Staminal Evolution In The Genus Salvia (Lamiaceae): Molecular Phylogenetic Evidence For Multiple Origins Of The Staminal Lever

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Annals of Botany (in press)

Abstract

• **Background and Aims** - The genus *Salvia* has traditionally included any member of the tribe Mentheae (Lamiaceae) with only two stamens and with each stamen expressing an elongate connective. The recent demonstration of the non-monophyly of the genus presents interesting implications for staminal evolution in the tribe Mentheae. In the context of a molecular phylogeny, we characterize the staminal morphology of the various lineages of *Salvia* and related genera and present an evolutionary interpretation of staminal variation within the tribe Mentheae.

• **Methods**. Two molecular analyses are presented in order to investigate phylogenetic relationships in the tribe Mentheae and the genus *Salvia*. The first presents a tribal survey of the Mentheae and the second concentrates on *Salvia* and related genera. Schematic sketches are presented for the staminal morphology of each major lineage of *Salvia* and related genera.

• **Key Results**. These analyses suggest an independent origin of the staminal elongate connective on at least three different occasions within the tribe Mentheae, each time with a distinct morphology. Each independent origin of the lever mechanism shows a similar progression of staminal change from slight elongation of the connective tissue separating two fertile thecae to abortion of the posterior thecae and fusion of adjacent posterior thecae. We characterize a monophyletic lineage within the Mentheae consisting of the genera *Lepechinia, Melissa, Salvia, Dorystaechas, Meriandra, Zhumeria, Perovskia*, and *Rosmarinus*.

• **Conclusions**. Based on these results, we can demonstrate (1) the independent origin of the staminal lever mechanism on at least three different occasions in *Salvia*, (2) *Salvia* is clearly polyphyletic, with five other genera intercalated within *Salvia*, and (3) staminal evolution has proceeded in different ways in each of the three lineages of *Salvia* but has resulted in remarkably similar staminal morphologies.

**Key words** – staminal morphology, *Salvia, Mentheae, Dorystaechas, Meriandra, Perovskia, Rosmarinus, Zhumeria, Lepechinia, Melissa*, key innovation, floral evolution.
The genus *Salvia* (Lamiaceae: tribe Mentheae) represents a cosmopolitan assemblage of nearly 1000 species displaying a remarkable range of variation in growth form, secondary compounds, floral form, and pollination biology. *Salvia* has radiated extensively in three regions of the world: Central and South America (500 spp.), Western Asia (200 spp.), and Eastern Asia (100 spp.). Each of these radiations displays the unusual morphological character which has led to the long standing assumption that *Salvia* is monophyletic: the significant elongation of the connective tissue of the two expressed anthers (Fig. 2). The demonstration of the non-monophyly of the genus (Walker et al., 2004) has led to a reinvestigation of the staminal morphology within *Salvia* and closely related genera in the Mentheae. This paper presents a molecular phylogeny of *Salvia* and related genera, characterizes the staminal morphology in the various lineages of the genus *Salvia* and closely related genera, and interprets that staminal morphology in a phylogenetic context.

*Salvia* is distinguished from the other 72 genera in the tribe Mentheae by having the two posterior stamens aborted, and the connective separating the thecae of the two expressed stamens significantly elongated (Fig. 2). It is the elongation of the staminal connective that enables the formation of the lever mechanism of pollination for which *Salvia* is best known (Fig. 1) (see Claßen-Bockhoff et al. 2003; 2004a for thorough reviews). The significant species radiations that are correlated with the presence of the lever mechanism in *Salvia* suggest it is the lever mechanism in a selective regime of pollination that is driving evolution in the group (Claßen-Bockhoff et al., 2004b). The significance of this lever mechanism to the reproductive biology in *Salvia*, first described by Sprengel (1793), has received considerable attention (Muller, 1873; Zalewska, 1928; Hruby, 1934; Werth, 1956; Baikova, 2002; 2004; Claßen-Bockhoff et al., 2003; 2004a; Wester and Claßen-Bockhoff, 2005). Himmelbaur and Stibal (1932-1934) directly addressed staminal evolution in *Salvia*, presenting a hypothesis of parallel evolution of the lever mechanism (from a common ancestor) in the New World and the Old World. We present here the first, robust, *Salvia-*wide molecular phylogeny with sampling across the tribe Mentheae to directly evaluate Himmelbaur and Stibal’s (1932-1934) hypothesis. Additionally, the following questions are addressed and answered: How many times has an elongate connective originated in *Salvia* and related genera? How many times has the staminal lever mechanism originated in Mentheae? What are the most closely related genera to *Salvia*? What are the trends in staminal evolution within *Salvia*?

The results will support independent origins of the staminal lever mechanism on at least three different occasions. From a common ancestor, we document remarkably similar - yet independent - progressions in staminal evolution, each presumably under pollinator selection, and each arriving at strikingly similar functional endpoints in a staminal lever.

**Materials and Methods**

**Taxa sampling**

Nomenclature for *Salvia* follows that suggested by Alziar (1988–1993). 144 trnL-F sequences, 139 nuclear rDNA ITS sequences and 85 psbA-trnH sequences representing...
38 genera and 144 species were obtained in this project (Table 1). Accessions, vouchers, locality and GenBank numbers are available in Table 1. The data matrix for the “Mentheae-wide analysis” combined ITS, psbA-trnH and trnL-F and consisted of 84 taxa. The data matrix for the “Salvia clade analysis” combined ITS, and trnL-F and consisted of 93 taxa. Outgroups chosen for the Mentheae-wide analysis were Ocimum basilicum and Hyptis alata, both from the tribe Ocimeae. Within the Mentheae, 34 genera were sampled that represented every subtribe within the Mentheae. Within the “Salvia clade analysis”, sampling concentrated on the genus Salvia (82 species sampled) and all genera indicated by the “Mentheae wide analysis” to be closely related to Salvia. Horminum pyrenaicum was selected as the outgroup for the “Salvia clade analysis” based on the results of the “Mentheae-wide analysis”.

**Extracts, amplification, and sequencing**

Total genomic DNA was extracted using DNeasy Plant Mini kits (Qiagen, Valencia, California, USA). Leaves used for DNA extractions were fresh, frozen, silica dried, or obtained from herbarium specimens. Polymerase chain amplification (PCR) and cycle sequencing followed the methods described elsewhere (Conti et al., 1996; Givnish et al., 2000). PCR product was purified either with QIAquick PCR purification kit (Qiagen) or with AmPure PCR purification kit (Agencourt, Beverly, Massachusetts, USA). Sequenced products were precipitated in ethanol and sodium acetate to remove excess dye terminators or cleaned with CleanSEQ Sequencing Reaction Clean-up system (Agencourt). Contiguous alignments were edited using Sequencher vs. 3.0 (Gene Codes, Ann Arbor, Michigan, USA).

Sequences were aligned visually in SeAl version 2.0a7 (Rambaut, 2001). Indels in the trnL-F data set were coded using the guidelines of Baum et al. (1994). Regions of ambiguous alignment were excluded from the analyses.

**Phylogenetic analysis**

Phylogenetic relationships within Salvia and Mentheae were evaluated in a two-step approach. The first involved a 84-taxon data set (37 species of Salvia) using sequences from the chloroplast regions psbA-trnH, and trnL-F, and the nuclear ITS region (“Mentheae-wide analysis”). The combined data sets were analyzed using maximum parsimony (MP). The heuristic MP analysis (Fitch, 1971) in PAUP* 4.0b10 (Swofford, 2002) used 100 random addition sequences, with 10 trees held at each step during stepwise addition, and tree bisection and reconnection (TBR) branch swapping to explore the possibility of multiple islands of most parsimonious trees (Maddison, 1991). To assess congruence between the three data sets, 100 replicates of the partition homogeneity test (Farris et al., 1995) were conducted using a full heuristic search, simple taxon addition, TBR branch swapping, and saving all most parsimonious trees. Although the partition homogeneity test has been criticized (Yoder et al., 2001), the test has merit as a first assessment for congruence of data sets (Hipp et al., 2004). Bootstrap (Felsenstein, 1985) support values were used to evaluate support for relationships within the resulting trees. Bootstrap values were obtained through a heuristic search on all characters, with 1000 replicates and 10 random addition sequences with TBR replicates with no more than 5000 trees saved per replicate.
The second approach (the “Salvia clade analysis”) involved an expanded sampling within the genus Salvia (83 species of Salvia) and 11 other species representing all closely related genera. This analysis used the chloroplast trnL-F and the nuclear rDNA ITS regions and with the same methodologies used in the “Mentheae-wide analysis” except for the inclusion of a maximum likelihood (ML) analysis in addition to MP. Maximum likelihood analyses were conducted on the “Salvia clade” data set as implemented in PAUP*. Optimality criteria were explored using Modeltest version 3.06 (Posada and Crandall, 1998). Heuristic ML searches with TBR branch-swapping were conducted.

Staminal morphological investigations

Stamens were examined for each species included within the analysis. Where fresh material was not available, literature was used to determine the staminal morphology in each species (Himmelbaur and Stibal, 1932-1934; Bokhari and Hedge, 1971, 1976; Hedge, 1974, 1982a, b; Claßen-Bockhoff et al., 2004a). General stamen types were characterized for each major clade suggested by the molecular results and mapped onto the terminals in the cladograms (Figs. 4, 5).

RESULTS

Analysis of Mentheae-wide data set

The aligned length of the trnL-F data set was 1137 base pairs. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 1062 base pairs. Twenty indel events were scored for the trnL-F data set, of which 18 were parsimony informative and included in the analysis. Of the 1082 characters in the analysis, 793 were constant, 117 variable characters were uninformative, and 172 were parsimony informative (15.9%). Fitch parsimony analysis of the trnL-F region (uninformative characters excluded) found 4399 equally parsimonious trees of 332 steps (CI=0.645, RI=0.913, RC=0.588).

The aligned length of the psbA-trnH data set was 624 base pairs. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 382 base pairs. Of the 382 characters in the analysis, 252 were constant, 58 variable characters were uninformative, and 72 were parsimony informative (18.8%). Fitch parsimony analysis of the psbA-trnH region (uninformative characters excluded) found 9470 equally parsimonious trees of 191 steps (CI=0.586, RI=0.864, RC=0.507).

Nuclear rDNA ITS sequences were not obtained from Salvia santolinifolia, S. tetradoanta, S. regla, Hoehnea epilobioides and Prunella vulgaris. The aligned length of the nuclear ITS data set was 811 base pairs. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 659 base pairs. Of the 659 characters in the analysis, 364 were constant, 98 variable characters were uninformative, and 197 were parsimony informative (29.9%). Fitch parsimony analysis of the ITS region found 5035 equally parsimonious trees of 1167 steps (CI=0.336 RI=0.652, RC=0.219)
The combined *trnL-F*, *psbA-trnH* and nuclear ITS analysis generated 2123 characters of which 1409 were constant, 273 were variable but uninformative, and 441 were parsimony informative (20.8%). Fitch parsimony analysis of the three regions found 2094 equally parsimonious trees of 1737 steps (CI=0.413, RI=0.755, RC=0.312).

The partition homogeneity test of the three data sets suggests significant incongruity between all three data sets (*trnL-F*, *psbA-trnH* and nuclear ITS) compared to random partitions of the same size (P<0.01). Despite the incongruence of the data sets, all three data sets independently support the integrity of the three clades of *Salvia* discussed in this paper. Further analyses of the specific topological differences found between individual data sets indicate that none of these incongruent clades have bootstrap support above 50% in the individual region analyses. Thus the combined data set approach is justified.

The tribe Mentheae is supported at 100% bootstrap in the strict consensus tree (Fig. 3). Within the Mentheae, a “*Salvia* clade” is moderately supported (64%) and with the genera *Lepechinia* and *Melissa* appearing as likely sister genera. For the purposes of this discussion, we use the term “*Salvia* clade” to refer to the least inclusive clade which contains all members of *Salvia*. In addition to all *Salvia*, also included in the “*Salvia* clade” are the genera *Dorystaechas*, *Meriandra*, *Perovskia*, *Rosmarinus*, and *Zhumeria* (see Fig. 3). At least three different clades contain species of *Salvia*; thus *Salvia* is not monophyletic. *Salvia* clade I is strongly supported as monophyletic and together with the genera *Rosmarinus* and *Perovskia* form a monophyletic lineage (94%). *Salvia* clade II, likewise, forms a well supported monophyletic lineage including two other genera, *Meriandra* and *Dorystaechas* (100%). Two remaining, well-supported lineages of *Salvia*, one of which includes the genus *Zhumeria*, occupy one of the few unresolved areas within the *Salvia* clade. These two are referred to as *Salvia* “clade III” and could be either monophyletic or form a paraphyletic grade leading to *Salvia* clade II (Fig. 3).

**Analysis of “Salvia clade” data set**

The aligned length of the *trnL-F* data set was 1019 base pairs. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 923 base pairs. Of the 1019 characters in the analysis, 755 were constant, 75 variable characters were uninformative, and 93 were parsimony informative (9.1%). Fitch parsimony analysis of the *trnL-F* region found 26007 equally parsimonious trees of 163 steps (CI=0.748, RI=0.971, RC=0.727).

No rDNA ITS was obtained for *Salvia tetradonta*, which was included in the combined analysis. The aligned length of the nuclear ITS data set (for the 93 included taxa) was 807 base pairs. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 762 base pairs. Of the 762 characters in the analysis, 428 were constant, 101 variable characters were uninformative, and 233 were parsimony informative (30.6%). Fitch parsimony analysis of the ITS region found over 230,000 equally parsimonious trees of 1286 steps (CI=0.341, RI=0.762, RC=0.260).
The combined *trnL-F* and nuclear ITS analysis generated 1698 characters of which 1183 were constant, 176 were variable but uninformative, and 339 were parsimony informative (20.0%). Fitch parsimony analysis of the *trnL-F* region (uninformative characters excluded) found over 100,000 equally parsimonious trees of 1489 steps (CI=0.376, RI=0.814, RC=0.306).

The partition homogeneity test of the two data sets suggests significant incongruity between the *trnL-F* and ITS data sets compared to random partitions of the same size (P<0.01). Despite the incongruence of the data sets, both data sets independently support the integrity of the three clades of *Salvia* discussed in this project. With regards to these main clades, the topology generated from the strict consensus of the *trnL-F* data set does not differ from the topology of the combined analysis (although polytomies found in the *trnL-F* strict consensus tree are resolved in the combined analysis). None of the examples of incongruence of the data sets that would affect the interpretations included in this paper found in the ITS strict consensus tree have bootstrap support above 50% in the ITS analysis.

Maximum likelihood produced a single tree with a log likelihood score \(-11859.60033\). The ML analyses were performed under the K80(K2P)+G+I model of evolution: ti/tv ratio = 1.683386; proportion invariable sites = 0.518164; nucleotide frequencies = 0.25; gamma shape parameter = 0.513370; substitution types = 2; rate categories = 4. All clades discussed in this paper were present in both the MP and ML trees and relationships among those clades, were identical under both assumptions. The only topological differences between the MP and ML trees were species relationships within the major lineages defined in this paper.

The strict consensus of all MP trees for the *Salvia* clade analysis (Fig. 4) exhibits the same, well-supported clades seen in the Mentheae-wide analysis. *Salvia*, likewise, is not monophyletic. *Lepechinia* together with *Melissa* form the sister group to the *Salvia* clade. *Salvia* “clade III” still appears as a paraphyletic grade, although the branch support for paraphyly (or monophyly) is weak. Within *Salvia* clade II, two moderately to well-supported subclades emerge with the increased taxa sampling: sect. *Audibertia* from Western North American sister to the large neotropical subg. *Calosphace*.

**Staminal morphology**

Two distinct stamen types were identified in *Salvia* clade I (stamen type A, B, Fig. 5). The two posterior thecae are expressed and not fused in stamen type A. In stamen type B, the two posterior thecae are entirely aborted, and the distal posterior ends of the adjacent connectives are fused into a complex structure blocking access to the base of the corolla. Five distinct stamen types were identified in *Salvia* clade II. In *Salvia axillaris* (stamen type G, Fig. 5), both posterior thecae are expressed, and not fused to one another. In sections *Standleyanana*, *Blakea*, and *Hastatae* (stamen type F, Figs. 4, 5), both posterior thecae are aborted, and the adjacent posterior thecae not fused. The remaining members of S. subg. *Calosphace* (stamen type E, Fig. 5) have both posterior thecae aborted and adjacent posterior connective branches fused. Two stamen types are described for *Salvia* sect. *Audibertia* (Fig. 4, 5): those that exhibit a reduced posterior theca (stamen type I),
and those with an entirely aborted posterior theca and connective arm (stamen type H).

Two stamen types were recognized in *Salvia* “clade III”. The first of these (stamen type M, Fig. 4, 5) has both posterior thecae expressed and not fused to one another. The second type of stamen found in *S.* “clade III” (stamen type N, Fig. 4, 5) has both posterior thecae aborted, or expressed and producing little or no pollen. The posterior thecae are flattened by growth on the abaxial side of the theca (Claßen-Bockhoff *et al.*, 2004a), resulting in a fan-shaped theca projected forward from the corolla throat. The two adjacent aborted thecae may be entirely fused, simply connivent, or even separated. Whereas access to the base of the corolla is not necessarily blocked, a lever mechanism is employed in this stamen type.

The inferred progression in staminal evolution within the *Salvia* clade is depicted in Fig. 6 based on the tree-mapping of these stamens types from *Salvia* and intercalated genera (Fig. 5). From the ancestral Mentheae stamen type without elongate connectives (stamen type O, Fig. 5), slightly elongate connectives evolved at least three times in the *Salvia* clade in lineages recognized as other genera (stamen types D, J, K, L, Fig. 5). The genera with these intermediate stamen types are either basal or sister to the three (or more depending on resolution within *Salvia* “clade III”) major clades of *Salvia* possessing the variety of stamen types described above. The final progression to the staminal lever mechanism has thus occurred in three similar, but parallel ways (Figs. 5, 6).

**DISCUSSION**

The molecular results presented here resolve a number of systematic questions within the tribe Mentheae, particularly the manner in which the lever mechanism has evolved within the *Salvia* clade. First, the genera *Lepechinia* and *Melissa* are closely related, and together with the “*Salvia* clade” form a monophyletic group within the Mentheae (Fig. 3). Second, as originally demonstrated by Walker *et al.* (2004), there exist three distinct lineages of *Salvia*, each lineage more closely related to other genera in the Mentheae than to the two other major lineages of *Salvia* (Figs. 3, 4). And third, the staminal lever mechanism has evolved three times independently, each time with a distinct morphology (Figs. 5, 6).

**Relationships within the Mentheae**

Mentheae (sensu Wagstaff *et al.*, 1995) is a well-supported monophyletic tribe containing 73 genera within the subfamily Nepetoideae (Cantino *et al.*, 1992; Wagstaff 1992; Wagstaff *et al.*, 1995; Walker *et al.*, 2004; Bräuchler *et al.*, 2005). This project has sampled all putative *Salvia* relatives, as well as representatives of all other major lineages within the tribe. Our purpose here is not to describe relationships between all genera of the Mentheae, but rather to describe the clade to which *Salvia* belongs. A thorough investigation into relationships within the tribe Mentheae, comprehensively sampling all genera within the tribe, is being addressed by Bräuchler *et al.* (2005). For the purposes of this paper, it suffices to say that our sampling within the Mentheae is thorough enough that we feel confident in identifying a monophyletic lineage consisting of the genera *Melissa, Lepechinia* (including Chaunostoma), *Salvia, Dorystaechas, Meriandra, Zhumeria, Perovskia,* and *Rosmarinus* (Fig. 3), a result also supported in part by
Staminal evolution in *Salvia* by Bräuchler et al. (2005). This finding is in agreement with the results of Wagstaff (1992) based on cpDNA restriction site analysis, although he did not sample *Meriandra*, or *Zhumeria*, and the placement of *Melissa* was unresolved. Within this clade, our data support a monophyletic lineage consisting of *Salvia*, *Dorystaechas*, *Meriandra*, *Zhumeria*, *Perovskia*, and *Rosmarinus* (the “Salvia clade”), a clade characterized morphologically by the abortion of the two posterior stamens. We are unable to resolve the exact relationship of *Lepechinia* and *Melissa* to the “Salvia clade”. It is unclear whether the genus *Melissa* is sister to (“Salvia clade” + *Lepechinia*), is sister to *Lepechinia*, or is sister to “Salvia clade”. However, in all analyses, “Salvia clade”, *Lepechinia*, and *Melissa* form a monophyletic clade (Fig. 3).

*Melissa* includes three species native to Iran and central Asia. *Lepechinia* is a New World group of approximately 40 species, historically presenting numerous taxonomic difficulties (Epling, 1944, 1948; Hart, 1983) owing to the fact that, “not one, but several characters hold most of the species together; not one species has all the characters, nor does any character occur in all species” (Hart, 1983). Hart (1983) suggested *Lepechinia* was polyphyletic based on this polythetic generic concept and the significant divergence evident in his cladistic morphological analysis. However, Hart’s (1983) work concentrated on one section of the genus *Lepechinia* and did not include putative outgroup genera in his analysis. Molecular work underway in our lab investigating the genus *Lepechinia* (Delventhal, et al., University of Wisconsin, unpubl. res.) supports the significant divergence of various lineages of *Lepechinia* from one-another. However, *Lepechinia* likely represents an ancient assemblage of at least two lineages of plants forming a basal grade in the larger clade discussed above. Our data suggest that the monotypic genus *Chaunostoma* belongs within the genus *Lepechinia*, a relationship also suggested by Epling (1948) and Croizat (1962). Both *Lepechinia* and *Melissa* have four expressed stamens, each with two parallel thecae and a connective not at all elongated.

In short, we informally recognize within the larger tribe Mentheae a lineage that would correspond to a subtribe consisting of the genera *Salvia*, *Dorystaechas*, *Meriandra*, *Zhumeria*, *Perovskia*, *Rosmarinus*, *Lepechinia* and *Melissa*. We feel this assemblage of genera warrants novel subtribal status as significant changes would have to be invoked to either Bentham’s (1876) or Wunderlich’s (1967) tribal and subtribal arrangements to accommodate all these genera. However, we choose to wait until relationships within the remainder of Mentheae are more completely known (e.g., Bräuchler et al., 2005) before formally naming this lineage. It is within this subtribe that we concentrate on staminal evolution within the three lineages of *Salvia* as suggested by the molecular phylogenetic data.

**Staminal evolution in Salvia clade I**

*Perovskia* and *Rosmarinus* together are well-supported as sister to *Salvia* clade I (Figs. 3, 4). Both analyses also place *Perovskia*+*Rosmarinus*+*Salvia* clade I sister to the remainder of the “Salvia clade”. *Perovskia* has a slightly elongate connective in its two expressed stamens (Bentham, 1876; Bokhari and Hedge, 1971; Wagstaff, 1992; stamen type D, Fig. 5). *Rosmarinus* has a significantly elongated connective in its two stamens, and a total abortion of the posterior branch of the connective and the posterior theca.
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(stamen type C, Fig. 5). The resulting morphology results in the stamen appearing essentially “normal” (i.e.- with no elongate connective), albeit with only one theca at the end, and a notch halfway up the “filament” representing where the filament ends and the connective begins. It is important to note, however, that this is the same morphology found in *Salvia* sect. *Audibertia* from western North America, and thus there is no morphological basis for why *Rosmarinus* should not be included in the genus *Salvia*.

Within *Salvia* clade I, two well-supported lineages are identified here, each with a distinct stamen morphology. The first well-supported clade within *Salvia* clade I consists of *S. daghestanica*, *S. canariensis*, *S. candidissima*, *S. verbascifolia*, *S. aethiopsis*, *S. austriaca*, and *S. sclarea* in our sampling. These species are the only species sampled here to display the staminal character of total fusion of the posterior thecae into what Bentham (1876) termed a glutinatorium, and what Claßen-Bockhoff *et al.* (2004a) and Himmelbaur and Stibal (1932-1934) described as “stamen type V” (stamen type B, Fig. 5; Fig. 1). This morphology creates the classic *Salvia* lever mechanism, where the pollinator is forced to push against the fused posterior connective tissue and activate the lever in order to access the nectar. Using the species groups established by Hedge (1974; 1982a, b) and the alliances suggested by Pobedimova (1954), it can be assumed this clade likely contains an additional 50 European and western Asian species.

All other taxa included in *Salvia* clade I produce rudimentary posterior thecae, sometimes with pollen produced, and never entirely fused to the adjacent posterior theca or connective arm. Field observations by the primary author suggest that a lever mechanism is often (although certainly not always) employed in these taxa, even with the posterior thecae not fused. Using the species groups established by Hedge based on morphological characters (1974; 1982a, b) and the alliances suggested by Pobedimova (1954), it can be assumed that essentially all central and southern African *Salvia* belong to this group, plus an additional at least 50 species from western Asia and the Mediterranean, and 8 species in the New World (Walker and Elisens, 2001; Walker *et al.*, 2004). These numbers would place the size of this group at over 100 species.

**Staminal evolution in *Salvia* clade II**

In both analyses, *Dorystaechas* and *Meriandra* are either sister to *Salvia* clade II, or represent a grade toward a monophyletic *Salvia* clade II – a large lineage of *Salvia* including the New World sect. *Audibertia* and subg. *Calosphae*. *Dorystaechas* and *Meriandra* have long been seen as somewhat anomalous genera in the Mentheae with no obvious affinities (Bokhari and Hedge, 1976). The two genera have been placed in the subtribe Meriandreae with *Perovskia* (Bentham, 1876), based on two expressed stamens and parallel thecae, in what Bokhari and Hedge (1976) describe as “…essentially an artificial assemblage of isolated relict genera united essentially only by the 2-staminate corollas”. Each of the genera also have slightly elongate connectives (in the case of *Perovskia* and *Dorystaechas* (stamen type K, Fig. 5), the connectives would probably be better described as swollen). *Dorystaechas* is a monotypic genus restricted to southwest Anatolia. *Meriandra* has slightly elongate connectives (stamen type J, Fig. 5) and consists of two species, one native to Ethiopia, and one to India (ironically, *Meriandra bengalensis* is the Ethiopian species).
Within the larger picture of the genus *Salvia*, S. sect. *Audibertia* represents an anomalous group restricted to the California Floristic Province and adjacent deserts. The separation of this group from other *Salvia* has been based on chemical compounds, stout woody habit, and most importantly, on the structure of its stamens. Sect. *Audibertia* is unique within *Salvia* in having the posterior branch of the connective entirely aborted (although the genus *Rosmarinus* shows a similar phenomenon, as do some individuals of the Old World *S. verticillata*). Whereas the anterior branch of the connective is still elongate, functionally it acts in the same manner as would a simple filament, albeit with only a single theca at its end (Bentham, 1876; Epling, 1938; Neisess, 1983) (stamen type H, Fig. 5). Worthy of note is a difference in staminal morphology seen between *Salvia* sect. *Audibertia* and the genus *Rosmarinus*. Whereas the “joint” between the filament and connective is indicated by a notch on the top of the stamen in *Rosmarinus*, an articulation circling the entire filament is found at that same “joint” in sect. *Audibertia*. Occasionally the posterior theca and connective branch is re-expressed in members of sect. *Audibertia*.

Contrary to the most recent treatment of the section (Neisess, 1983), our preliminary data suggest that sect. *Audibertia* (sensu Bentham) is a monophyletic lineage (Figs. 3, 4), and the species included in Neisess’ (1983) sect. *Echinosphace* likely represent a grade toward a monophyletic sect. *Audibertia* (sensu Neisess, 1983). The staminal morphology of sect. *Echinosphace* (4 spp.) is distinct from sect. *Audibertia* in that the posterior branch of the connective and the posterior theca are always expressed, albeit reduced (stamen type I, Fig. 5). Two species showing stamen type I, *S. carduacea* and *S. columbariae*, were sequenced as part of a project underway investigating relationships within sect. *Audibertia*, and form a grade toward sect. *Audibertia* sensu Neisess (1983) (Walker, University of Wisconsin, upubl. res.). As sect. *Echinosphace* represents a grade toward sect. *Audibertia*, a clear progression from both thecae being expressed to the entire abortion of the posterior theca is seen in the group.

*Salvia* subg. *Calosphace* consists of nearly 500 species and occurs throughout the New World, with centers of diversity in Mexico, the Andean region, and Southern Brazil and Argentina. Epling (1939) created the only comprehensive treatment of the subgenus, organizing 468 species into 91 sections (and in supplementary notes, an additional 71 species and 13 sections). A stumbling block to past and future work in subg. *Calosphace* are (1) the lack of knowledge of relationships between sections (an issue Epling did not address) and (2) the lack of faith in the monophyly of some of his larger sections. For these reasons, the only works to have been completed at the sectional level since Epling’s time have generally been limited to sections of five or fewer species (Peterson, 1978; Ahlenslager, 1984; Turner, 1996). In those revisions dealing with larger sections (Serna and Ramamoorthy, 1993 (11 species); Torke, 2000 (8 species)), the monophyly of those sections was not addressed. The sampling included with this paper is part of a larger project investigating large scale relationships within the subgenus *Calosphace*.

The typical staminal morphology for the subg. *Calosphace* consists of an elongation of the posterior connective branch, fusion of the two adjacent connective arms, and no differentiation of tissue at the distal end of the connective branch (stamen type E, Fig. 5).
As is well documented by Claßen-Bockhoff et al. (2004a), Baikova (2002, 2004) Epling (1939) and others, a tooth is often present on the lower side of the posterior connective branch. Claßen-Bockhoff et al. (2004a) clearly demonstrate anatomically that the aborted posterior theca may be either located at the distal end of the connective arm, or in some cases represented by the tooth on the lower side of the connective arm. Their finding suggests that the lever mechanisms found within subg. *Calosphace* may not all be homologous, and in fact the lever mechanism itself may have been derived at least twice within the subgenus. Despite that important difference, staminal morphology within the subgenus is uniform with respect to no posterior thecae expressed and the two posterior connective arms fused. This uniformity is true across the entirety of subg. *Calosphace* except for four of Epling’s sections. Sections *Hastatae* (7 spp.), *Blakea* (4 spp.) and *Standleyana* (1 sp.) all have a total abortion of the posterior thecae; however, the connective arms do not entirely fuse. These three sections are all included within the clade represented by stamen type F (Figs. 4, 5), and form a monophyletic group. *Salvia axillaris*, of monotypic section *Axillares*, is the only member of *Salvia* subg. *Calosphace* to have expressed posterior thecae (stamen type G, Fig. 5). The molecular phylogeny suggests that *S. axillaris* is sister to the remainder of subg. *Calosphace*. In turn, *Hastatae*, *Blakea* and *Standleyana* represent a monophyletic lineage sister to remaining members of the subgenus. These four sections thus depict an evolutionary “trail” of staminal morphology, showing a progression from both thecae expressed and no fusion of posterior connective branches, to abortion of posterior thecae and no fusion of posterior connective branches, and ultimately to the typical staminal morphology in subg. *Calosphace* of abortion of posterior thecae and fusion of connective branches (see Figs. 5, 6).

**Staminal evolution in *Salvia* “clade III”**

In addition to the clearly delineated *Salvia* clade I and *Salvia* clade II, there exists a group of *Salvia* that fit into neither of the above groups. The molecular and morphological evidence clearly supports *Salvia* “clade III” as having an independent origin of the lever mechanism (Fig. 5). However, this group of *Salvia* may represent a paraphyletic grade consisting of two monophyletic lineages rather than a single monophyletic clade III (Figs. 3, 4).

One of the two lineages consists of a group of western Asian and northern African species including *S. aristata*, *S. aegyptiaca*, *S. tetradonta*, *S. trichocalycina* and *Zhumeria majudae* (Fig. 4). The *Salvia* in this first lineage all have somewhat elongate connectives, both thecae producing pollen, and the posterior thecae never fused (stamen type M, Fig. 5). *Zhumeria majudae* is a shrub native to Iran with historically uncertain affinities (Bokhari and Hedge, 1976), but placed in our analyses as sister to this clade of *Salvia* (Fig. 4). *Zhumeria* is unusual within the broader “*Salvia* clade” in that, in addition to the two fertile stamens, two large staminodes are easily identified in the corolla (Bokhari and Hedge, 1976). The thecae of the two fertile stamens are somewhat separated, though without a distinct connective (stamen type L, Fig. 5). Using the species groups established by Hedge based on morphological characters (Hedge 1974; 1982a, b), in addition to the species sampled here, this first lineage of *Salvia* “clade III” likely also
includes *Salvia bazmanica, S. santolinifolia, S. macilenta, S. tebesana, S. eremophila, S. deserti, S. chudaei, S. pterocalyx,* and *S. rechingeri.*

The second lineage belonging to *Salvia* “clade III” consists of a group of Asian and Mediterranean species. This is the most poorly sampled clade of *Salvia* in this project but includes *S. glutinosa, S. miltiorrhiza, S. hians, S. cynica, S. przewalskii, S. digitaloides,* and *S. roborowskii* (from a group that likely contains nearly 100 species). *Salvia glutinosa* and *S. miltiorrhiza* are probably the best known members of this group, and each express the staminal morphology typical of all members of this group. The posterior thecae are rudimentary, and produce no or very little pollen. Often (although not always) in this group, the two adjacent posterior thecae post-genitally fuse (Claßen-Bockhoff et al., 2004a). These two posterior thecae are somewhat fan-shaped and are projected forward from the corolla throat (stamen type N, Fig. 5) and a lever mechanism is employed whether or not the posterior thecae fuse. Although we expect that this group of species probably includes nearly 100 species with a likely center of diversity in China, we are currently unable to define the exact extent of this clade owing to our unfamiliarity with *Salvia* of China and the fact that the particulars of staminal morphology are rarely included in species descriptions.

**Summary of staminal evolution in Salvia**

In hindsight, Himmelbaur and Stibal (1932-1934) presented a remarkably accurate assessment of staminal evolution in the genus *Salvia.* Working with limited material, and lacking the molecular evidence to suggest phylogenetic relatedness of *Dorystaechas, Meriandra, Zhumeria, Perovskia* and *Rosmarinus* to *Salvia,* the general progression in staminal evolution they suggested for the genus *Salvia* is similar in some fundamental points to what we present here. These points include their recognition of (1) the plesiomorphic staminal state as having two expressed thecae and no lever mechanism in each stamen and (2) parallel origins of the lever mechanism in the New World and the Old World. Some of the specific examples they suggest, such as *Salvia* sects. *Hastatae, Blakea,* and *Standleyana* being intermediate between the plesiomorphic state and derived state seen in core *S.* subg. *Calosphace,* are exactly the relationships suggested by the molecular data. The molecular approach employed here clarifies the phylogenetic relationships and thus the relationships of different stamen types. A good example of this is in the core *Salvia* “clade III” (stamen type N, Fig. 5) where in some species the aborted posterior thecae are free, in some they are connivent, and in some species they entirely fuse. Himmelbaur and Stibal (1932-1934) classified these stamen types as distinct from one another. Our molecular phylogeny indicates relatedness of species with or without fusion in this clade. Indeed, Claßen-Bockhoff et al.’s (2004a) developmental work demonstrate that the two posterior thecae in this group fuse post-genitally via fusion of papillate epidermal cells. Both the phylogenetic and developmental results indicate that the stamen types seen in core *Salvia* “clade III” are not as distinct as Himmelbaur and Stibal originally assumed.

The molecular data presented in this paper strongly support at least three independent origins of the lever mechanism in *Salvia.* However, Claßen-Bockhoff et al. (2004a) clearly demonstrated through developmental studies the homology of the staminal lever
mechanism across all major lineages of *Salvia* – that is, each type is derived from the elongation of the connective tissue. Do the findings of Claßen-Bockhoff *et al.* (2004a) concerning homology of the staminal lever contradict our findings of three separate origins of the staminal lever mechanism? Three lines of evidence strongly support that these staminal levers, although homologous at some level, represent the evolutionary products of three separate events. First, our findings suggest that whereas the lever mechanisms in *Salvia* are all derived from connective tissue, the precise staminal morphology of the lever mechanism in each of the three major lineages of *Salvia* supports three independent origins of the lever mechanism in different ways. The “gubernaculum” (Bentham 1876; Claßen-Bockhoff *et al.*, 2004a, stamen type III; stamen type B, Fig.5) seen in *Salvia* clade II is never found in *Salvia* clade I or III. The “glutinatorium” (Bentham 1876; Claßen-Bockhoff *et al.*, 2004a, stamen type V; stamen type E, Fig.5) seen in *Salvia* clade I is never found in *Salvia* clade II or III. The fan-shaped, connivent posterior thecae (stamen type N, Fig.5) seen in *Salvia* “clade III” are never found in *Salvia* clade I or II. Within each of the major lineages of *Salvia* described in this paper, the work of Claßen-Bockhoff *et al.* (2004a), Zalewska (1929), Himmelbaur and Stibal (1932-1934), and Hedge (1974; 1982a, b) have noted the uniformity of staminal morphology. Second, further support for three independent origins of the staminal lever mechanism comes from the molecular phylogeny that strongly places each of the three clades with a lever mechanism as sister to a group of *Salvia* with elongate connectives, but no lever mechanism. Third, and more significantly, each of these three more inclusive lineages of *Salvia* is in turn sister to genera without significantly elongate connectives (in the case of *Salvia* “clade III”, the genus Zhumeria is sister to one of the two groups in “clade III”).

It is not only trends in staminal evolution that are consistent across the various lineages in the “*Salvia clade*”, but some of the specific stamen types are surprising in their parallel recurrence. For example, stamen type A in *Salvia* clade I is scarcely distinguishable from stamen types G or M in *Salvia* clades II and III. Another striking example of parallel recurrence of similar stamen types, is the multiple origins of a stamen type exhibiting total abortion of the posterior theca and posterior connective branch. This stamen type has independently derived in *Salvia* sect. *Audibertia* (stamen type H), *Rosmarinus* (stamen type C), and in *Salvia verticillata* (not shown). *Salvia verticillata* belongs to the subclade of *Salvia* clade I expressing stamen type A (Figs. 4, 5), but itself often has the posterior branch of the connective aborted (Himmelbaur and Stibal, 1932-1934; Claßen-Bockhoff *et al.*, 2004a). In each of these three examples, the stamens have gone through a complicated evolutionary progression only to end up with a stamen that morphologically is scarcely distinguishable from the plesiomorphic state for the *Salvia* lineage, except in the fact that it has one theca instead of two.

This work demonstrates that the story of staminal evolution within the “*Salvia clade*” is remarkable in its recurrent nature. On three different occasions (*Salvia* clade I, clade II, and “clade III”) we see a four-step progression from slight elongation of the connective to significant elongation of the connective, to loss of fertility of the posterior thecae, and ultimately to the fusion of the posterior branches of the connectives (Fig. 5, 6).
**Issues in cases of parallel evolution**

That all *Salvia* belong to a single, well-defined lineage within the tribe Mentheae begs the question whether *Salvia* is truly polyphyletic or simply paraphyletic. To make the nearly 1000 species of *Salvia* monophyletic would require only the inclusion of thirteen species from the genera *Perovskia* (7 spp.), *Rosmarinus* (2 spp.), *Meriandra* (2 spp.), *Dorystaechas* (1 sp.) and *Zhumeria* (1 spp.). However, this paper demonstrates that the character that defines *Salvia* within the Mentheae (the significantly elongate connective) has independently originated in each of the three major *Salvia* lineages. The independent origin of the defining character for *Salvia* is supported by the molecular phylogeny, that each of the major clades of *Salvia* is associated with a genus that does not express the significantly elongate connective, and by the distinct staminal morphology in each of the major lineages of *Salvia*. Thus, this is not the case where 13 species not included in the genus *Salvia* represent anomalous members of the genus *Salvia* that have undergone character reversals (i.e., *Salvia* is paraphyletic). Rather, the significantly more parsimonious explanation is that the genera associated with *Salvia* never developed the character that defines the “genus” *Salvia*. That is, *Salvia* is polyphylectic in that it is defined by a convergent character. If the genera intercalating themselves within *Salvia* were larger in size, or if more genera were present in the *Salvia* lineage, it would not be difficult to accept the polyphyly of *Salvia*. If the other five genera had become extinct, one could engage in a philosophical discussion as to the monophyly of a clade whose defining character evolved multiple times. However, the *Salvia* clade represents a wonderful example of evolution leaving a “trail” as it progressed. Gould (1989) suggested that evolutionary novelties are chance occurrences, unlikely to be repeated in different times and places. This general philosophy no doubt played a role in the long held assumption of the monophyly of *Salvia* based on the “unlikely” origin of something as complex as the lever mechanism multiple times. However, the story we present of staminal evolution in *Salvia* suggests that in the context of a selective regime, Gould’s evolutionary “tape” can in fact repeat itself despite long odds – perhaps in response to similar genetic canalizations, phylogenetic constraint, similar pollination selective regimes, and/or convergent tendencies.

It is certainly worth noting that the large species radiations we see in each of the three clades of *Salvia* are associated with the formation of a lever mechanism. Functional analyses of the lever mechanism evolved in the various lineages of *Salvia*, currently being addressed by Claßen-Bockhoff et. al. (2004a), Wester and Claßen-Bockhoff (2005), Thimm *et al.* (2005), and Reith and Speck (2005), will shed light on the similarity of the functional aspects of the progression in staminal evolution seen in *Salvia*. These functional analyses, in concert with the phylogenetic data, we hope will ultimately afford the opportunity to address the suggestion of Claßen-Bockhoff *et al.* (2004b) that the lever mechanism is a key innovation driving species radiations within the genus *Salvia* (sensu Hodges and Arnold, 1995; Hodges, 1997; Barraclough et al., 1998; Pellmyr and Krenn, 2002).
ACKNOWLEDGEMENTS

The authors thank Mike Powell, Bart O’Brien, Janet Latham, Petra Wester, Regina Claßen-Bockhoff, Ian Hedge, and Richard Walker for help in obtaining plant material; Wisconsin State Herbarium, Royal Botanical Garden-Edinburgh, Field Museum Herbarium, and Missouri Botanical Gardens for access to herbarium material; Kandis Elliot for help with the stamen sketches; Petra Wester for insight into staminal morphology, critical discussions, and company in the field; and Jocelyn Hall and Naomi Delventhal for help with laboratory work. We gratefully acknowledge the support of Davis Grant funds, California Native Plant Society, National Science Foundation Dissertation Improvement Grant, and Botanical Society of America Karling Award for funds essential to this project.

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**Figure 1.** Flower and pollination of *Salvia pratensis* (*Salvia clade I*). A flower prior to the activation of the lever mechanism (A). The pollinator enters the flower and activates the lever mechanism (B), depositing pollen on the back of the pollinator. As the pollinator enters an older flower (stamens removed from sketch, but present in flower), pollen is transferred to the stigma (C). (Adapted from Claßen-Bockhoff et al., 2003).

**Figure 2.** The generalized trend in stamen morphology seen within tribe Mentheae leading to that seen in *Salvia*. Grey areas represent connective tissue. Step 2 (the functional loss of two of the four stamens) has apparently happened only once in the *Salvia* clade. The progression from step 2 to step 5 has happened on at least three independent occasions in the *Salvia* clade. Anterior thecae are on the top of each sketch, and the posterior thecae, which become entirely aborted and fused in step 5, are on the bottom of each sketch.

**Figure 3.** The “Mentheae-wide” analysis. A three region DNA combined parsimony analysis of the chloroplast regions *trnL-F, psbA-trnH*, and the nuclear rDNA ITS. Strict consensus of 2094 equally parsimonious trees of length 1737 steps. Bootstrap values above 50% are shown above the branches.

**Figure 4.** The “*Salvia clade*” analysis. A two region DNA combined parsimony analysis of the chloroplast region *trnL-F* and the nuclear rDNA ITS. Strict consensus of over 100,000 equally parsimonious trees of 1489 steps. Bootstrap values above 50% are shown above the branches. Stamen types corresponding to those in figure 5 are shown.

**Figure 5.** A summary of the cladogram shown in Fig. 4, with representations of the stamen types found in each clade. Grey areas of the sketches represent connective tissue. Grey lines in the cladogram represent branches in which significantly elongate connectives are seen. Dashed lines in the cladogram represent lineages in which a lever mechanism is found. Total abortion of the posterior thecae and total fusion of the posterior thecae occurs only in stamen types B, E, and N.

**Figure 6.** Hypothesis of evolutionary progression in the independent origin of the three different staminal lever mechanisms found in the tribe Mentheae. The three lever mechanisms (*Salvia clade I, clade II and “clade III”*) are homologous in that they are derived from the connective tissue of the stamen (shaded grey in this figure), but have been independently derived and are morphologically distinct from one another.
Salvia clade I
(cf. 250 spp.)

Rosmarinus (2 spp.)
stenmen type C

Perovskia (7 spp.)
stenmen type D

subg. Calosphace
(500 spp.)

Salvia clade II
(cf. 520 spp.)

sect. Audibertia
(20 spp.)

Meriandra (2 sp.)
stenmen type J

Dorystaechas (1 sp.)
stenmen type K

Zhumeria (1 sp.)

(cf. 15 spp)

Salvia “clade III”
(cf. 90 spp)

2 functional stamens only

stamen type O

significantly elongate connective tissue

lever mechanism

Fig. 5, p. 42
Salvia clade I (cf. 250 spp.)

Salvia clade II (cf. 520 spp.)

Salvia "clade III" (cf. 100 spp.)

Subg. Calosphace (500 spp.)

Sect. Audibertia (20 spp.)