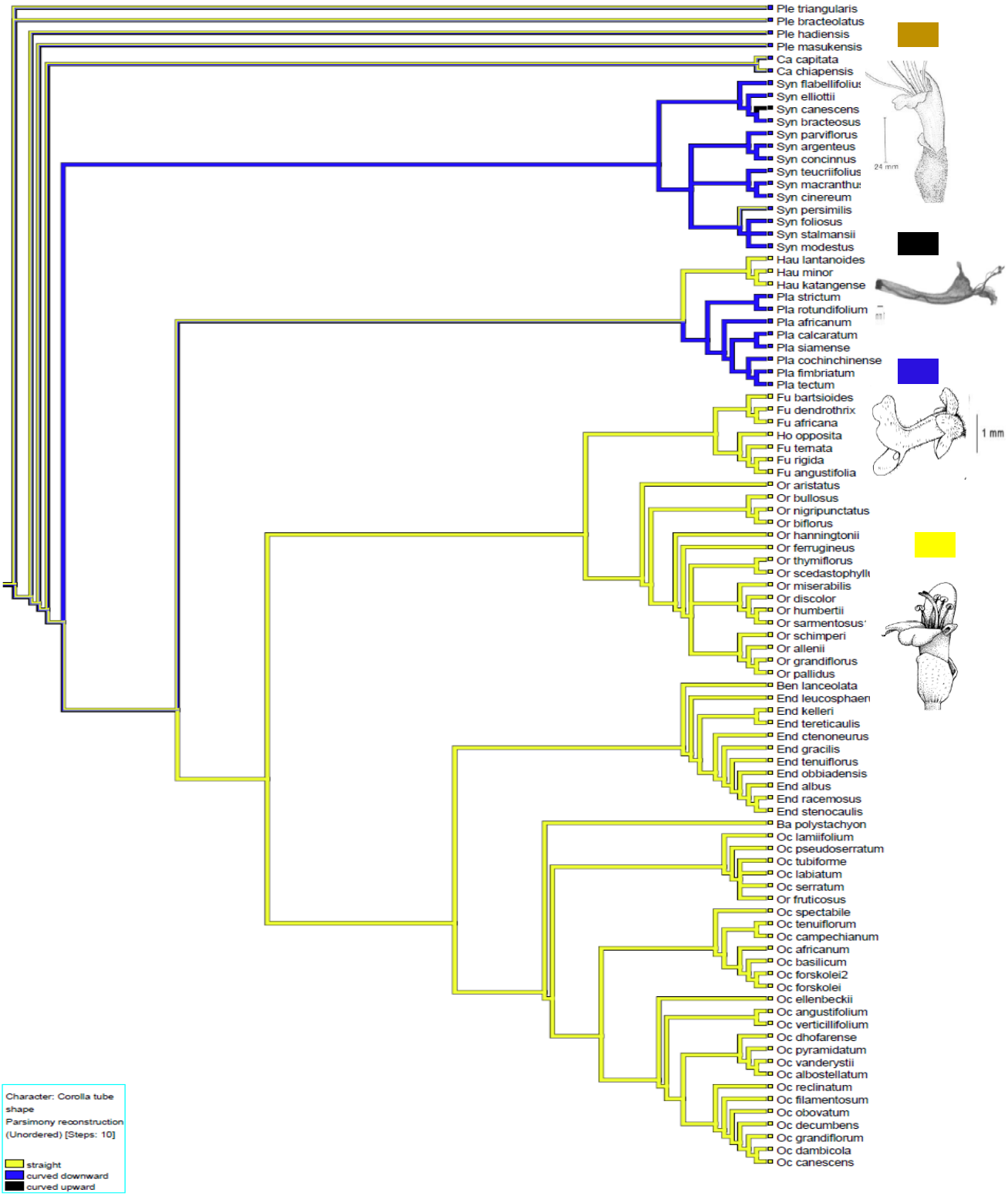


Figure 5.50 History of morphological character 7 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show straight status 0 is yellow (Paton,1997a), curved downward status 1 blue (Paton, 1997b), curved upward status 2 is black (Otieno et al., 2006) and straight to curved downward status 0 to 1 is brown (Ramamoorthy, 1986). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

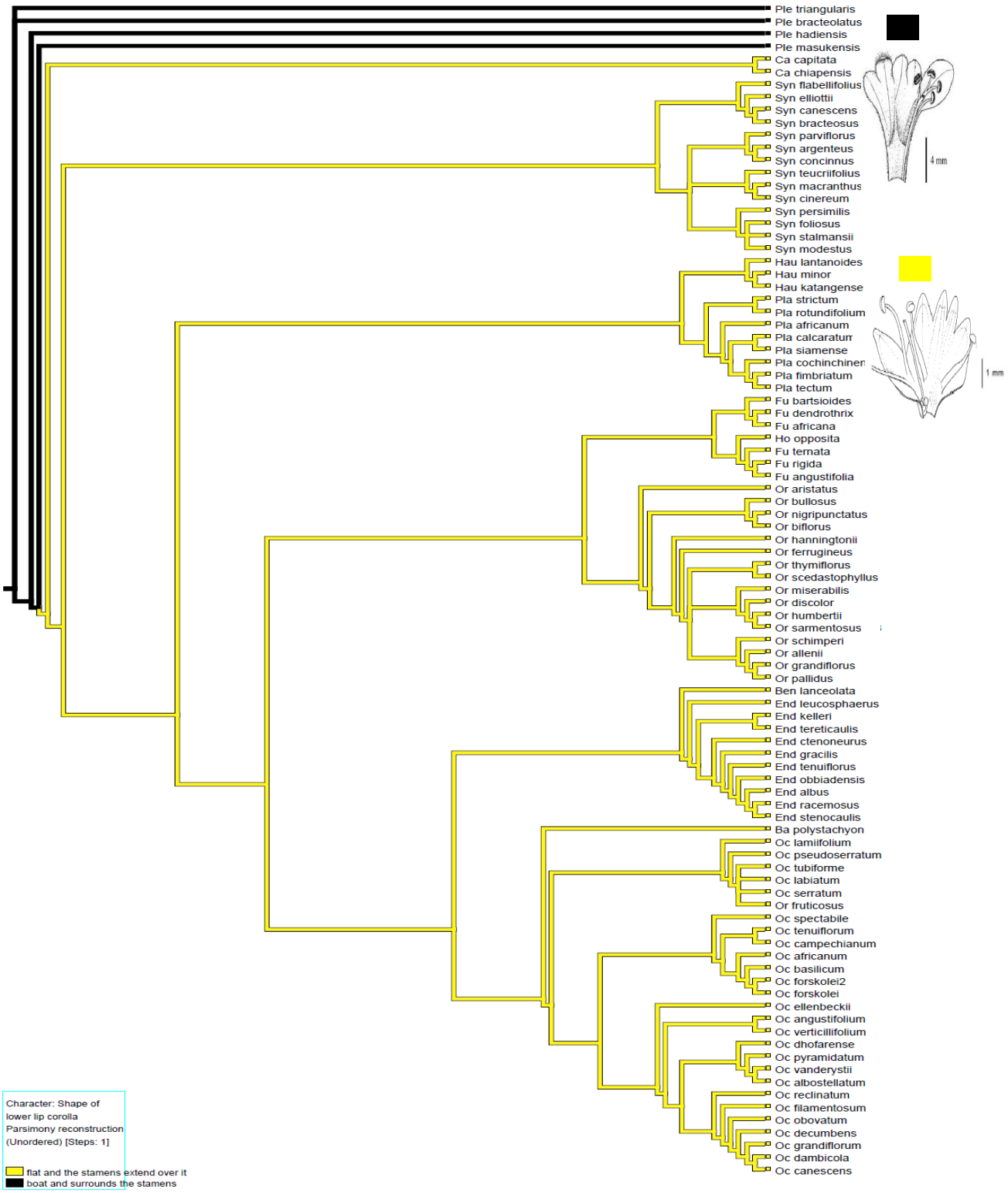


Figure 5.51 History of morphological character 8 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show flat and the stamens extend over it status 0 is yellow (Paton, 1997b) and boat-shaped and surrounds the stamens status 1 is black (Suddee et al., 2014). Ba = *Basilicum*, Ben = *Benguellia,* Ca = *Catoferia,* End = *Endostemon,* Fu = *Fuerstia,* Hau = *Haumaniastrum,* Ho = *Hoslundia,* Or = *Orthosiphon,* Pla = *Platostoma,* Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

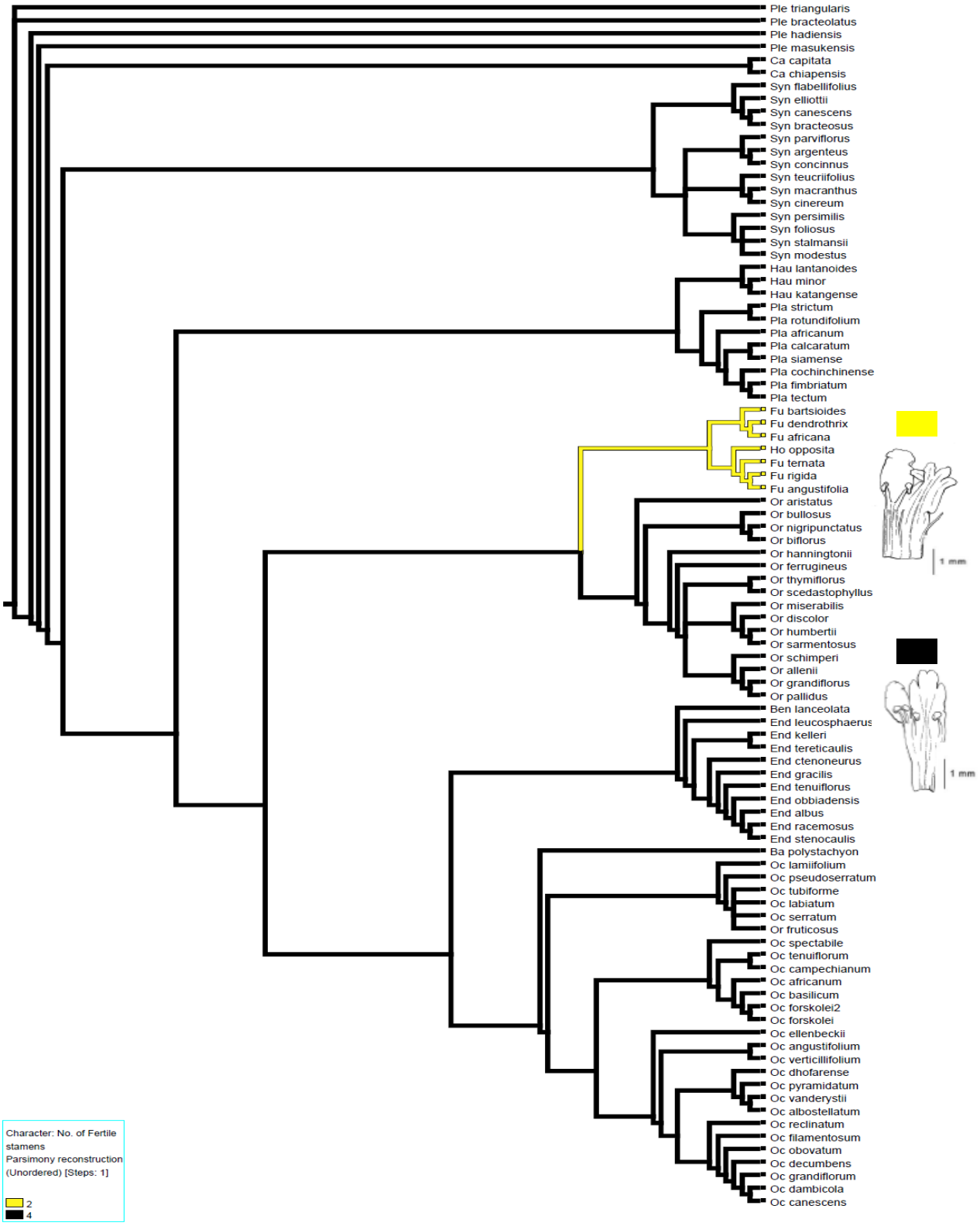


Figure 5.52 History of morphological character 9 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos shows 2 fertile stamens status 0 is yellow and 4 fertile stamens status 1 is black (Paton, 1993). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

Figure 5.53 History of morphological character 10 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show included stamen status 0 is yellow (Paton, 1993) and exserted stamen status 1 is black (Paton, 1997b). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

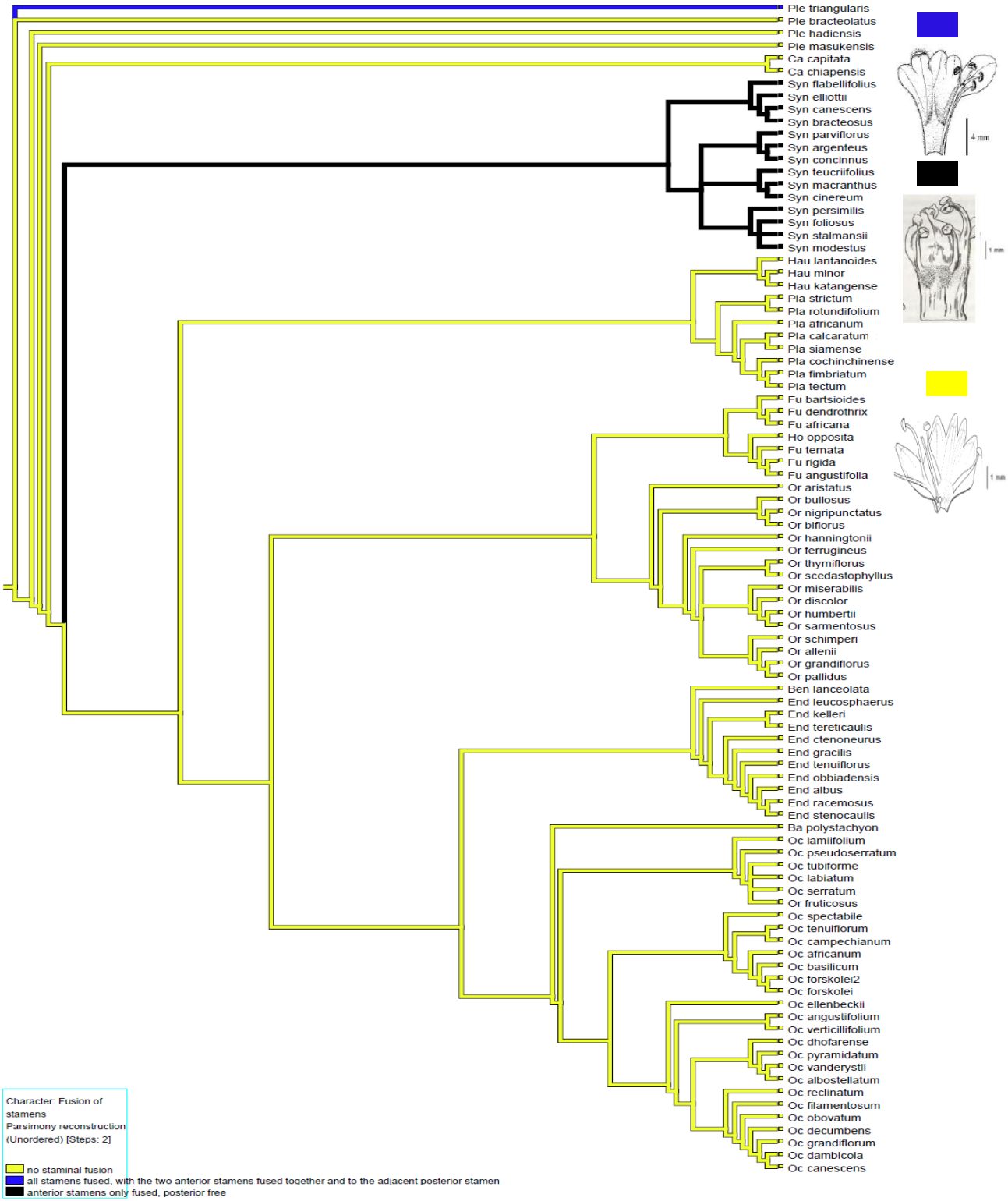


Figure 5.54 History of morphological character 11 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show no staminal fusion status 0 is yellow (Paton, 1997b) all stamens fused, with the two anterior stamens fused together and to the adjacent posterior stamen status 1 is blue (Suddee et al., 2014) and anterior stamens only fused, posterior free status 2 is black (Hedge et al., 1998). Ba = *Basilicum,* Ben = *Benguellia,* Ca = *Catoferia,* End = *Endostemon,* Fu = *Fuerstia,* Hau = *Haumaniastrum,* Ho = *Hoslundia,* Or = *Orthosiphon,* Pla = *Platostoma,* Ple = *Plectranthus,* Oc= *Ocimum* and Syn = *Syncolostemon.*

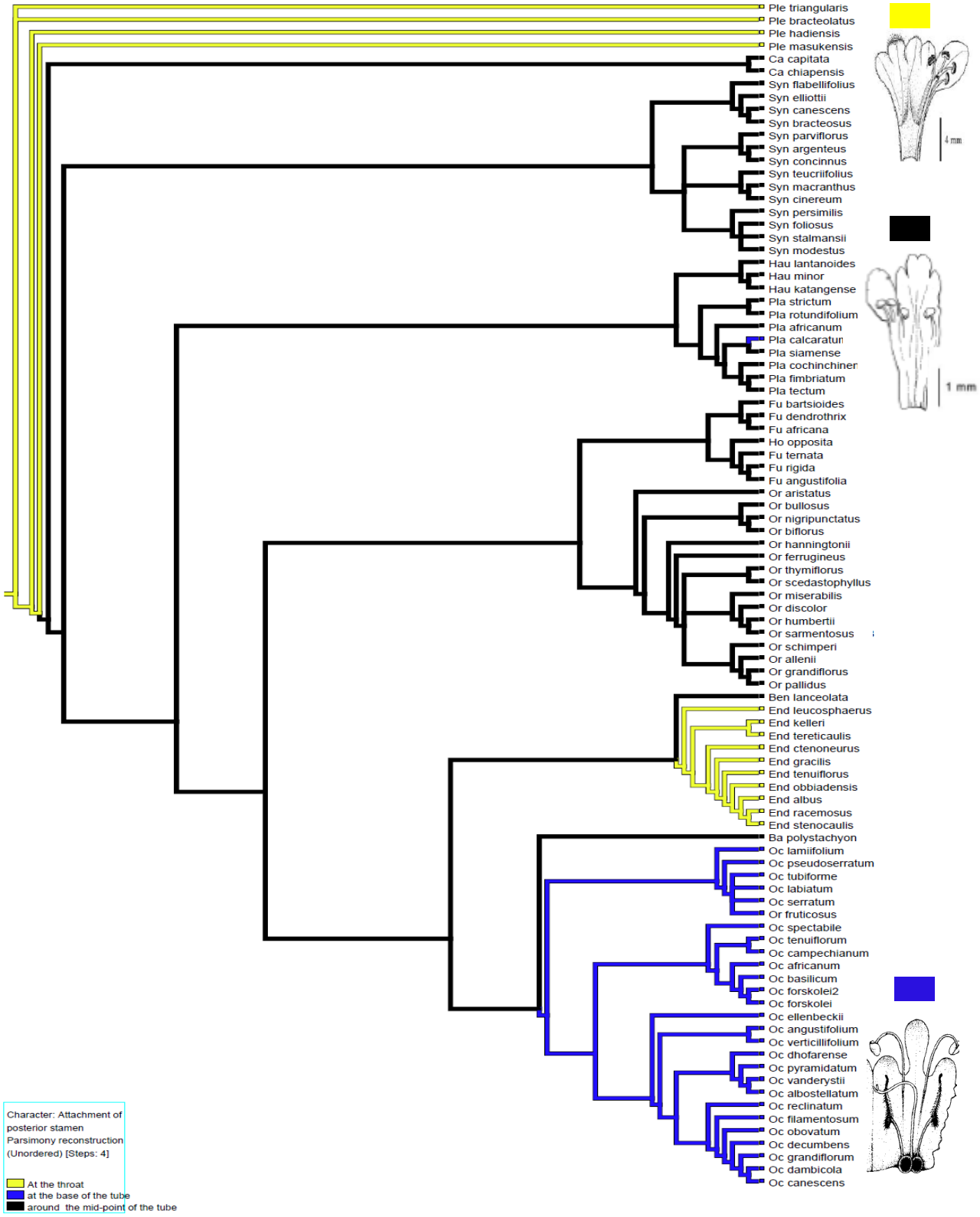


Figure 5.55 History of morphological character 12 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show attachment of posterior stamen at throat status 0 is yellow (Suddee et al., 2014) at the base of the tube status 1 is blue (Paton, 1992) and around the mid-point of the tube status 2 is black (Paton, 1993). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

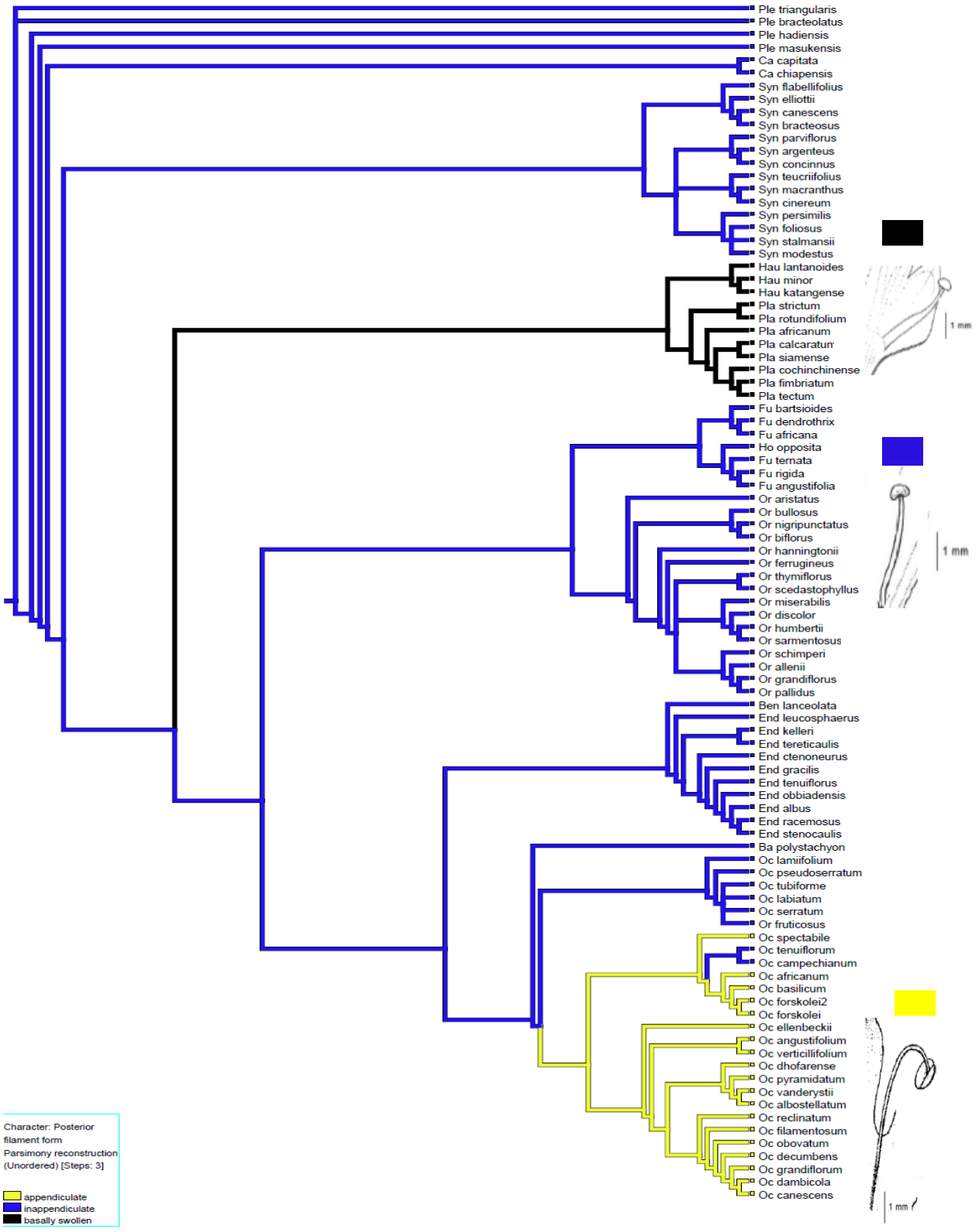


Figure 5.56 History of morphological character 13 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show appendiculate status 0 is yellow (O'Leary, 2017), inappendiculate status 1 is blue (Harley & Paton, 2012) and basally swollen status 2 is black (Paton, 1997b). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

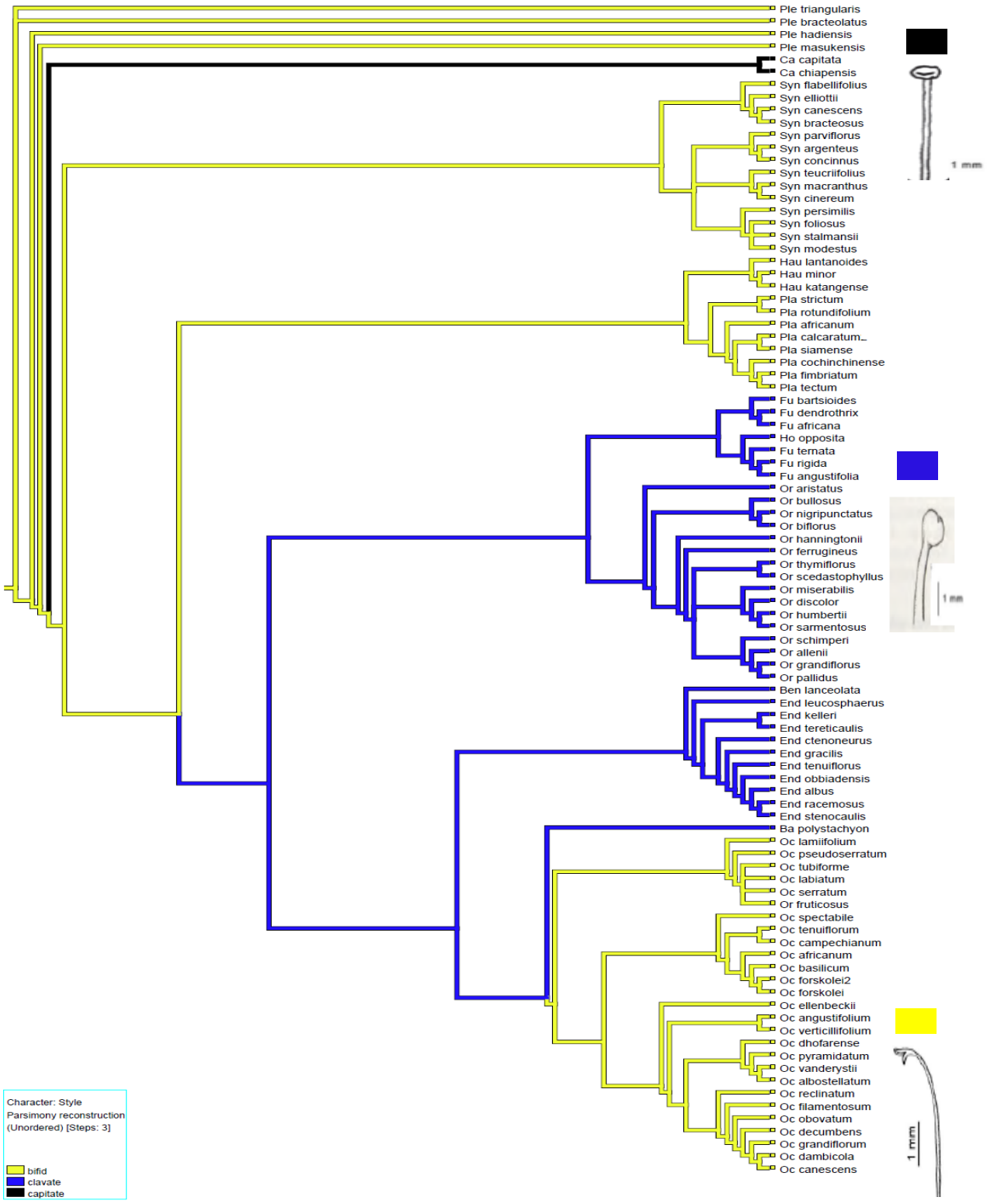


Figure 5.57 History of morphological character 14 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show bifid style status 0 is yellow (O'Leary, 2017), clavate status 1 is blue (Hedge et al., 1998) and capitate status 2 is black (Martínez-Gordillo et al., 2013). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

Figure 5.58 History of morphological character 15 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show style shield absent status 0 is yellow (O'Leary, 2017) and present status 1 is black (Ryding et al., 2003). Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

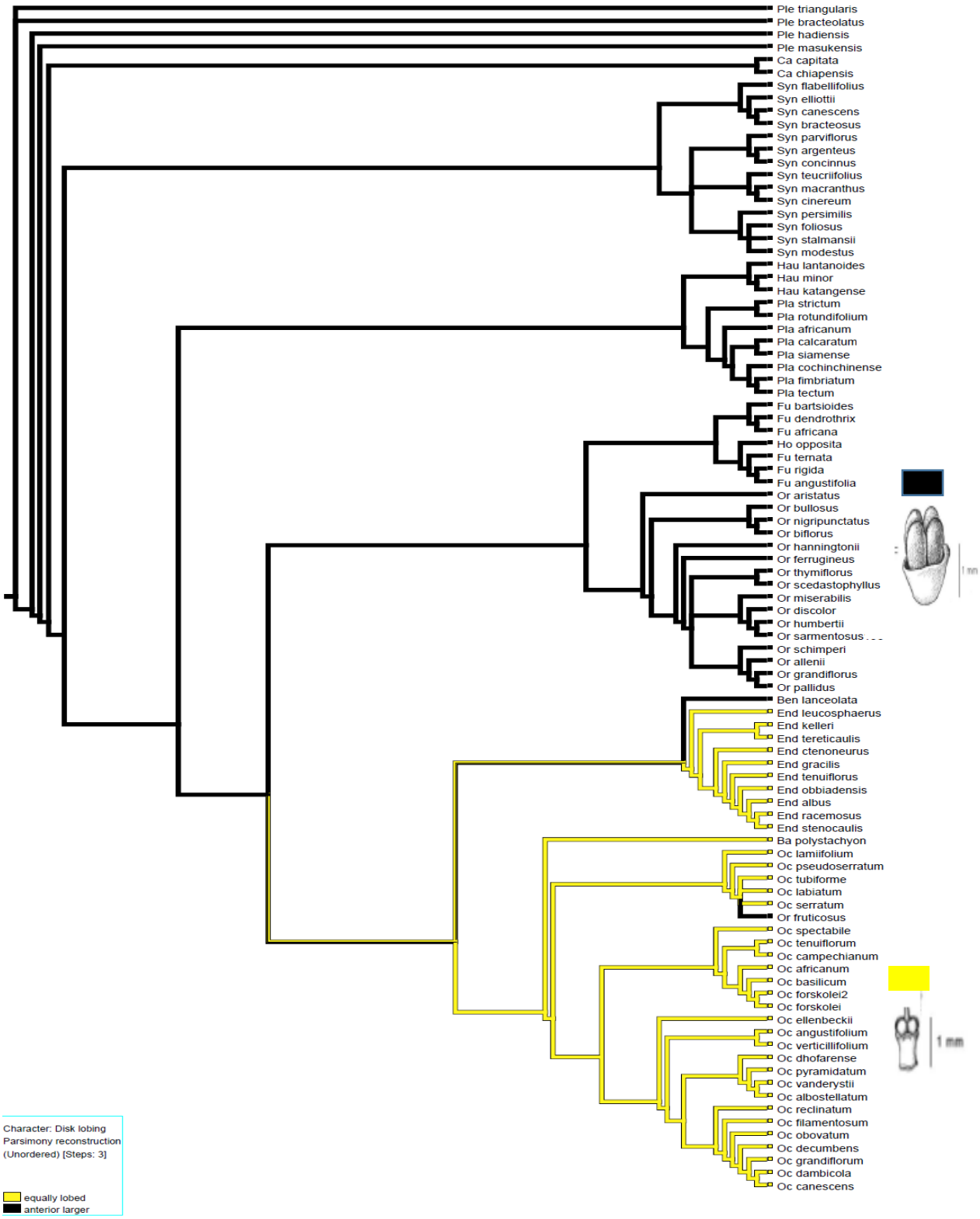


Figure 5.59 History of morphological character 16 traced onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Photos show equally lobed disk lobing status 0 is yellow (O'Leary, 2017) and anterior larger disk lobing status 1 is black (Harley & Paton, 2012). Ba = *Basilicum*, Ben = *Benguellia,* Ca = *Catoferia,* End = *Endostemon,* Fu = *Fuerstia,* Hau = *Haumaniastrum,* Ho = *Hoslundia,* Or = *Orthosiphon,* Pla = *Platostoma,* Ple = *Plectranthus*, Oc= *Ocimum* and Syn = *Syncolostemon*.

5.4.2 Geographical mapping analyses for continents and regions

Reconstruction of ancestral nodes using parsimony in Mesquite shows that the ancestor of subtribe Ociminae was in Africa (Figure 5.60). *Catoferia* has two species in Northern America and Southern America which is monophyletic. *Syncolostemon* from this study is monophyletic and widespread in Africa. The *Haumaniastrum* clade is found in Africa, and is closely related to *Platostoma* clade *Pla. strictum* and *Pla. rotundifolium* from Africa. *Platostoma* is found in Africa, Asia- Temperate and Asia-Tropical. *Fuerstia* and *Hoslundia* are found in Africa and is monophyletic; *Hoslundia* is also found in Western Indian Ocean Madagascar. These genera are the sister group to *Orthosiphon* which is found in Africa, Western Indian Ocean Madagascar, Asia-Temperate, Asia-Tropical and Australasia, with *Or. aristatus,* as a sister for all *Orthosiphon* species but it is not found in Africa as are the rest of these species. *Benguellia* is endemic to Angola (Africa); *Endostemon* is found in Africa, Western Indian Ocean Madagascar and Asia- Temperate and closely related to together phylogeny in this study. *Basilicum* has a close relationship to *Ocimum* which both are widespread in many continental such as in Africa, Asia- Temperate, Asia-Tropical and Australasia, but *Ocimum* is found also in Western Indian Ocean Madagascar, Pacific, Northern America and Southern America. The *Ocimum* subgenus *Nautochilus* is also found in Africa. *Oc. tenuiflorum* is closely related to *Oc. campechianum* and they are widespread but there is one continent shared between them is Southern America. *Oc. africanum* and *Oc. basilicum* related to other, both widespread and they are found in the same six continents (Africa, Asia-Temperate, Asia-Tropical, Northern America and Southern America). The outgroup of *Plectranthus* is widespread in Africa, Asia- Temperate and Asia-Tropical.

The distribution states of regions were mapped onto the phylogenetic results (Figure 5.61). The *Fuerstia* group can be divided into two groups: the first one is *Fu. bartsioides, Fu. dendrothrix,* and *Fu. africana,* which is found in Northeast Tropical Africa. The second group contains *Hoslundia opposita* which is widespread in West Tropical Africa, West-Central Tropical Africa, Northeast Tropical Africa, East Tropical Africa, South Tropical Africa, Southern Africa, and Madagascar. *Fu. ternata* is from East Tropical Africa, *Fu. rigida* and *Fu. angustifolia* are from South Tropical Africa and *Fu. angustifolia* is also found in East Tropical Africa. In *Orthosiphon* clade, *Or. aristatus* is a sister for all species of *Orthosiphon,* and it is widespread in China, Eastern Asia, the Indian subcontinent, Indo-China, Malesia, and Papuasia. *Or. miserabilis, Or. discolor, Or. sarmentosus,* and *Or. humbertii* formed a clade and they were from the same region (Madagascar).

The clade of *Ocimum* subgenus *Nautochilus* includes *Ocimum lamiifolium* from West-Central Tropical Africa, Northeast Tropical Africa, East Tropical Africa, and South Tropical Africa, close to *Oc. pseudoserratum, Or. fruticosus, Oc. serratum, Oc. labiatum* and

Oc. tubiforme which all these from Southern Africa. And also *Oc. labiatum* found in South Tropical Africa.

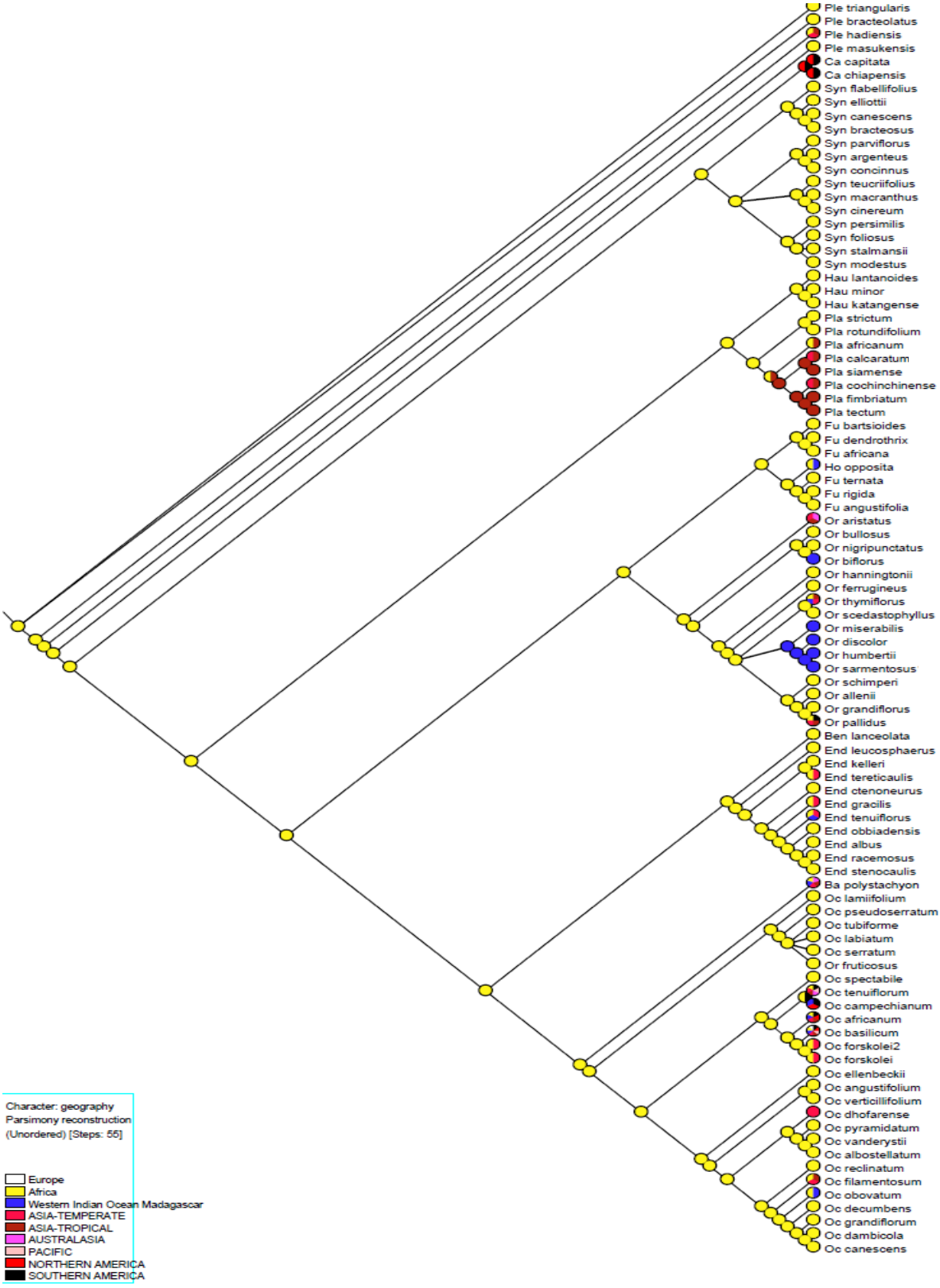


Figure 5.60 Geographic distribution of continents of subtribe Ociminae traced onto combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum*, Syn = *Syncolostemon*.

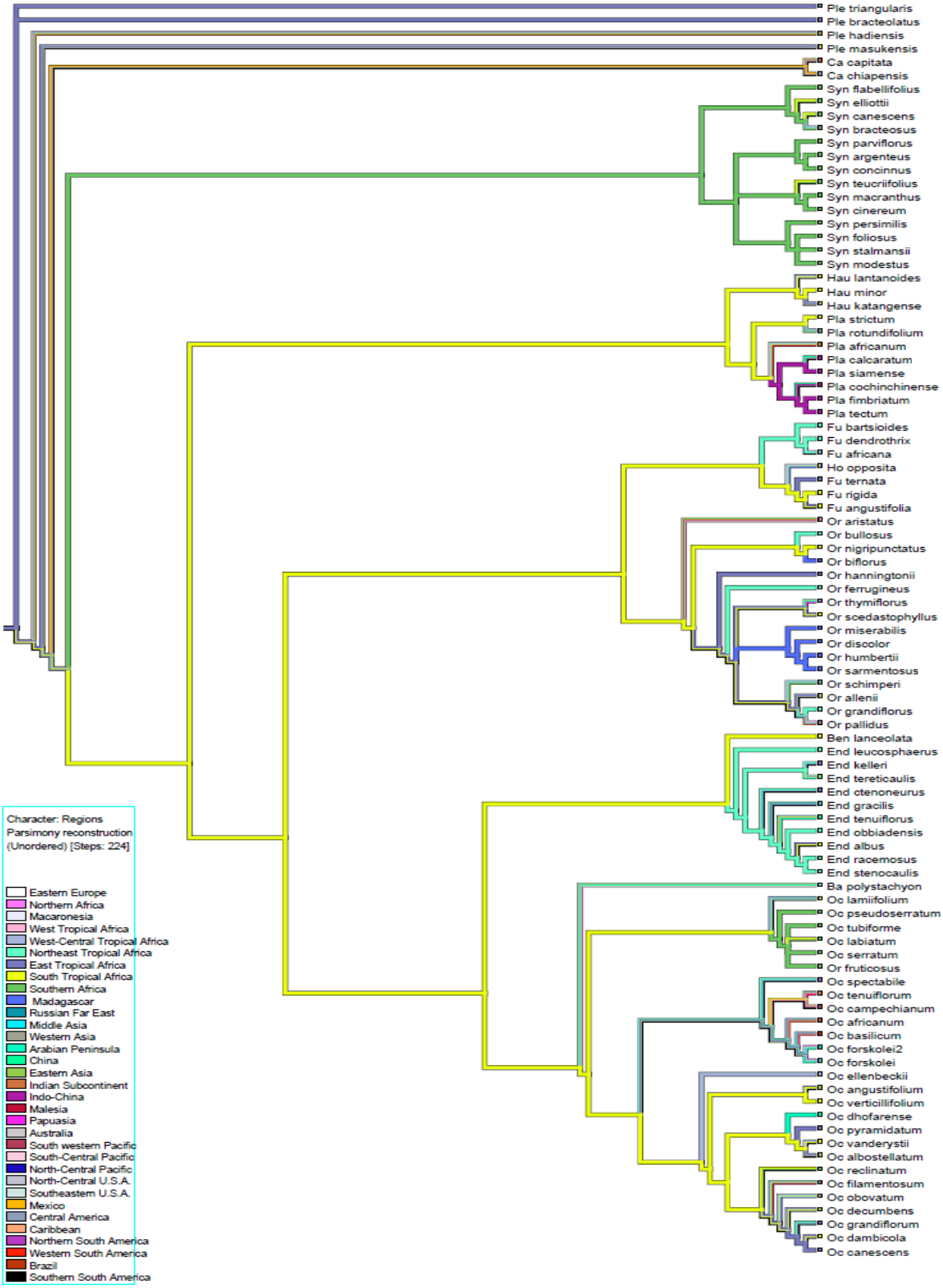


Figure 5.61 Geographic distribution of regions of subtribe Ociminae traced onto combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Ba = *Basilicum*, Ben = *Benguellia*, Ca = *Catoferia*, End = *Endostemon*, Fu = *Fuerstia*, Hau = *Haumaniastrum*, Ho = *Hoslundia*, Or = *Orthosiphon*, Pla = *Platostoma*, Ple = *Plectranthus*, Oc = *Ocimum*, Syn = *Syncolostemon*.

5.5 Discussion

Several morphological characters were used that were assumed to be phylogenetically informative or important for taxonomy. They were useful in the diagnostic of the genera of subtribe Ociminae when mapped onto the combinable component consensus tree from Bayesian analysis of the combined cpDNA and nrDNA regions. Table 5.1 is only giving diagnostic information on clades supported by all analyses. Biogeographic inferences based on dated phylogenetic trees have provided insights into distribution species of the subtribe Ociminae.

Table 5.1 Genera and diagnostic characters.

The genus	**The characters diagnose**
Catoferia	The position of the lateral calyx lobe is nearer the anterior lobes than the posterior lobe, capitate style and basally coloured fertile bracts.
Haumaniastrum* plus *Platostoma	Basally coloured fertile bracts, an emarginate shape to the anterior calyx lobes and basally swollen posterior filaments.
Haumaniastrum	Three flowers, the lateral calyx lobe close to posterior, in the same plane and have a straight corolla tube shape.
Platostoma	One to many flowers, the lateral calyx lobe close to posterior, not in the same plane except two species which have the lateral calyx lobe in the median position and have a downward curved corolla tube shape.
Syncolostemon	Anterior stamens are only fused, posterior free.
Orthosiphon	Three flowers, four fertile stamens, anterior larger disk lobing, subulate shape of anterior calyx lobes and attachment at the mid-point of the corolla tube.
Fuerstia* plus *Hoslundia	Opposite and ternate leaves or ternate leaves, one flower, two fertile stamens, subulate shape of anterior calyx lobes and included stamens.
Orthosiphon* plus *Fuerstia* plus *Hoslundia	Subulate anterior calyx lobes, inappendiculate of posterior filament form, clavate style, anterior larger disk lobing and attachment at the mid-point of the corolla tube.
Endostemon	Style shield.
Basilicum	Three flowers, lanceolate shape of anterior calyx lobes, exserted stamens, clavate style, attachment at the mid-point of the corolla tube, inappendiculate of posterior filament form and equal disk.
Ocimum	Bifid style, three flowers subgenus *Ocimum* and subgenus *Gymnocimum*, attachment of posterior stamen by the base, lanceolate shape of the anterior calyx lobes and appendiculate of posterior filament form of stamen.
Ocimum* plus *Ocimum* subgenus *Nautochilus	Exserted stamens, equal disk lobing, attachment of posterior stamen by the base and bifid style.
Ocimum* subgenus *Nautochilus	One to many flowers, subulate shape of the anterior calyx lobes, inappendiculate of posterior filament form of stamen.
Why *Orthosiphon fruticosus* becomes a part of *Ocimum* subgenus *Nautochilus*	One to many flowers, exserted stamens, attachment of posterior stamen by the base and bifid style.

The shape of the lower lip corolla is a very important characteristic, its boat shape and the fact that surrounds the stamens distinguishes the subtribe Plectranthinae from the subtribe Ociminae which has flat and the stamens extend over it, the two of which are sister groups as reported by Paton et al. (2004). The fusion of stamens can happen in the *Plectranthus* clade, it does not happen in the Ociminae though *Syncolostemon* shows fusion of the anterior stamens and free posterior stamens. There are main centres of diversity for the subtribe Ociminae from this study in Africa, with some species from the genera found in Western Indian Ocean Madagascar, Asia, and America. This is inconsistent with Paton et al. (1994) who showed that many genera in the subtribe Ociminae include a species which is found in India and Africa.

The genera were recognised in the subtribe Ociminae based on Table 5.2 *Catoferia, Syncolostemon, Haumaniastrum, Platostoma, Orthosiphon, Fuerstia, Hoslundia, Endostemon, Basilicum, Ocimum* and *Ocimum* subgenus *Nautochilus.*

Catoferia can be distinguished from other Ociminae by examining the position of the lateral calyx lobe which is nearer the anterior lobes than the posterior lobe and capitate style, and Harley and Paton (2012) reported that *"Catoferia* can be distinguished from *Orthosiphon* by having capitate style." Therefore, it is synapomorphic for the *Catoferia.* Basally coloured fertile bracts character are a diagnostic feature for *Catoferia, Haumaniastrum* and *Platostoma*. *Catoferia* is found in Mexico and Central America in the clade subtribe Ociminae, and Paton et al. (2004) reported that this genus possibly evolved from an African ancestor.

The fusion of stamens is a very significant characteristic in *Syncolostemon* because has fused anterior stamens, free posterior stamens and it is a diagnostic feature for this genus. Most of *Syncolostemon* species have 1–3 variable flowers, but one clade contains *Syn. parviflorus, Syn. argenteus,* and *Syn. concinnus,* and has a strongly supported phylogeny and only one flower. It can thus be suggested that this is one group with a strong relationship. *Syncolostemon* is widespread in Africa and most of them are found in Southern Africa in this study. However, it is also found in Madagascar; unfortunately, sequence data from Madagascan members of this clade is not available.

As mentioned in *Catoferia,* basally coloured fertile bracts character is a diagnostic feature for *Catoferia, Haumaniastrum* and *Platostoma* and this finding not consistent totally with Paton (1997b) who distinguished the *Platostoma* group sensu Ryding from other members of the subtribe Ociminae by basally colour bracts. The *Platostoma* group sensu Ryding is not shown to be monophyletic in the analyses of this study. Moreover, this character confirms the relationship between *Haumaniastrum* and *Platostoma* from the results of the phylogenetic tree in this study. *Haumaniastrum* and *Platostoma* have an emarginate shape to the anterior calyx lobes and basally swollen posterior filaments which make *Platostoma* and *Haumaniastrum* distinct from the rest of

the genera of the subtribe Ociminae. The number of flowers in the cyme, lateral calyx lobe and the corolla tube shape are very important to differentiate *Haumaniastrum* compared to *Platostoma* supporting the findings of Paton (1997a). *Haumaniastrum* and *Platostoma* attachment of posterior stamen by the mid-point of the corolla tube, however, *Pla. calcaratum* has the attachment of posterior stamen by the base of the tube. This evolution is not surprising because this species has a long corolla tube. *Haumaniastrum* is widespread in Africa. This African genus is closely associated with the African *Platostoma* sect. *Limniboza* instead of the Asiatic sect. *Acrocephalus* in this study, which support findings from other studies (Paton, 1997b). The Asiatic species of *Platostoma* formed one clade and this genus was also found in Madagascar, but there was a lack of sequence data available from Madagascar members in these clades.

Fuerstia, Hoslundia and *Orthosiphon* have subulate anterior calyx lobes, inappendiculate of posterior filament form, clavate style and included stamen for all these genera except two species from *Orthosiphon* which have exserted stamens, these similarities of characters confirm the relationship between these genera. Moreover, a clavate style characteristic differentiates *Orthosiphon*, *Fuerstia*, *Hoslundia* from *Ocimum* which has a bifid style, and this matches with Paton (1992), who indicated that *Orthosiphon* differs from *Ocimum* by having the clavate rather than the bifid style. Leaves occur as a diagnostic characteristic for small clades of *Fuerstia* and *Hoslundia*. *Fu. africana, Fu. bartsioides,* and *Fu. dendrothrix* have opposite leaves. *Ho. opposita* and *Fu. ternata* have opposite and ternate leaves, but *Fu. rigida,* and *Fu. angustifolia* have ternate leaves. And this confirms the results of this study regarding the relationship among *Fu. africana, Fu. bartsioides,* and *Fu. dendrothrix,* and between *Ho. opposita, Fu. ternata, Fu. angustifolia* and *Fu. rigida.* This finding supports the fact that *Hoslundia* is related to *Fuerstia* and should be *Fuerstia* merged under the earliest name *Hoslundia* with strong support from phylogenetic trees. However, in the *Orthosiphon* clade, *Or. nigripunctatus* has ternate leaves which Paton (1993) said closely linked *Fu. ternata* and *Or. nigripunctatus* but they are separated by the number of stamens. From this study, *Orthosiphon* is a sister group for *Fuerstia* and *Hoslundia.*

Orthosiphon has three flowers per inflorescence and four fertile stamens per flower but *Fuerstia* and *Hoslundia* have one flower and two fertile stamens. These characters support that the *Orthosiphon* is a sister group with *Fuerstia* and *Hoslundia* and confirms that *Hoslundia* is nested in *Fuerstia.* This result suggests that the significance of the number of stamens to distinguish *Fuerstia* and *Hoslundia* from *Orthosiphon* should be respected. The number of fertile stamens in the flower (two or four) is normally constant for the species in Lamiaceae (El‐Gazzar & Watson, 1970). The shift to two stamens is important in pollination and species proliferation (Claßen-Bockhoff et al., 2004).

The *Fuerstia*, *Hoslundia* and *Orthosiphon* clade is monophyletic in this study when *Orthosiphon fruticosus* is excluded and placed in *Ocimum* subgenus *Nautochilus*. However, this finding of the current study does not support the doubt Paton (1993) created which was that *Orthosiphon* might be paraphyletic by the exclusion of *Hoslundia* and *Fuerstia*. However, Ryding (1992) considered *Hoslundia* as a distinct genus due to the fleshy mature calyx. It is clear that this characteristic has been used by Ryding (1992) to separate *Hoslundia* from *Fuerstia* although similarities exist between them. They have identical nutlets, as well as corolla, inflorescence structure, and sterile posterior stamens (Ryding, 1992). Paton (1993) cast intense doubt based on morphology on the generic delimitation between *Fuerstia* and *Hoslundia*. However, *Fuerstia* and *Hoslundia* share several morphological characters, and the generic status of *Hoslundia* should be reconsidered.

In this study, there are some species are closely related to each other based on the phylogenetic trees, which according to the geographical data can be found in the same area. For example, the *Fuerstia* group is divided into two groups. The first group comprises *Fu. bartsioides, Fu. dendrothrix,* and *Fu. africana,* found in Northeast Tropical Africa and supports the relationship from this study. The second group contains *Hoslundia opposita* which is widespread in Africa and Madagascar but found in two regions in East Tropical Africa and South Tropical Africa, similar to *Fu. ternata, Fu. rigida,* and *Fu. angustifolia*. Geographic data support the division of the two clades of *Fuerstia,* one in Northeast Tropical Africa and the other in South Tropical Africa. It means there are two clades of *Fuerstia* with *Hoslundia;* it could all be one genus.

The geographic data show strong support for the *Orthosiphon* clade. *Orthosiphon aristatus,* includes all of the *Orthosiphon* species and is found in Asia- Temperate, Tropical Asia, and Australasia, but not in Africa; because of this, it becomes a sister to all species from Africa in the tree. There is a clade that contains all species of Western Indian Ocean Madagascar except *Or. biflorus* which is mentioned above (*Or. miserabilis, Or. discolor, Or. sarmentosus,* and *Or. humbertii*) and this data supports this clade.

Style shield is a diagnostic feature that's used to distinguish *Endostemon* from other members of the subtribe Ociminae, and this further supports Paton et al. (1994). It is clear to consider that style shield is synapomorphic for *Endostemon.* Most of the species of *Endostemon* clade are found in Africa and one species in Madagascar and Africa (*Endostemon tenuiflorus*). *Endostemon viscosus* from India was not included in this study and Paton et al. (1994), also mentioned that the only Indian species has a close relationship to an African one.

Basilicum polystachyon has three flowers as do *Ocimum* species subgenus *Ocimum* and subgenus *Gymnocimum.* The *Ocimum* subgenus *Nautochilus* (*Ocimum lamiifolium, Oc. pseudoserratum, Oc. serratum, Oc. labiatum,* and *Oc. tubiforme*) and *Orthosiphon fruticosus*

have one to many flowers and formed a clade together. This supports the fact that *Basilicum* is close to the *Ocimum* species. Therefore, the number of flowers can be used to distinguish the *Ocimum* subgenus *Nautochilus* from another subgenus of *Ocimum* and confirms the relationship between *Orthosiphon fruticosus* and *Nautochilus*. The subulate shape of anterior calyx lobes supports the relationship between *Basilicum polystachyon* and *Ocimum* subgenus *Nautochilus* and differentiate these from *Ocimum* species. Stamens are exserted in the *Ocimum* clade, which the morphology supports the relationship between *Ocimum* and *Basilicum* and between the *Ocimum* subgenus *Nautochilus* and the rest of *Ocimum* suggested by the analysis in this work. And also supports that *Orthosiphon fruticosus* is a member of *Ocimum* subgenus *Nautochilus* rather than *Orthosiphon*. Equal disk lobing helped to distinguish *Basilicum,* and *Ocimum* from other genera in Ociminae, which have the anterior lobes larger. Moreover, it confirms the relationship between *Basilicum* and *Ocimum* in this study, which supports Paton (1992) who observed that *Basilicum* is morphologically similar to *Ocimum.* Moreover, the equally four-lobed disk indicates that *Nautochilus* close to *Ocimum,* and this result further supports Paton (1992). However, it differentiates *Orthosiphon fruticosus* from *Ocimum* subgenus *Nautochilus.* It is clear, based on anterior larger disk lobing, that it falls in the *Orthosiphon* genus by traditional classification; however, the findings of the current study do not support this. Nevertheless, the importance of this characteristic used to distinguish *Orthosiphon* from *Ocimum* and this was found both in this study and that of Paton (1992). Attachment of posterior stamen by the base of the anterior lip distinguishes *Orthosiphon fruticosus* from *Orthosiphon* and includes it with the *Ocimum* subgenus *Nautochilus.* In addition, it confirms the relationship between *Ocimum* subgenus *Nautochilus* and *Ocimum* from this study and a study by Codd (1964). Moreover, this character sets apart *Basilicum* from *Ocimum* and *Orthosiphon* from *Ocimum.* This result is consistent with Paton (1992) when he found that attachment in *Orthosiphon* by the mid-point of the corolla tube, rather than the base in *Ocimum,* useful for separating these genera from each other. Bifid style distinguishes *Orthosiphon fruticosus* from *Orthosiphon* and confirms that it is nested within the *Ocimum* subgenus *Nautochilus.* It also differentiates *Ocimum* from *Basilicum,* which is the sister group in this study. Paton (1992) differentiates *Orthosiphon* from *Ocimum* by having inappendiculate posterior stamens rather than appendiculate in *Ocimum,* from this study exception some subgenus of *Ocimum* from that. Moreover, this character sets apart *Basilicum* from *Ocimum.*

5.6 Conclusion

In conclusion, this chapter mapped morphological and geographical distribution onto the combinable component consensus trees of the subtribe Ociminae from the combined cpDNA and

nrDNA regions. This provided a good investigation for genera and the relationship between them and corresponded to traditional classifications as well.

The subtribe Ociminae is monophyletic, easily diagnosable with morphological synapomorphy which is flat lower lip corolla and the stamens extend over it and has an Africa origin. All the morphological characters which were used in traditional classification are informative characteristics between the genera. The number of stamens is a diagnostic feature used to separate *Orthosiphon* from *Fuerstia* and *Hoslundia*; therefore, *Orthosiphon* is a sister to *Fuerstia* and *Hoslundia* while keeping *Orthosiphon* as a distinct genus. Moreover, this chapter confirms that *Hoslundia* has a close relation to *Fuerstia*; they all have the same morphology of 1 flowered cyme, two fertile stamens, opposite and ternate leaves or ternate leaves, subulate shape of anterior calyx lobes and included stamens and they formed a monophyletic group. Therefore, *Hoslundia* and *Fuerstia* will be merged under the earliest name *Hoslundia* later on in the paper, therefore, the name of *Fuerstia* should be changed to *Hoslundia*. *Ocimum* subgenus *Nautochilus* is a sister to the rest of *Ocimum* (subgenus *Ocimum* and subgenus *Gymnocimum*) because they have exserted stamens, equal disk lobing, attachment of posterior stamen by the base and bifid style. Due to the large morphological similarity (one to many flowers, exserted stamens, attachment of posterior stamen by the base and bifid style) and geographical data between *Orthosiphon fruticosus* and *Ocimum* subgenus *Nautochilus*, this study suggests considering this species as *Ocimum* subgenus *Nautochilus. Platostoma* is a sister to *Haumaniastrum* because they have basally coloured fertile bracts, an emarginate shape to the anterior calyx lobes and basally swollen posterior filaments but *Haumaniastrum* differs from *Platostoma* by three flowers than one to many flowers and the lateral calyx lobe close to posterior in the same plane and has a straight corolla rather than lateral calyx lobe close to posterior not in the same plane and a downward curved corolla.

Chapter 6 : DNA barcode techniques

6.1 Introduction

Identification of *Orthosiphon* species is very difficult because there are no keys to this genus and it has species delimitation. According to Paton et al. (2009) "*Orthosiphon rubicundus* is closely similar to the African material of *Or. schimperi*, but it seems clearly distinct, most notably on account of the regular formation of discrete tubers on the root system, but also with longer intervals between the verticils and a different aspect overall. In East Africa *Or. schimperi* can be distinguished most easily from *Or. thymiflorus* by its larger fruiting calyx (7–10 or more rather than 5–7 mm), flushed purplish red overall, rather than purple on the posterior lip and green underneath, and with the lateral lobes of the anterior lip of the fruiting calyx not much shorter than the lower lobes, but these distinctions do not always hold elsewhere". Because there is a confusion between these species they have been chosen for study plus other samples which were available in Kew.

Employing identification keys that have a basis in morphology characteristics may be problematic if there are certain features that cannot be seen, for example, if the specimen is outside specific life stages (e.g. flowering period), or is incomplete being old or broken. DNA barcoding offers a means of accurately identifying plant samples that cannot be distinguished through morphological means (Rydberg, 2010; Ghorbani et al., 2017).

DNA barcoding can identify and discriminate between species by employing short, variable, and standardized DNA regions (Hebert et al., 2003a). According to Chase et al. (2007), the most important characteristics of a universal barcode are to be able to be amplified for all taxa by the employment of standard primers; they should also be easy to sequence. Additionally, a barcode must have the requisite variability for species-level identification, ideally with high interspecific and low intraspecific sequence divergence. Further, for many potential applications, it must be possible to recover DNA from herbarium samples and other degraded samples (Group et al., 2009). The effectiveness of barcoding is reduced the more intraspecific and interspecific variations overlap (Meyer & Paulay, 2005).

The mitochondrial cytochrome c oxidasel (CO1) gene has been used as a universal barcode in animals. The low rate of nucleotide substitution in plant mitochondrial genomes precludes the use of CO1as a universal plant barcode (Fazekas et l., 2008). As CO1 was not useful in plants, many loci have been proposed as plant barcodes, including ITS (Chase & Fay, 2009; Kress et al., 2005), *trn*H-*psb*A (Chase et al., 2005; Kress et al., 2005), and *mat*K (Chase et al., 2005). The identification of high-resolution DNA barcodes at the species level is critical. The third International Barcode of Life Conference (CBOL) concluded with a remark that *mat*K and *rbc*L are sequences of the universal barcode sequence for land plants. Despite these useful

recommendations, debate continues as to identification and which combination of regions are most suitable for the DNA barcoding of plants (Bruni et al., 2010; Chen et al., 2010).

While scientific interest in this area has expanded, little research has been undertaken regarding barcoding for medicinal plants in the Lamiaceae. Several studies have demonstrated that *trn*H–*psb*A and *mat*K, and ITS regions are powerful barcodes for identifying most species in Lamiaceae.

For example, according to the findings of De Mattia et al. (2011), the most appropriate marker in six genera (*Ocimum, Mentha, Salvia, Origanum, Rosmarinus,* and *Thymus*) for molecular species recognition are the non-coding *trn*H–*psb*A intergenic spacer, with interspecific genetic distance values ranging from 0% to 7%, and *mat*K, at 0% to 5%. The very useful combination was the *mat*K + *tm*H–*psb*A and has a similarity of discriminatory performance as the *trn*H–*psb*A. Moreover, Theodoridis et al. (2012) examined the native plants of the Lamiaceae family on Chios Island (Greece) and the adjacent Çeşme-Karaburun Peninsula. According to the findings, the species of Lamiaceae were distinguished by *trn*H–*psb*A and *mat*K, as any multi-region combination. They suggested that "*mat*K and *trn*H-*psb*A could serve as single-region barcodes for Labiatae species contributing to the conservation and the trade control of valuable plant resources". Anbazhagan et al. (2014), confirmed that the *mat*K gene is an effective DNA barcode gene for *Ocimum sp* in plant DNA barcoding. Moreover, Aziz et al. (2015) pointed out that the best marker of DNA for identification of medicinal plants in Malaysia including *Orthosiphon stamineus* is *trn*H–*psb*A. It would be interesting to use these regions for DNA barcoding. Zahra et al. (2016) selected three plastid loci *rbc*L, *mat*K, and *trn*H–*psb*A to barcode herbal medicinal products from the Lamiaceae, including *Ocimum basilicum*, *Ocimum* x *africanum*, and *Ocimum tenuiflorum*. *mat*K and *trn*H–*psb*A were able to distinguish the species relatively better with a 40% success rate than *rbc*L, which had a 16% success rate. It is more crucial to test barcode effectiveness of *trn*H–*psb*A and *mat*K to identify unknown species from Lamiaceae.

In addition to plastid markers, the nuclear ribosomal internal transcribed spacer (ITS) region showed high levels of interspecific sequence variability (Cowan & Fay, 2012). This non-coding region has also been indicated as a barcoding region in Lamiaceae. For example, in *Salvia*, the best DNA barcoding choice for discriminating between species is ITS1 because other markers used (*rbc*L, *mat*K, *trn*L-F, and *psb*A-*trn*H from the chloroplast genome, and ITS2 from the nuclear genome) were less effective in discriminating between species (Wang et al., 2013). Al-Qurainy et al. (2014) wrote that nrDNA–ITS and chloroplast loci *rbc*L and *rpo*C1 in *Plectranthus asirensis* had little similarity via BLAST of the NCBI GenBank database to the rest of the species; hence they would be appropriate for DNA barcoding of this plant. Elsherbeny (2016) evaluated the utility of DNA barcoding in discriminating some Lamiaceae species present in Egypt by two cpDNA

regions (ITS and *rbc*L). He found that both ITS and *rbc*L were useful in discriminating among the collected species of the family Lamiaceae. In summary, several studies recommended the use of nuclear ribosomal ITS, *mat*K, and *tm*H–*psb*A spacer from the plastid genome for discriminating plant species in Lamiaceae.

Although successful validation for a number of plant families has been achieved by employing candidate DNA barcodes for chloroplast and nuclear regions (Christina & Annamalai, 2014), but, recently researchers have proposed the use of the whole-plastid genome sequence in plant identification (Erickson et al., 2008; Sucher & Carles, 2008; Parks et al., 2009; Nock et al., 2011; Yang et al., 2013). Because single-locus DNA barcodes lack adequate variations in the taxa that are closely linked it is presumed that multi-locus markers are more successful in determining species, but studies to date demonstrated that these are also not appropriate for universal plant identification (Li et al., 2015). Li et al. (2015) reported that a lot of potential has been demonstrated by whole-plastid-based barcodes in the discrimination of species, particularly for the taxa that are closely linked. Although, Hollingsworth, Graham, & Little (2011) argued that "the full plastid haplotype is not a good marker because it does not always track species boundaries".

DNA barcoding success depends on the distinctness of the clusters (Steinke et al., 2009). There are many methods of building the trees for clustering group. Unweighted Pair Group Method with Arithmetic mean (UPGMA) is one of the main phylogenetic approaches (Liberles, 2005). The UPGMA methodology produces rooted trees and has a constant-rate assumption, which assumes an ultrametric tree where the distances from the roots to all branch tips are equal (Sokal, 1958). The Neighbor-Joining (NJ) method is extremely helpful for estimating similarity among species (Erickson & Driskell, 2012). Therefore, it was used to test the DNA barcoding. The simple neighbour-joining method produces unrooted trees, however, it does not make the assumption of the constant evolution rate (i.e., molecular clock) across lineages (Saitou & Nei, 1987), meaning that the branch lengths are proportional to the change amount.

6.2 Aims and objectives

The first aim of the present study is to solve species boundary delimitation problems in *Orthosiphon* species by three candidate barcoding loci (*trn*H–*psb*A intergenic spacer, *mat*K, and ITS) and their combination for the first time. The second aim is to identify unknown species of *Orthosiphon* from the Kew Herbarium using these candidate DNA barcodes. The resolution of these potential barcoding regions will be tested with the following objectives:

1. To analyse levels of interspecific and intraspecific variation in the *Orthosiphon* genus.
2. To evaluate the species discrimination capability of DNA barcode markers.

3. To test the usefulness and effectiveness of *trn*H–*psb*A intergenic spacer, *mat*K, and ITS in identifying unknown species of *Orthosiphon.*

6.3 Materials and methods

6.3.1 Plant materials

Samples were collected for 32 species of *Orthosiphon* from Kew, with seven of them being unknown specimens which found in the index folder of *Orthosiphon* (Table 6.1) (Figure 6.1). Standard DNA barcoding methodology minimum ten samples, but in this study used under five samples that were available in Kew and they are broadly geographic samples to achievement that limitations

Table 6.1 List of plant samples of DNA barcoding from Kew.

Code	Species name	Collector	Collection date	Collection location
160A	*Orthosiphon allenii* (C.H.Wright) Codd	S. Bidgood et al. 2450	25/2/1994	Tanzania
161A	*Orthosiphon allenii* (C.H.Wright) Codd	M.G. Bingham 12636	10/12/2002	Zambia
162A	*Orthosiphon allenii* (C.H.Wright) Codd	L. Macuacua 1473	18/12/1980	Mozambique
192A	*Orthosiphon humbertii* Danguy	P. B. Phillipson et al. 4003	29/3/1992	Fianarantsoa/ Madagascar
194A	*Orthosiphon humbertii* Danguy	J. Leandri & P.Saboureau 4.225	2/12/1960	Andohahela/ Madagascar
195A	*Orthosiphon humbertii* Danguy	F. Ratovoson 1591	23/3/2010	Anosy region/ Madagascar
196A	*Orthosiphon humbertii* Danguy	H. Humbert et al. 29641	26/3/1955	Zombitsy (Sakarha)/ Madagascar
147A	*Orthosiphon pallidus* Royle ex Benth.	M. Thulin 11516	1/11/2006	Oman

Table 6.1 cont. List of plant samples of DNA barcoding from Kew.

Code	Species name	Collector	Collection date	Collection location
148A	*Orthosiphon pallidus* Royle ex Benth.	Korogo BUR-509	13/1/2007	Burkino Faso
149A	*Orthosiphon pallidus* Royle ex Benth.	Friis, et al. 10,387	19/9/2001	Ethiopia
152A	*Orthosiphon pallidus* Royle ex Benth.	A. Mwachala et al. EW1458	4/12/1998	Kenya
140A	*Orthosiphon rubicundus* (D.Don) Benth.	J. Olorunfeml 55795	24/4/1965	Nigeria
141A	*Orthosiphon rubicundus* (D.Don) Benth.	Hell & Enti Cc38733	1/8/1968	Ghana
142A	*Orthosiphon rubicundus* (D.Don) Benth.	M. Hakki 878	10/5/1978	Von Togo
144A	*Orthosiphon rubicundus* (D.Don) Benth.	S. Y. Hu & Y. C. Kong Y155	9/7/1999	Hong Kong
170A	*Orthosiphon schimperi* Benth	J. M. Fay 3318	9/1/1982	*Manovo-Gounda* St Floris *National Park*/ Central African
171A	*Orthosiphon schimperi* Benth.	Friis et al 9288	27/11/1998	Ethiopia
172A	*Orthosiphon schimperi* Benth.	C. Crawford FC242	26/11/2008	Mozambique
173A	*Orthosiphon schimperi* Benth.	D. J. Goyder & A. J. Paton 3523	16/1/1992	Malawi
174A	*Orthosiphon schimperi* Benth.	R. D. Kelly 54	10/1/1969	Salisbury/ Rhodesia
175A	*Orthosiphon sp* (Unknown)	R. A. Clement et al. 2097	23/3/1992	Madagascar
176A	*Orthosiphon sp* (Unknown)	R. A. Clement et al. 2056	15/3/1992	Madagascar
177A	*Orthosiphon sp* (Unknown)	M. Cherlet 54	16/2/1995	Eritrea
178A	*Orthosiphon sp* (Unknown)	C.F. Hemming &I. Deshumukh Jess/86/165-166	13/8/1986	Somalia

Table 6.1 cont. List of plant samples of DNA barcoding from Kew.

Code	Species name	Collector	Collection date	Collection location
184A	*Orthosiphon sp* (Unknown)	E. Werdermann et al. 2197	6/2/1959	Transvaal
185A	*Orthosiphon sp* (Unknown)	I. B. Pole Evans 3094 (31)	3/3/-	Pretoria
188A	*Orthosiphon sp* (Unknown)	Hemriques 738	10/9/1965	Angola
165A	*Orthosiphon thymiflorus* (Roth) Sleesen	Friis, et al. 14276	29/11/2011	Ethiopia
166A	*Orthosiphon thymiflorus* (Roth) Sleesen	M. Thulin 10979	18/5/2002	Somalia
167A	*Orthosiphon thymiflorus* (Roth) Sleesen	Friis, et al. 11,089	21/12/2002	Ethiopia
168A	*Orthosiphon thymiflorus* (Roth) Sleesen	M. G. & P. E. Bingham 11246	4/12/1996	Zambia
182A	*Orthosiphon thymiflorus* (Roth) Sleesen	B. Ntemi Sallu 239	27/1/1999	Tanzania

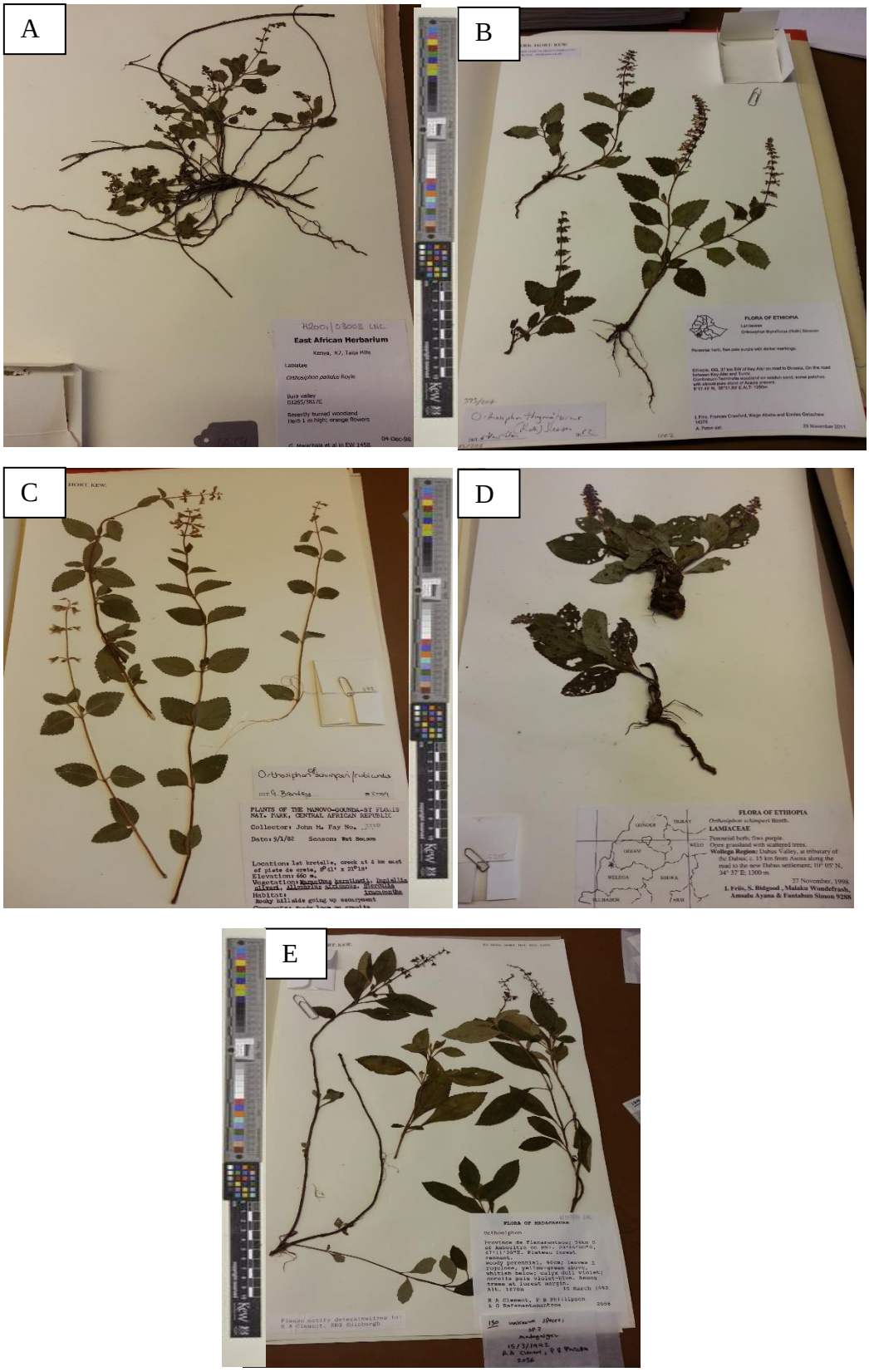


Figure 6.1 Pictures of herbarium specimens representing some samples in Table 6.2. A. *Orthosiphon pallidus* (152A). B. *Orthosiphon thymiflorus* (156A) C. *Orthosiphon schimperi* (170A). D. *Orthosiphon schimperi* (171A). E. *Orthosiphon sp* (Unknown) (176A).

6.3.2 DNA extraction, PCR and sequencing

The techniques are given in Chapter 2. Moreover, it has been tried to amplified the *rbc*L in this chapter with primers 1F ATGTCACCACAAACAGAAAC and 1460R TCCTTTTAGTAAAAGATTGGGCCGAG and 1F and 724R TCGCATGTACCTGCAGTAGC from Olmstead et al. (1992). Initial DNA denaturation 95 ℃ 3 minutes, 40 cycles, denaturation 95 ℃ 1 minute, annealing 56℃ 1 minute, extension 72 ℃ 1.5 minute and final extension 72 ℃ 7minutes.

6.3.3 DNA barcoding analysis

The dataset was imported into TaxonDNA/SpeciesIdentifier 1.8 (Meier et al., 2006). The Species Summary, Pairwise Summary, Pairwise Explorer, Distance Analysis, Extreme Pairwise, Best Match/Best Close Match, All Species Barcodes, Cluster, and Overlap Analysis were calculated with pairwise distances using Kimura 2-parameter corrected distances (K2P) (Kimura, 1980). Resultant data were stored in a Word document 2016. Pairwise Summary results were imported into Microsoft Excel 2016.

To evaluate the potential of barcoding regions to accurately identify species. It compares each sequence in the dataset based on their pairwise genetic distances and determines the sequences are likely to be conspecific. According, the “best match” and “best close match” functions based on barcoding gap were used to test the species discrimination rates for each and all combinations marker under the K2P-corrected distance using TaxonDNA (Meyer & Paulay, 2005). The relative distribution of pairwise genetic distances was calculated using TaxonDNA to evaluate the barcoding gap. Histograms were generated for the barcoding gaps to evaluate whether the interspecific distances were larger than the intraspecific distances.

6.3.4 Tree-based analysis

Different phylogenetic methods were selected to test the monophyletic relationship between species and to display the molecular identification results, such as the Neighbor-Joining (NJ) method under the K2P distance model and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree was used in MEGA 7.0 (Kumar et al., 2016b).

6.3.5 NCBI GenBank nucleotide BLAST

The sequences were blasted on GenBank to identify unknown species by matching to the submitted sequence there, by blastn tool.

6.4 Results

6.4.1 Sequencing

The sequencing of all DNA regions was successful except the *rbc*L. Overall, the aligned length included 812 bp (ITS), 451 bp (*trn*H-*psb*A), and 780 bp (*mat*K) for a single locus and 2043 bp (*matK*+ *trn*H-*psb*A +ITS) for all loci.

6.4.2 DNA barcoding analysis

According to the "Best Match" and "Best Close Match" in TaxonDNA method, the species discriminatory power was recorded as 100% and 100% for ITS, *mat*K and combination of *trn*H-*psb*A+*mat*K and ITS+*trn*H-*psb*A+*mat*K by applying the best match and the best close match criteria, respectively. However, *trn*H-*psb*A 91.66 and 91.66 % correct species values (Table 6.2).

Table 6.2 Species identification success rate based on TaxonDNA analysis.

Candidate barcodes	Best match (%)			Best close match (%)			No match (%)
	Correct	Ambiguous	Incorrect	Correct	Ambiguous	Incorrect	
ITS	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%
***trn*H-*psb*A**	91.66%	0.0%	8.33%	91.66%	0.0%	8.33%	0.0%
***mat*K**	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%
***trn*H-*psb*A+matK**	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%
Combined ITS+*trn*H-*psb*A+*mat*K	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%

Analysis of the relative distribution of K2P pairwise distances using TaxonDNA showed that single loci *matK*, ITS and combinations of ITS+*trn*H-*psb*A+*mat*K showed clear barcoding gaps in *Orthosiphon* species. *trn*H-*psb*A+*mat*K showed less gap. On the other hand, *trn*H-*psb*A lacked a barcoding gap (Figures 6.2-6.6).

In summary, *trn*H-*psb*A+*mat*K showed the least overlap followed by ITS, followed by ITS+*trn*H-*psb*A+*mat*K combinations followed by *mat*K and then *trn*H-*psb*A (Table 6.3).

Table 6.3 Shows the total overlap of the regions which were used in this study.

Region	**Total overlap**	**covering**
ITS	0.26% from 0.51% to 0.24%	0.0%
***trn*H-*psb*A**	0.48% from 0.48% to 0.96%	48.19%
***mat*K**	0.4% from 0.4% to 0.0%	0.0%
***trn*H-*psb*A+*mat*K**	0.08% from 0.43% to 0.34%	0.0%
ITS+*trn*H-*psb*A+*mat*K	0.3% from 0.52% to 0.21%	0.0%

As a single barcode, ITS showed the highest divergence in *Orthosiphon* species, followed by *trn*H-*psb*A, and combinations of ITS+*trn*H-*psb*A+*mat*K and followed by *trn*H-*psb*A+*mat*K, and after that *mat*K (Table 6.4).

Table 6.4 Shows the intraspecific and interspecific variation.

Region	**The intraspecific variation**	**The interspecific variation**	**Figure**
ITS	0.0% -0.5%	0.5% - 3%	6.2
***trn*H-*psb*A**	0.0% - 0.5%	0.5% - 2.0%	6.3
***mat*K**	0.0%	0.5% - 1.0%	6.4
***trn*H-*psb*A+*mat*K**	0.0% - 0.5%	0.5% - 1.5%	6.5
ITS+*trn*H-*psb*A+*mat*K	0.0%- 0.5%	0.5% - 2.0%	6.6

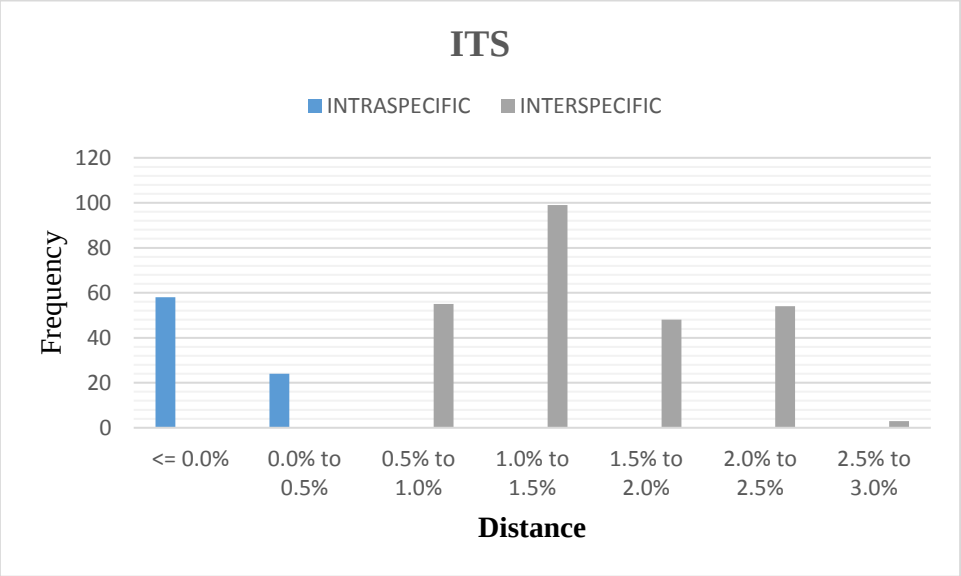


Figure 6.2 The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of ITS.

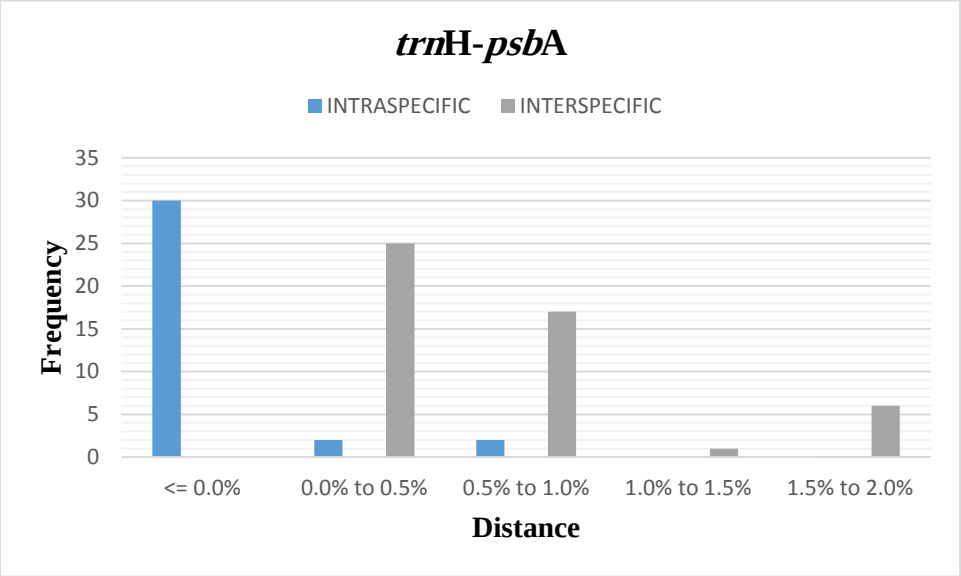


Figure 6.3 The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of *trn*H-*psb*A.

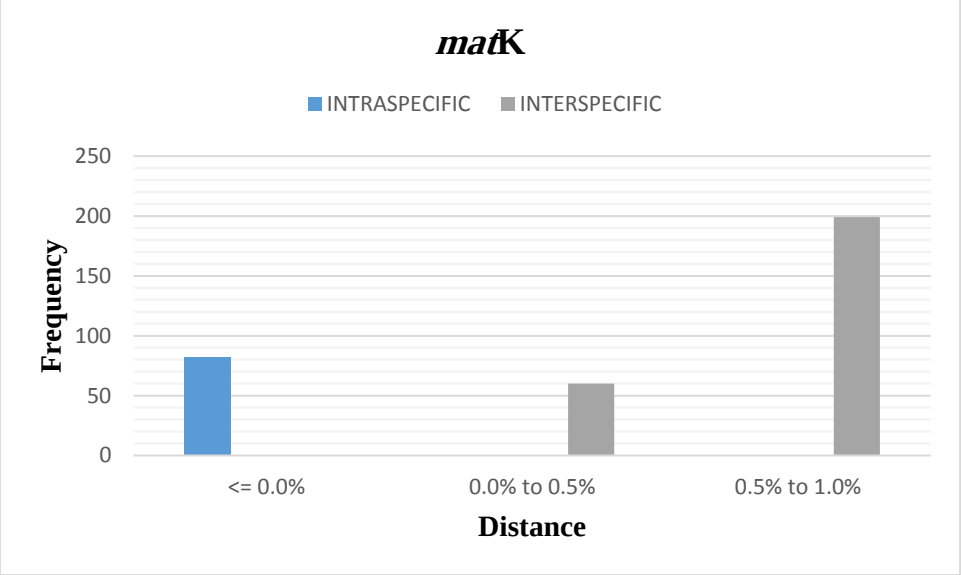


Figure 6.4 The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of *mat*K.

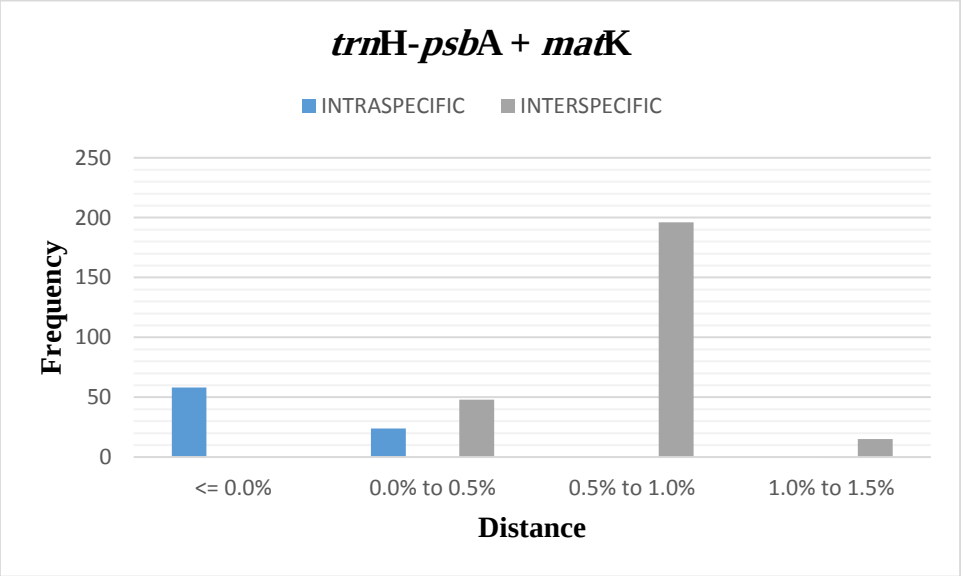


Figure 6.5 The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of *trn*H-*psb*A+*mat*K.

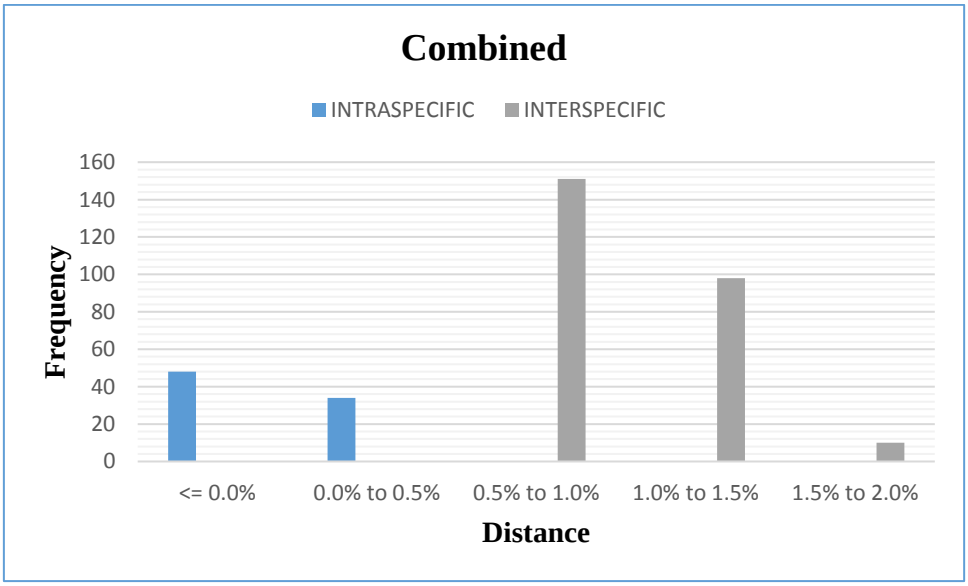


Figure 6.6 The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of ITS+*trn*H-*psb*A+*mat*K.

6.4.3 Tree-based analysis

The identification of single and combined barcodes was determined by evaluating the clustering properties of each species using the UPGMA analyses and NJ method.

All six of the *Orthosiphon* species sampled (100.0%) were successfully identified by ITS, *trn*H-*psb*A, *mat*K and combination of *trn*H-*psb*A+*mat*K and ITS+*trn*H-*psb*A+*mat*K formed a cluster in the UPGMA and NJ trees analysis namely: *Orthosiphon allenii, Orthosiphon humbertii, Orthosiphon pallidus, Orthosiphon rubicundus, Orthosiphon schimperi* and *Orthosiphon thymiflorus* (Figures 6.7-6.16).

Orthosiphon pallidus is closely related with *Or. allenii* by all the regions in UPGMA analyses and NJ method. *Or. schimperi, Or. rubicundus* and *Or. thymiflorus* are close to each other by ITS and *mat*K in UPGMA analyses and add *Or. humbertii* to this relation by *trn*H-*psb*A, *trn*H-*psb*A+*mat*K and ITS+*trn*H-*psb*A+*mat*K. NJ method confirms the relationship among *Or. schimperi, Or. rubicundus* and *Or. thymiflorus* by all the regions but *mat*K added *Orthosiphon humbertii* to this relation. All members of the species were clustered and differentiated from the other closely related species *Or. schimperi, Or. rubicundus* and *Or. thymiflorus* members of genus *Orthosiphon* by both analyses.

Seven unknown species could be discriminated in a UPGMA and NJ tree by three single markers and two combined region and are as follows: 178A and 188A are sister group with *Orthosiphon schimperi* and 177A with *Orthosiphon pallidus* group's. 185A is not with any clade with *Orthosiphon* but not far than this genus. 175A, 176A and 184A as the outgroup for *Orthosiphon* (Figures 6.7-6.16).

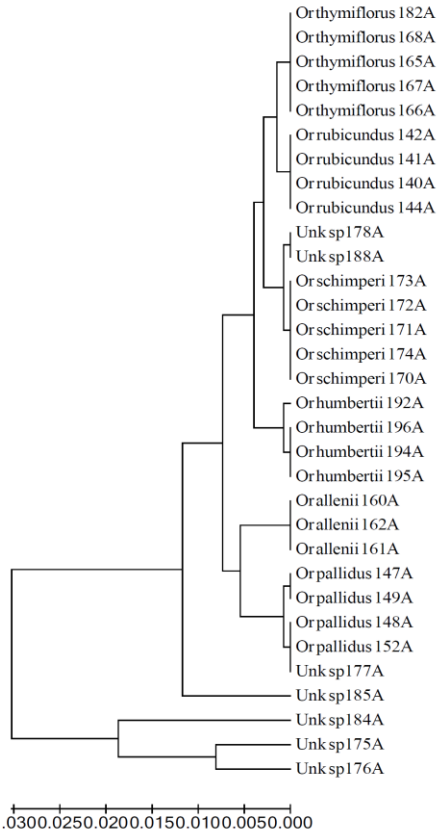


Figure 6.7 UPGMA tree based on ITS. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

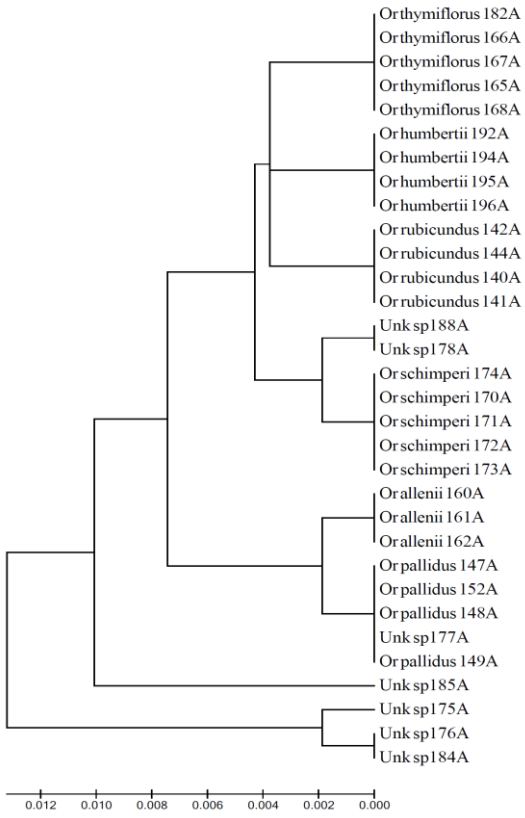


Figure 6.8 UPGMA tree based on *trn*H-*psb*A. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

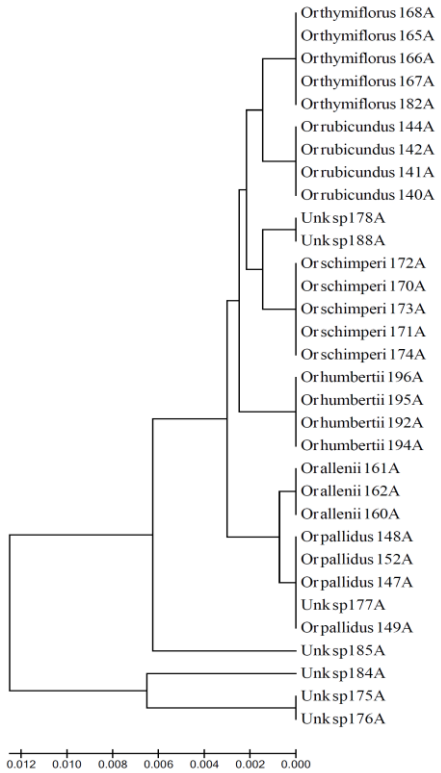


Figure 6.9 UPGMA tree based on *mat*K. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

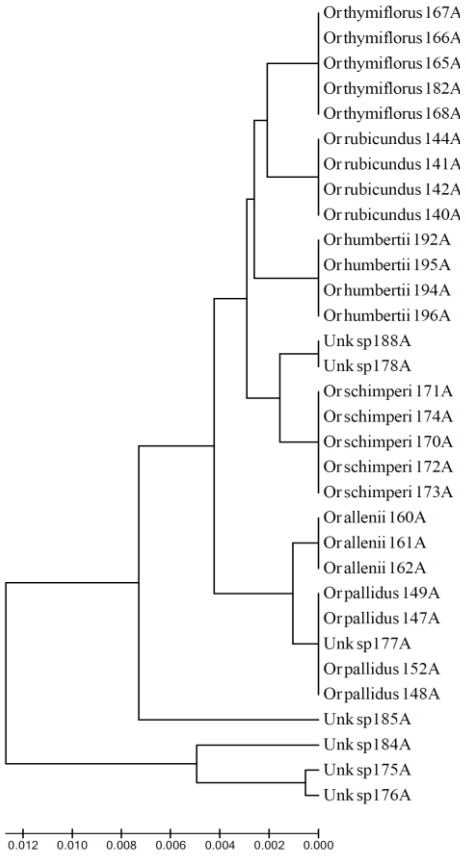


Figure 6.10 UPGMA tree based on *trn*H-*psb*A+ *mat*K. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

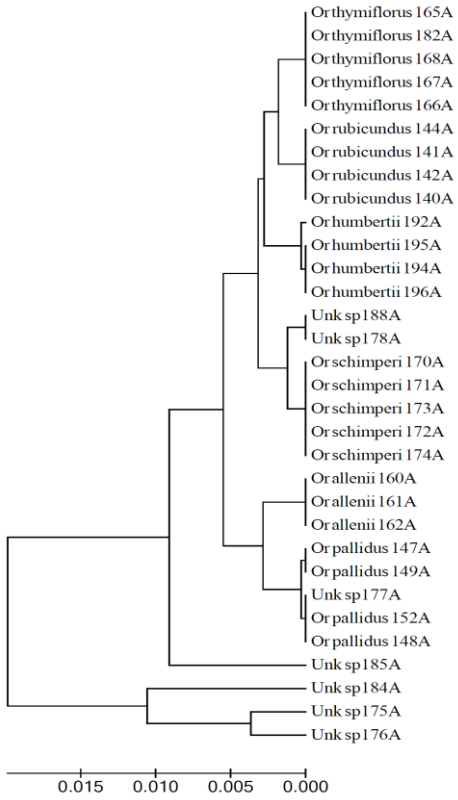


Figure 6.11 UPGMA trees based on ITS+*trn*H-*psb*A+ *mat*K. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

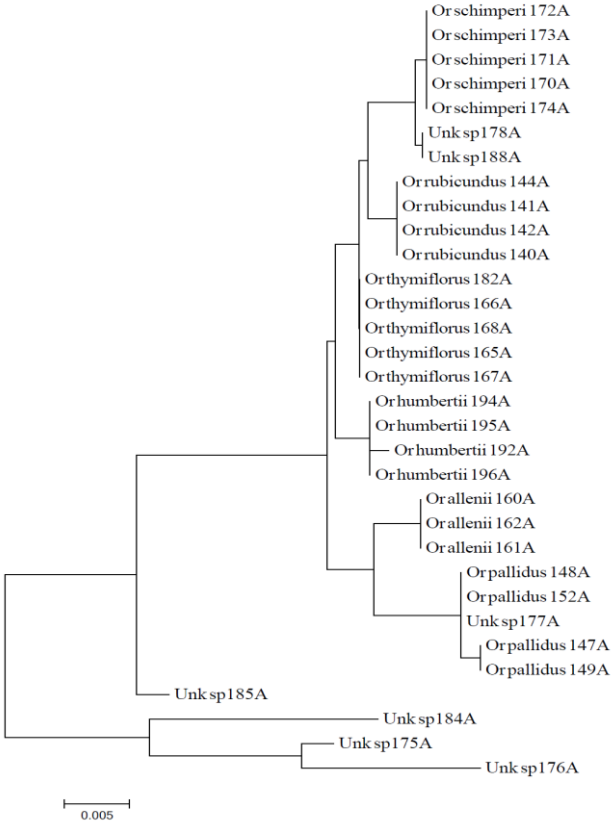


Figure 6.12 NJ tree based on ITS. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

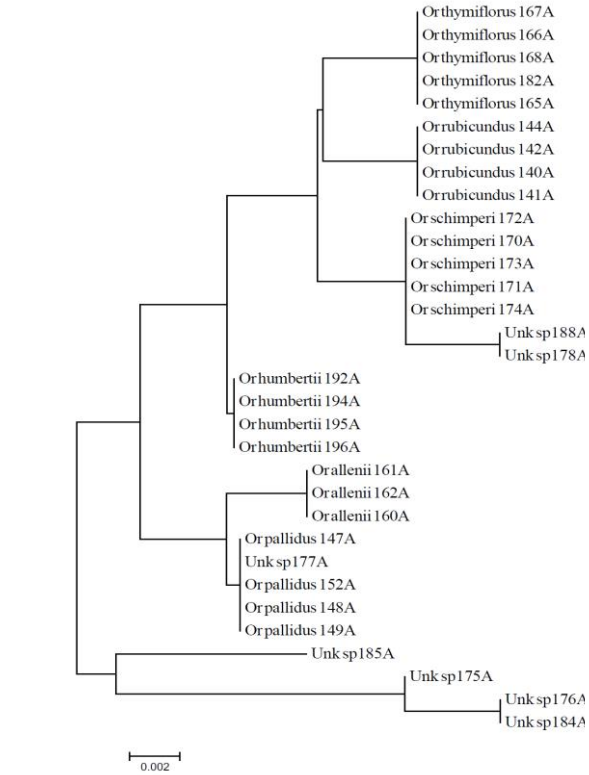


Figure 6.13 NJ tree based on *trn*H-*psb*A. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

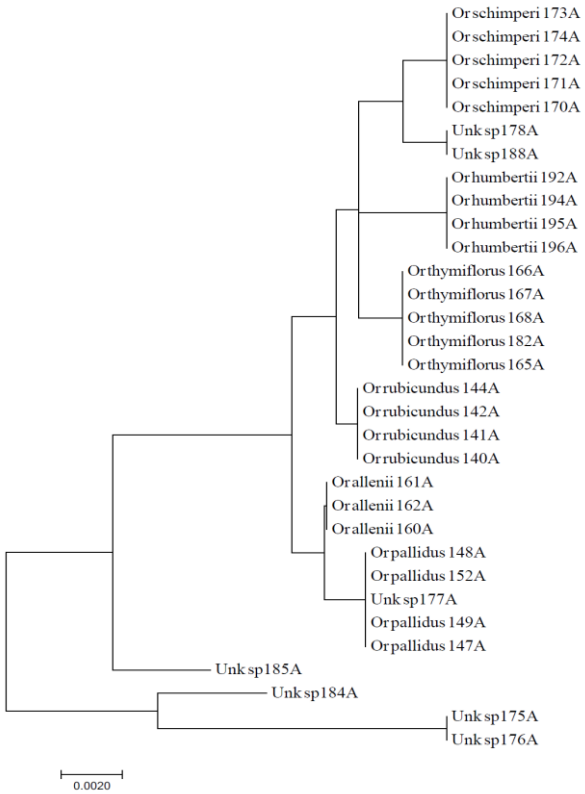


Figure 6.14 NJ tree based on *mat*K. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

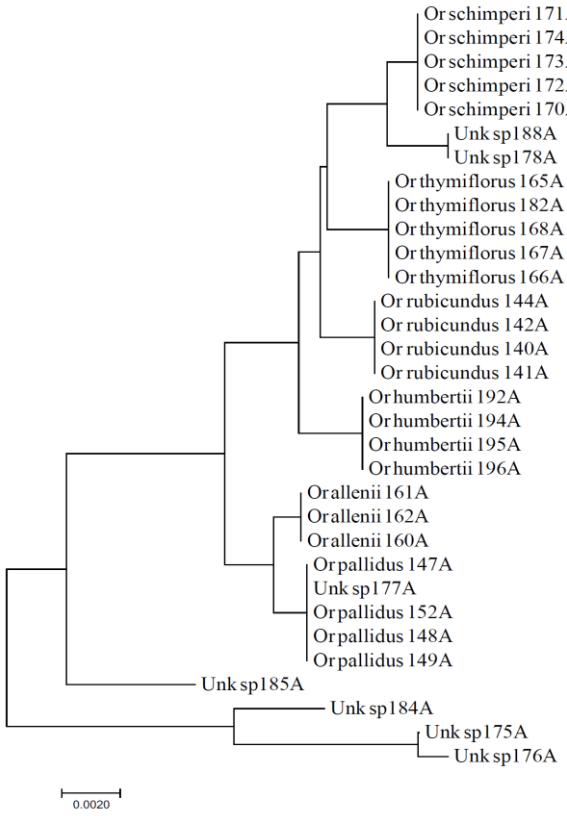


Figure 6.15 NJ tree based on *trn*H-*psb*A+ *mat*K. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

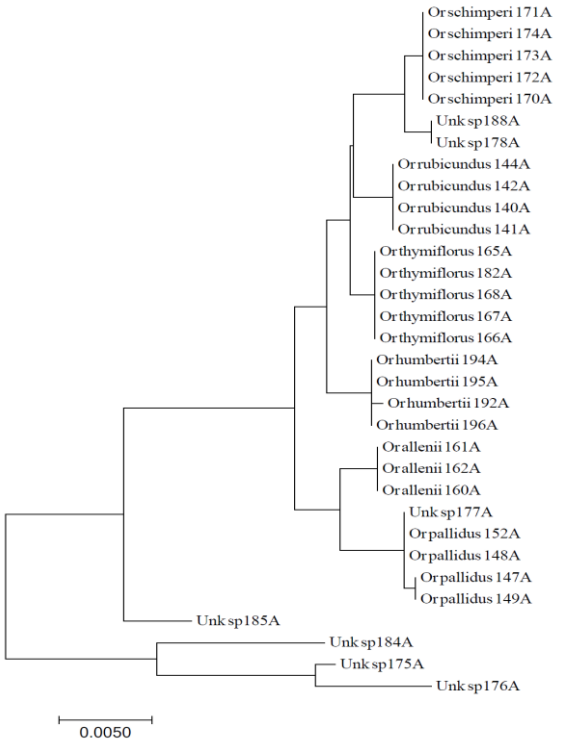


Figure 6.16 NJ tree based on ITS+*trn*H-*psb*A+ *mat*K. Or = *Orthosiphon* and Unk = Unknown specimens which found in the index folder of *Orthosiphon* at Kew.

6.4.4 NCBI GenBank nucleotide BLAST

To identify the unknown samples, especially that did not match with *Orthosiphon* species, the DNA sequences were blasted in NCBI GenBank. The blast result for unknown species can be found in Appendix 6 on the CD (Morgulis et al., 2008).

The data by blastn tool from GenBank identify 177A species belong to *Orthosiphon* genus but because the GenBank has limited sequences from *Orthosiphon* for example only two species available in *trn*H-*psb*A and *mat*K and three species of ITS, so the name of species not identified exactly. However, when blasted this with the data from this study 177A matched with *Orthosiphon pallidus*. 178A and 188A are *Orthosiphon* genus by blasted with limited data from GenBank but when Alan Paton examined these samples, he found these samples are *Orthosiphon cuanzae*. These results showed that unknown species of 185A was similar to *Ocimum* genus, but 175A, 176A and 184A were similar to *Plectranthus* genus. But again due to the limitation of the data available in GenBank, the name of species not has been confirmed exactly but the genera are agreed (Table 6.5).

Table 6.5 The blast result of unknown species in this study.

Unknown species no.	**Which species by blastn tool**	**The region**		
		ITS	***trn*H-*psb*A**	***mat*K**
175A	From genbank	*Plectranthus amboinicus* (99%)	*Plectranthus oertendahlii* (97%)	*Plectranthus malabaricus* (99%)
176A	From genbank	*Plectranthus malabaricus* (99%)	*Plectranthus oertendahlii* (97%)	*Plectranthus madagascariensis* (98%)
177A	From genbank	*Orthosiphon wulfenioides* (93%)	*Orthosiphon parvifolius* (96%)	*Orthosiphon aristatus* (99%)
	From this study	*Orthosiphon pallidus* (98%)	*Orthosiphon pallidus* (99%)	*Orthosiphon pallidus* (99%)
178A	From genbank	*Orthosiphon aristatus* (94%)	*Orthosiphon parvifolius* (96%)	*Orthosiphon aristatus* (99%)
	From this study	*Orthosiphon schimperi* (98%)	*Orthosiphon schimperi* (98%)	*Orthosiphon schimperi* (99%)
184A	From genbank	*Plectranthus caninus* (89%)	*Plectranthus oertendahlii* (96%)	*Plectranthus madagascariensis* (99%)
185A	From genbank	*Ocimum selloi* (91%),	*Ocimum sp.* (94%)	*Ocimum gratissimum* (99%)
	From this study	*Ocimum pseudoserratum* (95%)	*Ocimum pseudoserratum* (98%)	*Ocimum pseudoserratum* (99%)
188A	From genbank	*Orthosiphon wulfenioides* (98%)	*Orthosiphon parvifolius* (97%)	*Orthosiphon aristatus* (99%)
	From this study	*Orthosiphon schimperi* (99%)	*Orthosiphon schimperi* (99%)	*Orthosiphon schimperi* (99%)

6.5 Discussion

Sequencing success played a major role in determining the overall performance of DNA barcoding analysis. The results showed that ITS, *trn*H–*psb*A, and *mat*K were 100% successfully sequenced but *rbc*L failed to amplify. This is important for DNA barcoding because the most important characteristics of a universal barcode is to be recoverable from herbarium samples and other degraded samples (Group et al., 2009). This work was undertaken on twenty-five specimens of the medicinal plant of *Orthosiphon* as an example using DNA barcoding and seven unknown samples which they were putative *Orthosiphon* were identified by this method.

The usefulness of DNA barcodes has been proven for many of the animal groups (Fazekas, et al., 2008), but much work is still required before a core barcode region(s) is established for plants. Barcoding marker accuracy is dependent on how far intraspecific and interspecific divergence is separated (barcoding gap); Meyer and Paulay (2005) suggested that "barcoding technique becomes less effective by the increase of overlapping between intraspecific and interspecific variation". In this analysis of barcoding, comparing three barcode markers demonstrated that ITS may potentially be employed as a barcode region to identify species of the genus *Orthosiphon*. As this region has lower levels of intraspecies divergence and demonstrates sufficient genetic gaps between species. The discriminatory power of the ITS and *mat*K marker alone is the highest (100%) but *mat*K showed less interspecific variation. Therefore, it is suggested to use ITS as a single barcode rather than *mat*K, and so the ITS region exhibits superior performance to the *mat*K, *trn*H–*psb*A. Thus, this finding supports the use of ITS as the barcode marker for plants (Chase & Fay, 2009; Kress et al., 2005), and ITS showed high levels of interspecific sequence variability that is observed in Cowan and Fay (2012). Moreover, several studies support the ITS discriminatory performance in Lamiaceae (Wang et al., 2013; Al-Qurainy et al., 2014; Elsherbeny, 2016).

The combinations *mat*K+*trn*H–*psb*A and ITS+*mat*K+*trn*H–*psb*A, showed the highest discriminatory power (100%) by the best match criterion in TaxonDNA method. ITS+*mat*K+*trnH–psb*A showed higher interspecific variation than *mat*K+*trn*H–*psb*A, although *mat*K+*trn*H–*psb*A has the lowest overlap of all barcoding markers. Overall, *mat*K+*trn*H–*psb*A as a barcoding marker has a good resolution as was found by studied of De Mattia et al. (2011), Theodoridis et al. (2012), and Zahra et al. (2016), but this study recommends using ITS+*mat*K+*trn*H–*psb*A to get the most efficiency for discrimination of DNA barcoding in *Orthosiphon* as the example of one genus of Lamiaceae.

DNA barcoding success lies in the distinctness of the clusters (Steinke et al., 2009). The UPGMA and NJ trees, which were inferred by using the sequence data of region ITS, *trn*H–*psb*A, *mat*K, and a combination of *trn*H–*psb*A+*mat*K and ITS+*trn*H-*psb*A+*mat*K, resolved the

relationship between the members of *Orthosiphon.* All members of the species were clustered and differentiated from the other closely related species *Or. schimperi*, *Or. rubicundus* and *Or. thymiflorus* members of genus *Orthosiphon.* This confirms the NJ method is highly useful for estimating relatedness among species (Erickson & Driskell, 2012) and also UPGMA method as well.

All the *Orthosiphon* sampled were successfully identified by the markers used in this study for identifying unknown samples successfully by tree and blast. Based on phylogenetic position, the unknown species of 177A is *Orthosiphon pallidus* and 178A and 188A are sister groups with *Orthosiphon schimperi*. Paton confirmed that unknown species 178A and 188A are *Orthosiphon cuanzae* and that they are morphologically very similar to *Orthosiphon schimperi*. According to unknown species 175A, 176A and 184 are outgroups for *Orthosiphon*. The unknown species 185A is not with any clade with *Orthosiphon* but not far than this genus. For this situation, the blast tool may help to identify these.

In terms of the blast tool, ITS, *trn*H–*psb*A, and *mat*K support that the 185A unknown species is closely related to *Ocimum* species rather than *Orthosiphon* species. The unknown species 175A, 176A, and 184A are similar to *Plectranthus* species. Regardless of exactly what is the name due to the limited data on *Ocimum* and *Plectranthus,* blast tool can be used to help identify unknown species but it should prior knowledge of barcode boundaries and all the relevant taxa have been sampled. The result from the blastn tool is significant and supports the results of UPGMA and NJ trees. Additionally, this strong evidence indicates that unknown species 185A, 176A, 176A, and 184A are not *Orthosiphon* species and this important to separate them from *Orthosiphon* file at Kew.

6.6 Conclusion

The results from this study indicated that the highest discriminatory resolution for *Orthosiphon* species identification is provided by a single marker: ITS. In addition, ITS+*mat*K+*trn*H–*psb*A is a good combination of markers. Moreover, the species of *Orthosiphon* were successfully identified by the candidate DNA barcodes (ITS, *trn*H–*psb*A, *mat*K, *mat*K+*trn*H–*psb*A and ITS+*mat*K+*trn*H–*psb*A). The second major finding was that ITS, *mat*K, and *trn*H–*psb*A can identify unknown species of *Orthosiphon* by UPGMA and NJ trees and blastn tool at the NCBI.

Chapter 7 : General discussion

7.1 Introduction

Several vital points have been put forward by Paton (1993) which raised doubts of morphology on the generic delimitation of *Fuerstia* from *Orthosiphon*. There is a close relationship between *Orthosiphon* and *Fuerstia* as they have an identically shaped calyx and corolla. Within *Orthosiphon*, *Fuerstia* shows the greatest similarity to subgenus *Orthosiphon* that also exhibits a clavate style with small rounded lobes, a 4-lobed disk having the largest anterior lobe and posterior stamens are adnate to the corolla around the middle of the tube. In 1929, Fries separated *Fuerstia* from *Orthosiphon* on the basis of the fact that *Fuerstia* possesses sterile posterior stamens. Paton (1993) suggested the *Fuerstia* and *Orthosiphon* should be in the same genus and even within the subgenus *Orthosiphon* because of the resemblance between them, but at this time formal changes were not made. He argued against the use of two sterile posterior stamens for identifying and delimiting *Fuerstia* and *Orthosiphon* since the number of fertile stamens is inconsistent in Lamiaceae. The four stamens are usually found in the Ocimeae, however, two sterile stamens rarely occur in the Ocimeae such as in one species in *Plectranthus* (Codd, 1975) and *Ocimum* (Paton et al., 1999). This generic complex also includes *Hoslundia*. There are identical nutlets in this species (Ryding, 1992), as well as corolla, inflorescence structure, and sterile posterior stamens, in comparison with *Fuerstia*. However, *Hoslundia* has a fleshy mature calyx that distinguishes it from other genera. Paton et al. (2004) studied the tribe Ocimeae but this relationship was left unresolved.

Another issue concerns the position of *Nautochilus* which has been placed in *Orthosiphon* sect. *Serrati* previously by Codd (1964), because they have similarity in two characters; the pair of upper filaments is attached close to the base of the corolla tube and the disk is annular but Paton (1992) moved this taxon into the *Ocimum* based on the disk being equally four-lobed, whereas in the remainder of *Orthosiphon* the anterior lobe is usually larger than the others and the style is bifid rather than clavate with rounded lobes in *Orthosiphon*; the posterior stamens are adnate near the base of the corolla tube and are basally ciliate rather than attaching to the middle of the tube and being glabrous in *Orthosiphon*. However, Paton et al. (2004) did not resolve the relationship between *Ocimum* and *Nautochilus.*

The principal aim of this thesis was to undertake a detailed study of the generic boundaries and the relationship in the subtribe Ociminae, using DNA sequencing to provide first phylogenetic tree and investigate the morphological character and geographical distribution in the context of phylogeny. The second aim evaluates the utility of DNA barcoding in species delimitation of *Orthosiphon.* There has been no molecular phylogeny focusing on the Ociminae except for some genera such as *Syncolostemon* (Otieno et al., 2006), and *Ocimum* (Kumar et al., 2016a), therefore this project used the Ociminae as an example to explore generic circumscription in the subtribe

Ociminae. This leads to similar methods being applied to observe to what extent generic delimitation could be resolved.

The taxonomic position of *Orthosiphon*, *Fuerstia*, and *Hoslundia* has been questioned because of their close relationship and morphological similarity. This study was based on chloroplast, nuclear, combined chloroplast, combined nuclear and combined chloroplast and nuclear DNA regions and the distribution of morphological characters and geographical distribution onto the combinable component consensus trees of subtribe Ociminae from combined regions. The results suggest that *Hoslundia* is embedded in *Fuerstia* with strong support, and *Fuerstia* and *Hoslundia* group is a sister with *Orthosiphon*. The clade of *Fuerstia* and *Hoslundia* can be distinguished from clade *Orthosiphon* by having two fertile stamens and one-flowered-cymes, while *Orthosiphon* has four fertile stamens, and mostly three-flowered cymes. These genera share the same characters such as the attachment of posterior stamen by the mid-point of the corolla tube or nearer the anterior lip, posterior filament inappendiculate, clavate style, subulate anterior calyx lobes and the disk lobing with the anterior lobe larger. A fleshy mature calyx can be used to distinguish *Hoslundia* from *Fuerstia*, however, from this study, this character was a weak argument for separating this genus. The geographical distribution showed Northeast Tropical Africa and South Tropical Africa divide of *Fuerstia*. It means there are two clades of *Fuerstia* with *Hoslundia*, it could all be one genus. Because these genera form a monophyletic group, *Hoslundia* and *Fuerstia* will be merged under the earliest name, *Hoslundia*. But before that, it is very important to indicate the relevant principles of nomenclature.

Article 11.4 of the International Code of Nomenclature for algae, fungi, and plants, describes the principles of priority of names and states that taxa that are below generic rank should be named in such a way that combines the final epithet of the earliest legitimate name of the taxon that is of the same rank with the correct name of the species or genus that it is assigned to (Turland et al., 2018). The only exception to this rule concerns cases in which there is a limitation of priority (Turland et al., 2018). New combinations must follow Article 6.10 which states that "A new combination (combinatio nova, comb. nov.) or name at a new rank (status novus, stat. nov.) is a new name based on a legitimate, previously published name, which is its basionym" (Turland et al., 2018). Thus the nine species of *Fuerstia* will be transferred to *Hoslundia* and make new combinations by following article 6.10. However, the new names necessary here will be published in a separate paper so, to avoid confusion the names below should not be considered effectively published (Article 29, Turland et al. 2018). Ineffective publication of examples below serve to demonstrate how these new combinations will be made. *Hoslundia adpressa* ((A. J. Paton) Althobaiti, A.J.Paton & Culham comb. nov. (not effectively published here)). Basionym: *Fuerstia adpressa* A.J.Paton in Kew Bull. 48: 132 (1993). Type: Angola, Benguella, between Ganda and

Caconda, Oct. 1933, Hundt 700 (holotypus BM). *Hoslundia dendrothrix* ((A. J. Paton) Althobaiti, A.J.Paton & Culham comb. nov. (not effectively published here)). Basionym: *Fuerstia dendrothrix* A. J. Paton, Kew Bull. 48(1): 135 (1993). Type: Somalia, Togdheer, Alla Ully near Sheikh, Nov. 1972, Wood 5/72/139 (holotypus K). *Hoslundia ternata* ((A. J. Paton) Althobaiti, A.J.Paton & Culham comb. nov. (not effectively published here)). Basionym: *Fuerstia ternata* A. J. Paton, Kew Bull. 48(1): 130 (1993). Type: Tanzania, Mpanda District, 8 Nov. 1959, Richards 11735 (holotypus K).

In addition, the relationship between *Ocimum* and *Nautochilus* was unresolved before. *Ocimum* is monophyletic with the inclusion of the *Nautochilus*. *Ocimum* subgenus *Nautochilus* is a sister to the rest of *Ocimum.* The *Ocimum* subgenus *Nautochilus* and the rest of *Ocimum* formed a clade strongly supported by exserted stamens, equal disk lobing and attachment of posterior stamen by the base of the corolla tube and bifid style. However, subgenus *Nautochilus* differs from the other subgenus of *Ocimum* by having cymes one to many-flowered (rather than three-flowered), subulate shape of the anterior calyx lobes (rather than lanceolate), and inappendiculate posterior staminal filaments (rather than appendiculate), Surprisingly, *Orthosiphon fruticosus* was found to be included in the subgenus *Nautochilus* clade. These results are strongly supported by morphological and geographical distribution on phylogenetic trees of the subtribe Ociminae. *Orthosiphon fruticosus* shares the morphological characteristics of *Ocimum* subgenus *Nautochilus*. The attachment of the posterior stamens at the throat around the base of the anterior lip distinguishes *Orthosiphon fruticosus* from *Orthosiphon* which has posterior stamens attached at the mid-point of the corolla tube, and this is additional support for including it with *Nautochilus*. The third characteristic is the bifid style of *Orthosiphon fruticosus* rather than the clavate style seen in the remainder of *Orthosiphon*., distinguishing *Orthosiphon fruticosus* from *Orthosiphon* and confirmation of it being nested within the *Nautochilus* The fourth characteristic is that the stamens are exserted rather than included as in most of *Orthosiphon*. Therefore, this study suggests considering this species to be within *Ocimum* subgenus *Nautochilus.* Regarding geographical distribution, *Ocimum* subgenus *Nautochilus* clade includes *Ocimum lamiifolium* as a sister to all species of *Nautochilus. Ocimum lamiifolium* is found in different regions in Africa, comparing by other species in *Ocimum* subgenus *Nautochilus* and they are: *Oc. pseudoserratum, Or. fruticosus, Oc. serratum, Oc. labiatum,* and *Oc. tubiforme* which are found in one region Southern Africa and this is supported by phylogenetic results in this study. *Orthosiphon fruticosus* will be transferred to *Ocimum* and requires a new name because *Ocimum fruticosum* (Ryding) A.J.Paton is already in use. Because of this, construction of the new name will follow Article 6.11 (Turland et al. 2018) as the name *Ocimum fruticosum* is not available. The new name will be *Ocimum glomeratum* Althobaiti, A.J.Paton & Culham, nom. nov. (not to be considered effectively published here).

Basionym: *Orthosiphon fruticosus* Codd, Bothalia viii. 153 (1964). Type: South Africa, Lydenburg Dist.; Steelpoort Sta.; 6.5 Mi. SW. of Steelpoort Sta., 14 Oct. 1957, Codd 9777, (holotypus PRE). This name is used because the leaves of this species are clustered (*glomeratum* in Latin). This name and the new combinations above will be made in a separate publication.

Overall, based on the results of this study I have identified that the monophyly of subtribe Ociminae is well-supported. When the geographic distribution was mapped onto a phylogenetic tree, support was offered to the hypothesis that the Ociminae has an African origin. Within the Ociminae there exist monophyletic clades that, in broad terms, have a correspondence with genera that have already been recognised: *Platostoma*, *Haumaniastrum*, *Catoferia*, *Endostemon*, and *Syncolostemon*. There has been debated about the treatment of the *Nautochilus* group and this work supports its recognition as a subgenus of *Ocimum.* However, this study suggests that, *Or. fruticosus* needs to be moved into *Ocimum* subgen *Nautochilus*, and *Fuerstia* should be included in *Hoslundia*,

The current study evaluated the performance of three candidate barcoding loci and their combination and efficiency for discrimination of the different *Orthosiphon* species. The results revealed that of the three barcode regions under investigation, ITS had, comparatively, the lowest level of overlap in the distribution for interspecific and intraspecific divergences. Additionally, this region contained the greatest discriminatory ability for identifying species correctly. Thus it is concluded that ITS is a significant barcode region when identifying *Orthosiphon* species as a single marker and, for the combination, ITS+*mat*K+*trn*H-*psb*A provided.

7.2 General conclusion

Molecular phylogeny is used to inform the understanding of the evolution of morphological characters in the subtribe Ociminae. A better understanding of the evolution of these characters helped to integrate the systematics of Ociminae. It demonstrated how the combined use of morphological, geographical, and molecular tools hold great promise for ending confusion in Ociminae systematics and hold promise for enhancing the resolution of *Orthosiphon, Fuerstia*, and *Hoslundia* relationships. It also clarified the relationship between *Ocimum* and the *Ocimum* subgenus *Nautochilus.*

The study used DNA sequence data based on *trn*L–*trn*F, *trn*H–*psb*A, *rps*16, *mat*K, ETS, ITS, combined chloroplast, nuclear, and combined chloroplast and nuclear regions. The successful method of DNA extraction with modification was determined for herbarium specimens.

Monophyly of *Catoferia, Endostemon, Syncolostemon, Platostoma,* and *Haumaniastrum* is supportive of current classification. *Orthosiphon* is monophyletic with exclusion *Or. fruticosus,*

Orthosiphon fruticosus is a member of *Ocimum* subgenus *Nautochilus. Fuerstia* is monophyletic with inclusion *Hoslundia,* so *Hoslundia* and *Fuerstia* will be merged under the earliest name *Hoslundia* later on in the paper. *Ocimum* is monophyletic with the inclusion subgenus *Nautochilus* after including *Orthosiphon fruticosus.* The current data highlight the importance of nomenclatural changes that are necessary.

According to DNA barcoding, the higher discriminatory resolution for *Orthosiphon* species identification is provided by a single marker (ITS) more than other single markers which are used in this study. In addition, ITS+*mat*K+*trn*H–*psb*A is a good combination marker. Moreover, successful identification of the species of *Orthosiphon* was done by these candidate DNA barcodes (ITS, *trn*H–*psb*A, *mat*K, *mat*K+*trn*H–*psb*A, and ITS+*mat*K+*trn*H–*psb*A). The second major finding was that ITS, *mat*K, and *trn*H–*psb*A can identify unknown species of *Orthosiphon* and their combination by UPGMA and NJ trees and blastn tool at the NCBI.

7.3 Planned publications

1. Phylogeny and evolution of the subtribe Ociminae (Lamiaceae) based on four plastid DNA regions and two nuclear DNA regions.
2. The first initiative of DNA barcoding of species delimitation of *Orthosiphon* (Lamiaceae).
3. Identifying unknown *Orthosiphon* species in Kew through DNA barcodes techniques, using three candidate DNA barcoding markers.

7.4 Future work

Further research should be undertaken on the genera of the subtribe Ociminae as next-generation sequencing is used to solve phylogenetic incongruences. Further work would require samples from different geographical regions: Madagascan *Platostoma,* Indian *Endostemon*, and Madagascan *Syncolostemon.* From DNA barcoding in this study, it is suggested to extended sampling for each species of *Orthosiphon* to test barcodes (ITS and combination ITS+*mat*K+*trn*H–*psb*A) effectively. The sequences generated from this study will be deposited in the NCBI GenBank and will serve as references for future *Orthosiphon* identifications.

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Appendices

Appendix 1 Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Endostemon stenocaulis (Hedge) Ryding, A.J.Paton & Thulin	M. Thulin et al. 10528	10/5/2001	Somalia	18	√	√	√	√	√	√
Endostemon tenuiflorus (Benth.) M.R.Ashby	Radcliffe-Smith 5168	22/9/1977	Oman, Dhofar Province	42	√	√	√	√	√	√
Endostemon tereticaulis (Poir.) M.R.Ashby	G. S. Collenette 3277	19/2/1982	Saudi Arabia	37	√	√	√	√	√	√
Fuerstia africana T.C.E.Fr.	Ib Friis, Wege Abebe & Ermias Getachew. 12,479	16/10/1906	Ethiopia	113	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Fuerstia angustifolia G.Taylor	S. B. Boaler. 742	22/11/1962	Tanzania	57	√	√	x	x	√	√
Fuerstia bartsioides (Baker) G.Taylor	G. Schweinfurth, 2932	-	Sudan	-	√	√	x	√	√	√
Fuerstia dendrothrix A.J.Paton	J. B.Gillett & B. M Watson. 23603	23/06/1981	Northern Somalia	38	√	√	√	√	√	√
*Fuerstia rigida (*Benth.) A.J.Paton	G. Barbosa. 9407	09/11/1961	Angola	58	√	√	√	√	√	√
Fuerstia ternata A.J.Paton	MRS. H. Richards 7197	9/12/1956	Tanzania	63	√	√	√	√	√	√
Haumaniastrum katangense (S.Moore) P.A.Duvign. & Plancke	F. Malaisse & P. Goetghebeur 108	19/4/1985	Zaire-Shaba	34	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Haumaniastrum lantanoides (S.Moore) P.A.Duvign. & Plancke	DE Willde 3792	6/5/1948	Zaire	71	√	√	√	√	√	√
Haumaniastrum minor (Briq.) A.J.Paton	K. Harder et al. 671	5/3/1995	Zambia	24	√	√	√	√	√	√
Hoslundia opposita Vahl	Parkes, C. 2929	11/10/1984	Sotuba Exptl station. Nr Bamako Mali	35	-	-	-	√	√	√
Hoslundia opposita Vahl	Michael Jensen et al. B1/23	-/5/1997	Ethiopia	22	√	√	√	√	√	√
Platostoma africanum P.Beauv.	W. R. Q. Luke 16023	30/10/2012	Mali	7	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H - *psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Platostoma calcaratum (Hemsl.) A.J.Paton var *calcaratum*	Henry 12339	1900	Yunnan	119	√	√	√	√	√	√
Platostoma cochinchinense (Lour.) A.J.Paton	Chinese collector 45/Oomm O. Ford 1895	-/10/1893	Hong Kong	126	√	√	√	√	√	√
Platostoma fimbriatum A.J.Paton	P. Puudjaa & T. Chongnuruk 438	4/11/1997	Thailand	22	√	√	√	√	√	√
Platostoma rotundifolium (Briq.) A.J.Paton	L. Festo 1161	1/4/2001	Tanzania	18	√	√	√	√	√	x
Platostoma siamense (Murata) A.J.Paton	V. Lamxay et al VL1898	10/5/2009	Lao Pdr, Vietnam.	10	√	√	√	√	√	√
Platostoma strictum (Hiern) A.J.Paton	J. Goyder, A. J. Paton & E. J. Tawakali 3552	23/1/1992	Malawi	27	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Platostoma tectum A.J.Paton	David, et al. CL1185	16/11/2009	Cambodge	10	√	√	√	√	√	√
Plectranthus bracteolatus A.J.Paton	Bridson, D.	08/09/1984	Tanzania	35	√	√	x	x	x	x
Plectranthus hadiensis (Forssk.) Schweinf. ex Sprenger	Chase, N.C.	-	Zimbabwe	-	√	√	x	√	√	x
Plectranthus masukensis Baker	Richards, H.M.	-	Tanzania	-	√	√	x	√	√	√
Plectranthus triangularis A.J.Paton	Mlangwa, J.A.	-	Tanzania	-	√	x	x	√	√	√
Syncolostemon argenteus N.E.Br.	T. Edwards & C. Potgieter 1920	-/3/2000	Natal	19	√	√	√	√	√	√
Syncolostemon bracteosus (Benth.) D.F.Otieno	D. K. Harder, et al 352	20/3/1996	Zambia	23	√	√	√	√	√	√
Syncolostemon canescens (Gürke) D.F.Otieno	P.Wilkin 743	1/1/1995	Zimbabwe	24	√	√	√	√	√	√
Syncolostemon cinereum (Codd) D.F.Otieno & Retief	T. Edwards 1977	-/3/2000	Natal	19	√	√	√	√	√	√
Syncolostemon concinnus N.E.Br.	K. Balkwill & K. Changwe 12213	12/4/2002	Kwazulu/ Natal	17	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Syncolostemon elliottii (Baker) D.F.Otieno	M. Kruger-Gaadingwe, MSB 467	28/11/2007	Botswana	12	√	√	√	√	√	√
Syncolostemon flabellifolius (S.Moore) A.J.Paton	A. Mapaura 715	29/10/2014	Mozambique	5	√	√	√	√	√	√
Syncolostemon foliosus (S.Moore) D.F.Otieno	K. Balkwill 12067	22/11/2000	Mpumalanga	19	√	√	√	√	√	√
Syncolostemon macranthus (Gürke) Ashby	F. W. Mabatha 2580	29/5/2009	South Africa	10	√	√	√	√	√	√
Syncolostemon modestus (Codd) D.F.Otieno	A. McCallum & M-J. Balkwill 300	2/10/1997	Swaziland	22	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Syncolostemon parviflorus E.Mey. ex Benth. var *parviflorus*	K. Balkwill et al. 1877	1/2/1999	Mpumalanga	20	√	√	√	√	√	√
Syncolostemon persimilis (N.E.Br.) D.F.Otieno	S. D. Williamson 743	7/8/1998	Mpumalanga	21	√	√	√	√	√	√
Syncolostemon stalmansii (A.J.Paton & K.Balkwill) D.F.Otieno	K. Balkwill et al 10898	2/2/1999	Kangwane	20	√	√	√	√	√	√
Syncolostemon teucriifolius (Hochst.) D.F.Otieno	E. Van Wyk 12527	24/11/1994	Natal	25	√	√	√	√	√	√
Orthosiphon allenii (C.H.Wright) Codd	H. T. Chapama 1169	1/3/2017	Malawi	2	√	√	√	√	√	√
Orthosiphon aristatus (Blume) Miq.	Barbon et al. 13125	12/7/1994	Philippine	25	√	√	√	√	√	√
Orthosiphon biflorus A.J.Paton & Hedge	Sussct M39	23/11/1987	Madagascar	32	√	√	√	√	√	√
Orthosiphon bullosus Chiov.	M. Thulin et al. 7672	21/5/1990	Somalia	29	√	√	√	√	√	√
Orthosiphon discolor A.J.Paton & Hedge	P.Boiteau 2074	10/4/1970	Madagascar	49	√	√	√	√	√	√
Orthosiphon ferrugineus Balf.f.	G. Miller et al M.100064	26/1/1990	Yemen	29	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Orthosiphon fruticosus Codd	L. E. Codd 9843	11/3/1958	Preroria	61	√	√	√	√	√	√
Orthosiphon grandiflorus A.Terracc	M. Thulin et al. 11339	25/5/2006	Ethiopia	13	√	√	√	√	√	√
Orthosiphon hanningtonii (Baker) A.J.Paton	P.L.Luke et al. 6307	10/7/2000	Kenya	19	√	√	√	√	√	√
Orthosiphon humbertii Danguy	P. B. Phillipson, R.A. Clementand & G. Rafamantansoa. 4003	29/03/1992	Madagascar	27	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Orthosiphon miserabilis A.J.Paton & Hedge	Paul Smith & Solofo Rakotoarisoa. 1817	15/01/1903	Madagascar	116	√	√	√	√	√	√
Orthosiphon nigripunctatus G.Taylor	D. B. Fanshawe. 391	12/10/1953	Northern Rhodesia (Zambia)	66	√	√	x	x	x	√
Orthosiphon pallidus Royle ex Benth.	Parkes, C. E.39	25/09/1969	Ethiopia-Blue Nile Gorge.	50	√	√	√	√	√	√
Orthosiphon sarmentosus A.J.Paton & Hedge	R. A. Clement et al. 2028	12/3/1992	Fianarantsoa/ Madagascar	27	√	√	√	√	√	√
Orthosiphon scedastophyllus A.J.Paton	D. J. Goyder et al. 6112	22/11/2009	Mozambique	10	√	√	√	√	√	√
Orthosiphon schimperi Benth.	Sally Bidgood, F. Mbago & K. Vollesen. 2253	13/02/1994	Tanzania	25	√	√	√	√	√	√
Orthosiphon thymiflorus (Roth) Sleesen	C. P. Clarke. 138	23/02/1909	Northern Mozambique	110	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Ocimum africanum Lour.	W. R. Q. Luke et al. 7237	19/1/2001	Kenya	18	√	√	√	√	x	√
Ocimum albostellatum (Verdc.) A.J.Paton	S. Bidgood et al. 7653	4/2/2009	Tanzania	10	√	√	√	√	√	√
Ocimum angustifolium Benth.	S. Bidgood et al. 2596	4/3/1994	Tanzania	25	√	√	√	√	√	√
Ocimum basilicum L.	T. Al- Abbasi MSTAAW 1541	30/4/2004	Saudi Arabia	15	√	√	√	√	√	√
Ocimum campechianum Mill.	M. Giuliett et al 4/2003 74876	21/4/2003	Brazil	16	√	√	√	√	√	√
Ocimum canescens A.J.Paton	P. Kuchar 22842	27/1/2000	Tanzania	19	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Ocimum cufodontii (Lanza) A.J.Paton	Q. Luke et al. 14081	12/2/2010	Kenya	9	√	√	√	x	√	x
Ocimum dambicola A.J.Paton	S. Bidgood et al. 7995	3/3/2009	Tanzania	10	√	√	√	√	√	√
Ocimum decumbens Gürke	R. K. Brummitt et al. 18046	18/11/1986	Tanzania	33	√	√	√	√	√	√
Ocimum dhofarense (Sebald) A.J.Paton	R. M. Lawton 2388	28/8/1982	Oman	37	√	√	√	√	√	√
Ocimum ellenbeckii Gürke	Friis et al. 14801	6/12/2012	Ethiopia	7	√	√	√	√	√	√
Ocimum filamentosum Forssk.	S. Collenette 9272	22/2/1995	Saudi Arabia	24	√	√	√	√	√	√
Ocimum forskolei Benth.	L. Boulos et al. 16645	17/7/1987	South Yemen	32	√	√	√	√	√	√
Ocimum forskolei Benth.	P. Kuchar 16591	7/11/1984	Somalia	35	√	√	√	√	x	√
Ocimum grandiflorum Lam.	S. Bidgood et al. 6234	27/5/2006	Tanzania	13	√	√	√	√	√	√
Ocimum labiatum (N.E.Br.) A.J.Paton	L. A. Nkuna 2840	26/2/2013	South Africa	6	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Ocimum lamiifolium Hochst. ex Benth.	D. Sheil 1539	8/5/1993	Uganda	26	√	√	√	x	√	√
Ocimum obovatum E.Mey. ex Benth var. obovatum	P.P. Smith 0702	12/12/1994	Zambia	25	√	√	√	√	√	√
Ocimum pseudoserratum (M.R.Ashby) A.J.Paton	Galpins 133155	13/12/1934	Pretoria	85	√	√	√	√	√	√
Ocimum pyramidatum (A.J.Paton) A.J.Paton	W. R.Q. Luke 16812	18/3/2015	Tanzania	4	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Ocimum reclinatum (S.D.Williams & M.Balkwill) A.J.Paton	J. Goyder 5021	2/12/2001	Mozambique	18	√	√	√	√	√	√
Ocimum serratum (Schltr.) A.J. Paton	Barell, S.C.H. 135	6/11/1968	Tshaneni hubaubo distvict. Houe vela, Swaziland.	51	√	√	√	-	-	-
Ocimum serratum (Schltr.) A.J.Paton	Dibakwane 6004	20/12/2013	South Africa/ KwaZulu-Natal	6	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 1 cont. Samples used in this study and PCR results.

Species name	**Collector**	**Collection date**	**Collection location**	**The age of specimens/ years**	***trn*L-F**	***trn*H-*psb*A**	***rps*16**	***mat*k**	**ITS**	**ETS**
Ocimum spectabile (Gürke) A.J.Paton	J. Goyder & P. Siro Masinde 3955	7/1/1995	Kenya	24	√	√	√	√	√	√
Ocimum tenuiflorum L.	W. R .Q. Luke & PA 3840	10/9/1993	Kenya	26	√	√	√	x	√	√
Ocimum tubiforme (R.D.Good) A.J.Paton	Hilliard & Burtt, 6034	21/1/1969	Natal	50	√	√	√	√	√	√
Ocimum vanderystii (De Wild.) A.W.Hill	Parmentier & Kila 4752	31/8/2007	Katanga/ Congo	12	√	√	√	√	√	√
Ocimum verticillifolium Baker	M.Thulin et al. 8963	10/1/1995	Somalia	24	√	√	√	√	√	√

√ Good, x Failed, – There is another specimen and √ The whole fragment did not amplify so it was amplified in two parts sequencing.

Appendix 2 The method of CTAB DNA extraction protocol (Doyle and Doyle, 1990), with long isoproponl precipitation with cleaned up CTAB by DNeasy Plant Mini Kit from Qiagen.

Initial preparation

● Check supply of chemical reagents; CTAB, sarkosyl, chloroform Isolamyl alcohol (24:1), 0.1xTE buffer, 3M sodium acetate pH.2, β-Mercaptoethanol (B-ME), isopropanol (propan-2-ol), 100% alcohol, 70% alcohol, PCR grade water.

● Check supply of clean pestle and mortar (P&M) or tungsten beads

● Pre-heat water bath/dry heater block to 65ºC.

● Fill ice-box

● Pre sample combine 700µl CTAB + 10µl β-ME [optional 70µl 10% sarkosyl sol.], heat to 65ºC.

Procedure

Grinding

Pulverisation with sand by pestle and mortar.

Place 0.01-0.02g dried leaf into mortar, add 1-3 small spatulas of acid washed sand, grind leaf to fine ash with pestle. Scrape powder to 1.5 ml Eppendorf using clean spatula. Add 710µl of 65ºC CTAB/β-ME or 780µl of CTAB/Sarkosyl/β-ME, vortex, incubate at 65ºC for 2 hours.

Working in fumehood

1. Precipitate proteins and carbohydrates. Add 700µl of chloroform:Isolamyl (Cl), vortex until uniformly suspended.
2. Fractionate cell debris, proteins and carbs. Centrifuge for 3 mins at 13,000 rpm. The tube now has three layers, lower=solvent, middle=cell debris, upper=DNA and buffer.
3. Transfer upper layer to new 1.5ml Eppendorf. Pour waste Cl into collection bottle [use fumehood]. Add an equal-volume of fresh Cl, repeat centrifugation, transfer upper layer to new 1.5ml Eppendorf. Pour waste Cl into fumehood collection bottle.

Working in an open lab bench

4. Precipitate DNA. Add 2/3 volume (~350µl) of ice-cold iso-propanol, mix by inversion, and leave on ice for one week in the -20ºC freezer.
5. Concentrate DNA. Centrifuge for 3 minutes at 13,000 rpm [tube hinges outwards]. Using a p200 tip, remove the liquid into beaker, store in iso-propanol waste container. A pellet will be stuck to wall [tube hinge side] and maybe loose and colourless.
6. Wash DNA. Add 500µl of 70% ethanol, centrifuge for 1 min at 13,000 rpm. Decant ethanol to waste beaker, store in ethanol waste in container.

7. Resuspend the DNA. Adding 180µl of 0.1xTE buffer and 20µl of 3M sodium acetate, mix until DNA is suspended. This may require gentle heating on a dry block. Add 600µl of ice-old 100% ethanol mix by inversion, place on ice for 60 minutes.
8. Concentrate DNA. Centrifuge for 3 mins at 13,000 rpm. Using a p200 tip, removed the liquid into beaker, store in ethanol waste in container.
9. Wash DNA. Add 600µl of 70% ethanol to wash pellet, flicking to dislodge pellet from tube wall. Centrifuge for 3 mins at 13,000 rpm and using a p200 tip remove the liquid into beaker, store in ethanol waste in container.
10. Remove any residual ethanol using p10 tip. Pellet must now dry until No ethanol smell remains. This can be achieved by overnight drying (ideal route), or by brief drying on the dry-block (50^{o}C).
11. Re-suspend DNA. Dissolve pellet in 30µl of PCR grade water, or for long-term storage 0.1 TE or 0.1xTris, both at pH 8.0.
12. Ensure sample is labelled, store at -20^{o}C for 2-3 days.
13. Clean up the CTAB using by DNeasy® Plant Mini Kit and following the instructions manual**.**

Appendix 3 Data matrix used for mapping morphological and distribution characters onto phylogenetic trees of subtribe Ociminae. Outgroup species is denoted with *. Character states are scored 0 to 3, see below the table.

Species name	**characters**															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Basilicum polystachyon (L.) Moench	1	0	2	1	0	1	0	0	1	1	0	2	1	1	0	0
Benguellia lanceolata (Gürke) G.Taylor	1	0	2	1	0	0	0	0	1	1	0	2	1	1	0	1
Catoferia capitata (Benth.) Hemsl.	1	1	2	1	3	0	0&1	0	1	1	0	2	1	2	1	1
Catoferia chiapensis A.Gray ex Benth.	1	1	2	1	3	0	0&1	0	1	1	0	2	1	2	0	1
Endostemon albus A.J.Paton, Harley & M.M.Harley	1	0	3	1	0	0	0	0	1	0	0	0	1	1	1	0
Endostemon ctenoneurus Harley	1	0	3	1	0	0	0	0	1	0	0	0	1	1	1	0
Endostemon gracilis (Benth.) M.R.Ashby	1	0	3	1	0	0	0	0	1	0	0	0	1	1	1	0
Endostemon kelleri (Briq.) Ryding ex A.J.Paton & Harley	1	0	3	1	0	0	0	0	1	0	0	0	1	1	1	0
Endostemon leucosphaerus (Briq.) A.J.Paton, Harley & M.M.Harley	1	0	0	1	0	0	0	0	1	0	0	0	1	1	1	0
Endostemon obbiadensis (Chiov.) M.R.Ashby	1	0	3	1	0	0	0	0	1	0	0	0	1	1	1	0
Endostemon racemosus Ryding, A.J.Paton & Thulin	1	0	3	1	0	0	0	0	1	0	0	0	1	1	1	0
Endostemon stenocaulis (Hedge) Ryding, A.J.Paton & Thulin	1	0	3	1	0	0	0	0	1	0	0	0	1	1	1	0
Endostemon tenuiflorus (Benth.) M.R.Ashby	1	0	3	1	0	0	0	0	1	0	0	0	1	1	1	0
Endostemon tereticaulis (Poir.) M.R.Ashby	1	0	3	1	0	0	0	0	1	0	0	0	1	1	1	0
Fuerstia africana T.C.E.Fr.	1	0	0	1	0	1	0	0	0	0	0	2	1	1	0	1
Fuerstia angustifolia G.Taylor	0	0	0	1	0	1	0	0	0	0	0	2	1	1	0	1

Fuerstia bartsioides (Baker) G.Taylor	1	0	0	1	0	1	0	0	0	0	0	2	1	1	0	1
Fuerstia dendrothrix A.J.Paton	1	0	0	1	0	1	0	0	0	0	0	2	1	1	0	1
*Fuerstia rigida (*Benth.) A.J.Paton	0	0	0	1	0	1	0	0	0	0	0	2	1	1	0	1
Fuerstia ternata A.J.Paton	0&1	0	0	1	0	1	0	0	0	0	0	2	1	1	0	1
Haumaniastrum katangense (S.Moore) P.A.Duvign. & Plancke	1	1	2	0	2	2	0	0	1	1	0	2	2	0	0	1
Haumaniastrum lantanoides (S.Moore) P.A.Duvign. & Plancke	1	1	2	1	2	2	0	0	1	1	0	2	2	0	0	1
Haumaniastrum minor (Briq.) A.J.Paton	1	1	2	1	2	2	0	0	1	1	0	2	2	0	0	1
Hoslundia opposita Vahl	0&1	0	0	1	0	1	0	0	0	0	0	2	2	1	0	1
Platostoma africanum P.Beauv.	1	1	3	1	1	2	1	0	1	1	0	2	2	0	0	1
Platostoma calcaratum (Hemsl.) A.J.Paton var calcaratum	1	1	3	1	0	2	1	0	1	1	0	1	2	0	0	1
Platostoma cochinchinense (Lour.) A.J.Paton	1	1	3	1	1	2	1	0	1	1	0	2	2	0	0	1
Platostoma fimbriatum A.J.Paton	1	1	3	1	1	2	1	0	1	1	0	2	2	0	0	1
Platostoma rotundifolium (Briq.) A.J.Paton	1	1	3	1	1	2	1	0	1	1	0	2	2	0	0	1
Platostoma siamense (Murata) A.J.Paton	1	1	3	1	0	2	1	0	1	1	0	2	2	0	0	1
Platostoma strictum (Hiern) A.J.Paton	1	1	3	1	1	2	1	0	1	1	0	2	2	0	0	1
Platostoma tectum A.J.Paton	1	1	5	1	1	2	1	0	1	1	0	2	2	0	0	1
Plectranthus bracteolatus A.J.Paton*	1	0	3	1	0	0	0&1	1	1	0	0	0	4	0	0	1
Plectranthus hadiensis (Forssk.) Schweinf. ex Sprenger*	1	0	3	1	0	0	0&1	1	1	1	0	0	1	0	0	1
Plectranthus masukensis Baker*	1	0	3	1	0	0	0&1	1	1	0	0	0	1	0	0	1
Plectranthus triangularis A.J.Paton*	1	0	3	1	0	0	0&1	1	1	0	1	0	1	0	0	1
Syncolostemon argenteus N.E.Br.	1	0	0	1	0	0	1	0	1	1	2	0	1	0	0	01
Syncolostemon bracteosus (Benth.) D.F.Otieno	1	0	1	1	0	0	1	0	1	1	2	2	1	0	0	1
Syncolostemon canescens (Gürke) D.F.Otieno	1	0	1	1	0	0	2	0	1	1	2	2	1	0	0	1
Syncolostemon cinereum (Codd) D.F.Otieno & Retief	1	0	1	1	0	0	1	0	1	1	2	2	1	0	0	1
Syncolostemon concinnus N.E.Br.	1	0	0	1	0	0	1	0	1	1	2	2	1	0	0	1

Syncolostemon elliottii (Baker) D.F.Otieno	1	0	1	1	0	0	1	0	1	1	2	2	1	0	0	1
Syncolostemon flabellifolius (S.Moore) A.J.Paton	1	0	1	1	0	0	1	0	1	1	2	2	1	0	0	1
Syncolostemon foliosus (S.Moore) D.F.Otieno	1	0	0	1	0	0	1	0	1	1	2	2	1	0	0	1
Syncolostemon macranthus (Gürke) Ashby	1	0	1	1	0	0	1	0	1	1	2	2	1	0	0	1
Syncolostemon modestus (Codd) D.F.Otieno	1	0	0	1	0	0	1	0	1	1	2	2	1	0	0	1
Syncolostemon parviflorus E.Mey. ex Benth. var parviflorus	1	0	0	1	0	0	1	0	1	1	2	2	1	0	0	1
Syncolostemon persimilis (N.E.Br.) D.F.Otieno	1	0	1	1	0	0	0&1	0	1	1	2	2	1	0	0	1
Syncolostemon stalmansii (A.J.Paton & K.Balkwill) D.F.Otieno	1	0	1	1	0	0	1	0	1	1	2	2	1	0	0	1
Syncolostemon teucriifolius (Hochst.) D.F.Otieno	1	0	1	1	0	0	1	0	1	1	2	2	1	0	0	1
Orthosiphon allenii (C.H.Wright) Codd	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon aristatus (Blume) Miq.	1	0	2	1	0	1	0	0	1	1	0	2	1	1	0	1
Orthosiphon biflorus A.J.Paton & Hedge	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon bullosus Chiov.	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon discolor A.J.Paton & Hedge	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon ferrugineus Balf.f.	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon fruticosus Codd	1	0	2	1	0	1	0	0	1	1	0	2	1	0	0	1
Orthosiphon grandiflorus A.Terracc	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon hanningtonii (Baker) A.J.Paton	1	0	2	1	0	1	0	0	1	1	0	2	1	1	0	1
Orthosiphon humbertii Danguy	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon miserabilis A.J.Paton & Hedge	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon nigripunctatus G.Taylor	0	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon pallidus Royle ex Benth.	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon sarmentosus A.J.Paton & Hedge	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon scedastophyllus A.J.Paton	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Orthosiphon schimperi Benth.	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1

*Orthosiphon thymiflorus (*Roth) Sleesen	1	0	2	1	0	1	0	0	1	0	0	2	1	1	0	1
Ocimum africanum Lour.	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum albostellatum (Verdc.) A.J.Paton	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum angustifolium Benth.	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum basilicum L.	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum campechianum Mill.	1	0	2	1	0	0	0	0	1	1	0	1	1	0	0	0
Ocimum canescens A.J.Paton	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum dambicola A.J.Paton	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum decumbens Gürke	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum dhofarense (Sebald) A.J.Paton	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum ellenbeckii Gürke	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum filamentosum Forssk.	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum forskolei Benth.	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum forskolei Benth.	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum grandiflorum Lam.	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum labiatum (N.E.Br.) A.J.Paton	1	0	3	1	0	1	0	0	1	1	0	1	1	0	0	0
Ocimum lamiifolium Hochst. ex Benth.	1	0	3	1	0	1	0	0	1	1	0	1	1	0	0	0
Ocimum obovatum E.Mey. ex Benth var. obovatum	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum pseudoserratum (M.R.Ashby) A.J.Paton	1	0	3	1	0	1	0	0	1	1	0	1	1	0	0	0
Ocimum pyramidatum (A.J.Paton) A.J.Paton	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum reclinatum (S.D.Williams & M.Balkwill) A.J.Paton	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum serratum (Schltr.) A.J. Paton	1	0	3	1	0	1	0	0	1	1	0	1	1	0	0	0
Ocimum spectabile (Gürke) A.J.Paton	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum tenuiflorum L.	1	0	2	1	0	0	0	0	1	1	0	1	1	0	0	0

Ocimum tubiforme (R.D.Good) A.J.Paton	1	0	3	1	0	1	0	0	1	1	0	1	1	0	0	0
Ocimum vanderystii (De Wild.) A.W.Hill	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0
Ocimum verticillifolium Baker	1	0	2	1	0	0	0	0	1	1	0	1	0	0	0	0

No.	Morphological characters	Codes
1	**Leaves**	Ternate (0); opposite (1).
2	**Fertile bracts**	Uniformly green (0); basally coloured (1).
3	**No. of flowers in cyme**	1 (0); 1-3 a variable (1); 3 (2); 1-many (3).
4	**Calyx**	Fleshy mature (0); not fleshy mature (1).
5	**Position of lateral calyx lobe**	Median, between posterior and anterior (0); close to posterior, not in same plane (1); close to posterior, in same plane (2); nearer the anterior lobes than posterior lobe (3).
6	**Shape of anterior calyx lobes**	Lanceolate (0); subulate (1); emarginate (2).
7	**Corolla tube shape**	Straight (0); curved downward (1); curved upward (2).
8	**Shape of lower lip corolla**	Flat and the stamens extend over it (0); boat-shaped and surrounds the stamens (1).
9	**No. of fertile stamens**	2 (0), 4 (1).
10	**Stamen**	Included (0); exserted (1).
11	**Fusion of stamens**	No staminal fusion (0); all stamens fused, with the two anterior stamens fused together and to the adjacent posterior stamen (1); anterior stamens only fused, posterior free (2).
12	**Attachment of posterior stamen**	At the throat (0) at the base of the tube (1); around the mid-point of the tube (2).
13	**Posterior filament form**	Appendiculate (0); inappendiculate (1); basally swollen (2).
14	**Style**	Bifid (0); clavate (1); capitate (2).
15	**Style shield**	Absent (0); present (1).
16	**Disk lobing**	Equally lobed (0); anterior larger (1).

Appendix 4 Data matrix used for mapping geographical continental distribution onto phylogenetic trees of subtribe Ociminae. Outgroup species are denoted with *. Continents codes are scored 0 to 8, see below the table.

Species name	**Continents codes**
Basilicum polystachyon (L.) Moench	1&2&3&4&5
Benguellia lanceolata (Gürke) G.Taylor	1
Catoferia capitata (Benth.) Hemsl.	7&8
Catoferia chiapensis A.Gray ex Benth.	7&8
Endostemon albus A.J.Paton, Harley & M.M.Harley	1
Endostemon ctenoneurus Harley	1
Endostemon gracilis (Benth.) M.R.Ashby	1&3
Endostemon kelleri (Briq.) Ryding ex A.J.Paton & Harley	1
Endostemon leucosphaerus (Briq.) A.J.Paton, Harley & M.M.Harley	1
Endostemon obbiadensis (Chiov.) M.R.Ashby	1
Endostemon racemosus Ryding, A.J.Paton & Thulin	1
Endostemon stenocaulis (Hedge) Ryding, A.J.Paton & Thulin	1
Endostemon tenuiflorus (Benth.) M.R.Ashby	1&2&3
Endostemon tereticaulis (Poir.) M.R.Ashby	1&3
Fuerstia africana T.C.E.Fr.	1
Fuerstia angustifolia G.Taylor	1
Fuerstia bartsioides (Baker) G.Taylor	1
Fuerstia dendrothrix A.J.Paton	1
Fuerstia rigida (Benth.) A.J.Paton	1
Fuerstia ternata A.J.Paton	1
Haumaniastrum katangense (S.Moore) P.A.Duvign. & Plancke	1
Haumaniastrum lantanoides (S.Moore) P.A.Duvign. & Plancke	1
Haumaniastrum minor (Briq.) A.J.Paton	1
Hoslundia opposita Vahl	1&2
Platostoma africanum P.Beauv.	1&4
Platostoma calcaratum (Hemsl.) A.J.Paton var calcaratum	3&4
Platostoma cochinchinense (Lour.) A.J.Paton	3&4
Platostoma fimbriatum A.J.Paton	4
Platostoma rotundifolium (Briq.) A.J.Paton	1

Platostoma siamense (Murata) A.J.Paton	4
Platostoma strictum (Hiern) A.J.Paton	1
Platostoma tectum A.J.Paton	4
Plectranthus bracteolatus A.J.Paton*	1
Plectranthus hadiensis (Forssk.) Schweinf. ex Sprenger *	1&3&4
Plectranthus masukensis Baker*	1
Plectranthus triangularis A.J.Paton*	1
Plectranthus thyrsoideus (Baker) B.Mathew (from Genbank)*	1
Syncolostemon argenteus N.E.Br.	1
Syncolostemon bracteosus (Benth.) D.F.Otieno	1
Syncolostemon canescens (Gürke) D.F.Otieno	1
Syncolostemon cinereum (Codd) D.F.Otieno & Retief	1
Syncolostemon concinnus N.E.Br.	1
Syncolostemon elliottii (Baker) D.F.Otieno	1
Syncolostemon flabellifolius (S.Moore) A.J.Paton	1
Syncolostemon foliosus (S.Moore) D.F.Otieno	1
Syncolostemon macranthus (Gürke) Ashby	1
Syncolostemon modestus (Codd) D.F.Otieno	1
Syncolostemon parviflorus E.Mey. ex Benth. var parviflorus	1
Syncolostemon persimilis (N.E.Br.) D.F.Otieno	1
Syncolostemon stalmansii (A.J.Paton & K.Balkwill) D.F.Otieno	1
Syncolostemon teucriifolius (Hochst.) D.F.Otieno	1
Orthosiphon allenii (C.H.Wright) Codd	1
Orthosiphon aristatus (Blume) Miq.	3&4&5
Orthosiphon biflorus A.J.Paton & Hedge	1&2
Orthosiphon bullosus Chiov.	1
Orthosiphon discolor A.J.Paton & Hedge	2
Orthosiphon ferrugineus Balf.f.	0
Orthosiphon fruticosus Codd	0
Orthosiphon grandiflorus A.Terracc	1
Orthosiphon hanningtonii (Baker) A.J.Paton	1
Orthosiphon humbertii Danguy	2
Orthosiphon miserabilis A.J.Paton & Hedge	2
Orthosiphon nigripunctatus G.Taylor	1
Orthosiphon pallidus Royle ex Benth.	1&3&4
Orthosiphon sarmentosus A.J.Paton & Hedge	2

Orthosiphon scedastophyllus A.J.Paton	1
Orthosiphon schimperi Benth.	1
*Orthosiphon thymiflorus (*Roth) Sleesen	1&2&3&4
Ocimum africanum Lour.	1&2&34&6&7&8
Ocimum albostellatum (Verdc.) A.J.Paton	1
Ocimum angustifolium Benth.	1
Ocimum basilicum L.	0&1&2&&34&6&7&8
Ocimum campechianum Mill.	7&8
Ocimum canescens A.J.Paton	1
Ocimum dambicola A.J.Paton	1
Ocimum decumbens Gürke	1
Ocimum dhofarense (Sebald) A.J.Paton	3
Ocimum ellenbeckii Gürke	1
Ocimum filamentosum Forssk.	1&3&4
Ocimum forskolei Benth.	1&3
Ocimum forskolei Benth.	1&3
Ocimum grandiflorum Lam.	1
Ocimum labiatum (N.E.Br.) A.J.Paton	1
Ocimum lamiifolium Hochst. ex Benth.	1
Ocimum obovatum E.Mey. ex Benth var. obovatum	1
Ocimum pseudoserratum (M.R.Ashby) A.J.Paton	1
Ocimum pyramidatum (A.J.Paton) A.J.Paton	1
Ocimum reclinatum (S.D.Williams & M.Balkwill) A.J.Paton	1
Ocimum serratum (Schltr.) A.J. Paton	1
Ocimum spectabile (Gürke) A.J.Paton	1
Ocimum tenuiflorum L.	1&3&4&5&6&8
Ocimum tubiforme (R.D.Good) A.J.Paton	1
Ocimum vanderystii (De Wild.) A.W.Hill	1
Ocimum verticillifolium Baker	1

1. Europe= 0, 2. Africa= 1, 3. Western Indian Ocean Madagascar=2 4. Asia-Temperate= 3, 5. Asia-Tropical= 4, 6. Australasia= 5, 7. Pacific= 6, 8. Northern America=7, 9. Southern, America= 8.

Appendix 5 Data matrix used for mapping geographical regional distribution onto phylogenetic trees of subtribe Ociminae . Outgroup species are denoted with *. Regions codes are scored 0 to 9, A-H, K-N, P-Z and a, see below the table.

Species name	**Regions**
Basilicum polystachyon (L.) Moench	3&4&5&6&7&8&9& D&E&G& H&J&K&M& N
Benguellia lanceolata (Gürke) G.Taylor	7
*Catoferia capitata (*Benth.) Hemsl.	U &V&Y
Catoferia chiapensis A.Gray ex Benth.	U&V
Endostemon albus A.J.Paton, Harley & M.M.Harley	6&7
Endostemon ctenoneurus Harley	5&6
Endostemon gracilis (Benth.) M.R.Ashby	5&6& D
Endostemon kelleri (Briq.) Ryding ex A.J.Paton & Harley	5&6
Endostemon leucosphaerus (Briq.) A.J.Paton, Harley & M.M.Harley	5
Endostemon obbiadensis (Chiov.) M.R.Ashby	5
Endostemon racemosus Ryding, A.J.Paton & Thulin	5
Endostemon stenocaulis (Hedge) Ryding, A.J.Paton & Thulin	5
Endostemon tenuiflorus (Benth.) M.R.Ashby	5&6&7&8&9& D
Endostemon tereticaulis (Poir.) M.R.Ashby	3&5&6&7&8& D
Fuerstia africana T.C.E.Fr.	4&5&6
Fuerstia angustifolia G.Taylor	6&7
Fuerstia bartsioides (Baker) G.Taylor	5
Fuerstia dendrothrix A.J.Paton	5
*Fuerstia rigida (*Benth.) A.J.Paton	7
Fuerstia ternata A.J.Paton	6
Haumaniastrum katangense (S.Moore) P.A.Duvign. & Plancke	4&6&7
Haumaniastrum lantanoides (S.Moore) P.A.Duvign. & Plancke	4&7
Haumaniastrum minor (Briq.) A.J.Paton	7
Hoslundia opposita Vahl	3&4&5&6&7&8&9
Platostoma africanum P.Beauv.	3&4&5&6&7& H&K
Platostoma calcaratum (Hemsl.) A.J.Paton var calcaratum	E& J
Platostoma cochinchinense (Lour.) A.J.Paton	E& J&K
Platostoma fimbriatum A.J.Paton	J
Platostoma rotundifolium (Briq.) A.J.Paton	3&4&5&6&7&8

Platostoma siamense (Murata) A.J.Paton	J
Platostoma strictum (Hiern) A.J.Paton	7
Platostoma tectum A.J.Paton	J
Plectranthus bracteolatus A.J.Paton*	6
Plectranthus hadiensis (Forssk.) Schweinf. ex Sprenger *	1&4&5&6&7&8& D& H
Plectranthus masukensis Baker*	4&6&7
Plectranthus triangularis A.J.Paton*	6
Plectranthus thyrsoideus (Baker) B.Mathew (from Genbank)*	7
Syncolostemon argenteus N.E.Br.	8
Syncolostemon bracteosus (Benth.) D.F.Otieno	3&4&5&6&7&8
Syncolostemon canescens (Gürke) D.F.Otieno	7&8
Syncolostemon cinereum (Codd) D.F.Otieno & Retief	8
Syncolostemon concinnus N.E.Br.	8
Syncolostemon elliottii (Baker) D.F.Otieno	7&8
Syncolostemon flabellifolius (S.Moore) A.J.Paton	7
Syncolostemon foliosus (S.Moore) D.F.Otieno	8
Syncolostemon macranthus (Gürke) Ashby	8
Syncolostemon modestus (Codd) D.F.Otieno	8
Syncolostemon parviflorus E.Mey. ex Benth. var parviflorus	8
Syncolostemon persimilis (N.E.Br.) D.F.Otieno	8
Syncolostemon stalmansii (A.J.Paton & K.Balkwill) D.F.Otieno	8
Syncolostemon teucriifolius (Hochst.) D.F.Otieno	7&8
Orthosiphon allenii (C.H.Wright) Codd	4&6&7
Orthosiphon aristatus (Blume) Miq.	E&G& H&J&K&M& N
Orthosiphon biflorus A.J.Paton & Hedge	9
Orthosiphon bullosus Chiov.	5
Orthosiphon discolor A.J.Paton & Hedge	9
Orthosiphon ferrugineus Balf.f.	5
Orthosiphon fruticosus Codd	8
Orthosiphon grandiflorus A.Terracc	5
Orthosiphon hanningtonii (Baker) A.J.Paton	6
Orthosiphon humbertii Danguy	9
Orthosiphon miserabilis A.J.Paton & Hedge	9
Orthosiphon nigripunctatus G.Taylor	7
Orthosiphon pallidus Royle ex Benth.	1&3&4&5&6&9&D&H
Orthosiphon sarmentosus A.J.Paton & Hedge	9

Orthosiphon scedastophyllus A.J.Paton	6&7
Orthosiphon schimperi Benth.	3&4&5&6&7&8
*Orthosiphon thymiflorus (*Roth) Sleesen	3&4&5&6&7&8&9& D&E& H&J&K&M
Ocimum africanum Lour.	4&5&6&7&8&9& E&G& H&J&K& U& V&W&Z
Ocimum albostellatum (Verdc.) A.J.Paton	4&6&7
Ocimum angustifolium Benth.	6&7&8
Ocimum basilicum L.	0&2&3&4&5&6&7&8&9& A&B&C&E&G& H&J&K&M& P&Q&R& S&U& V&W&X&Z
Ocimum campechianum Mill.	T&U& V&W&X&Z&a
Ocimum canescens A.J.Paton	6
Ocimum dambicola A.J.Paton	6&7
Ocimum decumbens Gürke	4&6&7&8
Ocimum dhofarense (Sebald) A.J.Paton	D
Ocimum ellenbeckii Gürke	4
Ocimum filamentosum Forssk.	5&6&7&8 &D& H&K
Ocimum forskolei Benth.	1&5&6&7& D
Ocimum forskolei Benth.	1&5&6&7& D
Ocimum grandiflorum Lam.	5&6
Ocimum labiatum (N.E.Br.) A.J.Paton	7&8
Ocimum lamiifolium Hochst. ex Benth.	4&5&6&7
Ocimum obovatum E.Mey. ex Benth var. obovatum	3&4&5&6&7&8&9
Ocimum pseudoserratum (M.R.Ashby) A.J.Paton	8
Ocimum pyramidatum (A.J.Paton) A.J.Paton	6
Ocimum reclinatum (S.D.Williams & M.Balkwill) A.J.Paton	7&8
Ocimum serratum (Schltr.) A.J. Paton	8
Ocimum spectabile (Gürke) A.J.Paton	5&6
Ocimum tenuiflorum L.	6&7& E&G& H&J&K&M& N& P&Q& W&X&Y
Ocimum tubiforme (R.D.Good) A.J.Paton	8
Ocimum vanderystii (De Wild.) A.W.Hill	4&7
Ocimum verticillifolium Baker	7

Eastern Europe= 0; Northern Africa= 1; Macaronesia= 2; West Tropical Africa= 3; West-Central Tropical Africa= 4; Northeast Tropical Africa= 5; East Tropical Africa= 6; South Tropical Africa= 7; Southern Africa= 8; Madagascar= 9; Russian Far East= A; Middle Asia= B; Western Asia= C; Arabian Peninsula= D; China= E; Mongolia= F; Eastern Asia= G; Indian Subcontinent= H; Indo-China= J; Malesia= K; Papuasia= M; Australia= N; South-Western Pacific= P; South-Central Pacific= Q; North-Central Pacific= R; North-Central U.S.A.= S; South-Eastern U.S.A.= T; Mexico= U; Central America= V; Caribbean= W; Northern South America= X; Western South America= Y; Brazil= Z and Southern South America= a.