

SALVIA (LAMIACEAE) IS NOT MONOPHYLETIC: IMPLICATIONS FOR THE SYSTEMATICS, RADIATION, AND ECOLOGICAL SPECIALIZATIONS OF SALVIA AND TRIBE MENTHEAE¹

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Salvia, with over 900 species from both the Old and New World, is the largest genus in the Lamiaceae. Unlike most members of the subfamily Nepetoideae to which it belongs, only two stamens are expressed in *Salvia*. Although the structure of these stamens is remarkably variable across the genus, generally each stamen has an elongate connective and divergent anther thecae, which form a lever mechanism important in pollination. In a preliminary investigation of infrageneric relationships within *Salvia*, the monophyly of the genus and its relationship to other members of the tribe Mentheae were investigated using the chloroplast DNA regions *rbcL* and *trnL-F*. Significant conclusions drawn from the data include: *Salvia* is not monophyletic, *Rosmarinus* and *Perovskia* together are sister to an Old World clade of *Salvia*, the section *Audibertia* is sister to subgenus *Calosphace* or the monotypic Asian genus *Dorystaechas*, and the New World members of section *Heterosphace* are sister to section *Salviastrum*. Owing to the non-monophyly of *Salvia*, relationships at the next clearly monophyletic level, tribe Mentheae, were investigated.

Key words: Lamiaceae; Mentheae; phylogenetics; *rbcL*; *Rosmarinus*; *Salvia*; stamen; *trnL-F*.

The genus *Salvia* (tribe Mentheae, Lamiaceae) represents an enormous and cosmopolitan assemblage of nearly 1000 species displaying a remarkable range of variation. *Salvia* has undergone marked species radiations in three regions of the world: Central and South America (500 spp.), central Asia/Mediterranean (250 spp.), and eastern Asia (90 spp.) (Fig. 1). *Salvia* is separated from other members of tribe Mentheae on the basis of its unusual staminal structure. Whereas most Mentheae have four stamens, *Salvia* expresses only two, and the two thecae on each stamen are separated by an elongate connective (Figs. 2–3). The staminal structure in *Salvia* is often associated with an unusual pollination syndrome in which the pollinator pushes against the posterior anther theca while accessing a nectar reward at the base of the corolla tube, causing the anterior theca to deposit pollen on the pollinator via a lever-like mechanism (Fig. 2) (Sprengel, 1793; Himmelbaur and Stibal, 1933–1935; Claßen-Bockhoff et al., 2003). The assumption among previous researchers has been that this peculiar pollination mechanism has only evolved once within the Mentheae, and thus, *Salvia* is monophyletic.

Although the genus as a whole has long been presumed to be monophyletic based on the unusual structure of its stamens (Fig. 2), the tremendous diversity of staminal structure (Fig. 3), floral morphology, and vegetative habit found across the species of *Salvia* has led to considerable confusion regarding infrageneric boundaries. Bentham's (1848) study of *Salvia* sep-

arated the genus into 12 sections and remains the most widely accepted treatment of *Salvia*. No comprehensive treatment of the genus has been completed since then, despite the recognition of over 500 new species of *Salvia*. Many researchers have modified Bentham's (1876) ultimate subgeneric arrangement (Briquet, 1897; Stibal, 1934, 1935; Pobedimova, 1954; Hruby, 1962; El-Gazzar and Watson, 1968), but no easily employable, clearly defined subgeneric arrangement exists (Hedge, 1974). Many recent researchers have avoided the troublesome issue of Bentham's subgeneric groupings by describing "species-groups" or small (often monotypic) sections (Epling, 1939; Hedge, 1974, 1982a, b).

Thus *Salvia* raises a number of important systematic and evolutionary questions. Is *Salvia* monophyletic? How are other genera of Mentheae related to *Salvia*? How many major lineages are contained within *Salvia*? What are the large-scale relationships of New World *Salvia* lineages? Where did the major lineages originate? We address these questions with a molecular systematic investigation of all major groups within *Salvia* and allied genera of Mentheae.

MATERIALS AND METHODS

Taxa sampling—Nomenclature for this project follows that suggested by Alziar (1988–1993). Earlier work investigating *rbcL* in the Lamiaceae (Kaufmann and Wink, 1994; Wink and Kaufmann, 1996) resulted in *rbcL* sequences being obtained for over 80 species of Nepetoideae. This project expands on that sampling and presents 51 new *trnL-F* sequences and a total of 127 *rbcL* sequences. Accessions, vouchers, and GenBank numbers are available in the Appendix (see Supplemental Data accompanying the online version of this article). The data matrix for the *rbcL* analysis consists of 127 taxa, and the combined analysis of *rbcL* and *trnL-F* consists of 55 taxa. Attempts were made to use the same DNA for the *rbcL* and *trnL-F* sequencing; however, this was not possible for 19 of the taxa sampled. In four cases in the combined analysis, the *rbcL* sequence was obtained from a different species than the *trnL-F* sequence (*Thymus serpyllum* [*trnL-F*]/*T. vulgaris* [*rbcL*], *Agastache urticifolia* [*trnL-F*]/*A. foeniculum* [*rbcL*], *Marrubium supinum* [*trnL-F*]/*M. in-*

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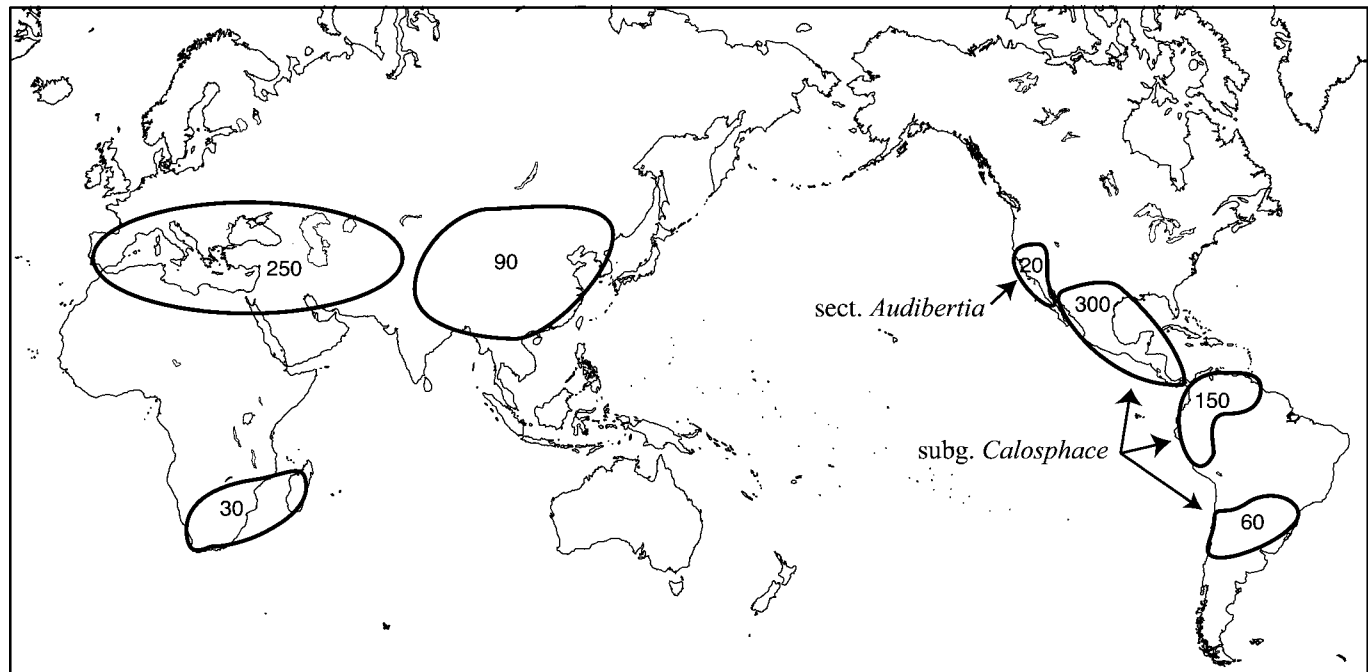


Fig. 1. Centers of diversity for the genus *Salvia*. Approximate numbers of species in each region are listed inside each area.

canum [*rbcL*], *Mentha spicata* [*trnL-F*]/*M. longifolia* [*rbcL*]). Although combining data from different members of a genus is not ideal, the placement of the four genera relative their sister groups did not change in single region analyses. This, in concert with the fact that our goal was not defining the placement of these genera but defining the lineages of *Salvia*, we feel justifies our decision to combine these sequences. The sampling of taxa is concentrated in the subfamily Nepetoideae (112 species sampled, representing 22 genera), particularly tribe Mentheae (105 species sampled, representing 18 genera) and the genus *Salvia* (70 species sampled). The extensive sampling within *Salvia*

was designed to sample across the morphological diversity of the genus. *Verbena officinalis* of the Verbenaceae was chosen as an outgroup.

Extractions, amplification, and sequencing—Total genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Valencia, California, USA). Extractions were made from fresh leaves, leaves dried in silica gel, leaves from herbarium material, and frozen leaves. Polymerase chain reaction (PCR) amplification and cycle sequencing followed the methods described elsewhere (Conti et al., 1996; Givnish et al., 2000). The PCR product was purified either with a QIAquick PCR purification kit (Qiagen) or with an 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 v. 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 55-taxon data set (36 species of *Salvia*) using *rbcL* and *trnL-F* sequences. The combined data set was analyzed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses. 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 *rbcL* and *trnL-F* 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). Topological constraints based on previous systematic hypotheses were used in full heuristic searches with 100 random addition replicates to evaluate the number of extra steps required to force taxa together. Bootstrap (Felsenstein, 1985) and Bremer support values (Bremer, 1988; Don-

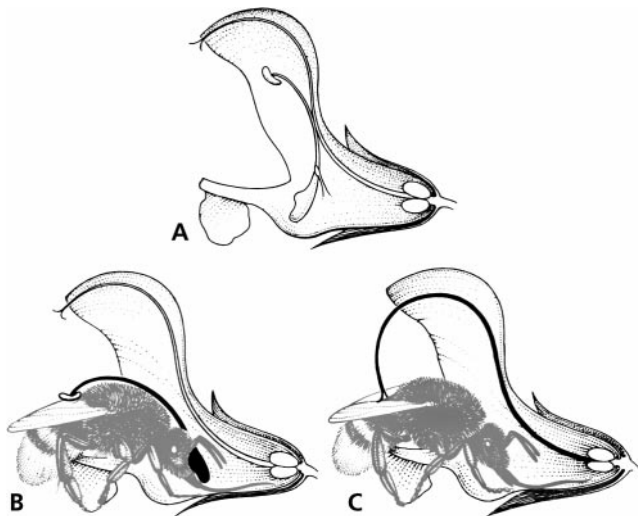


Fig. 2. Flower and pollination of *Salvia pratensis* (*Salvia* clade I). A flower without the lever mechanism activated (A). As the pollinator enters the flower (B), the pollen is deposited on the back of the pollinator. As the pollinator enters an older flower (stamens removed from sketch, but remain present in flower) pollen is transferred (C). The posterior anther thecae forming the lever can be fused or free and in the subg. *Leonia*, produce fertile pollen (from Claßen-Bockhoff et al., 2003).

Bentham's (1876) *Salvia*

(did not include *Audibertia*, *Salviastrum*)

Subgenus *Salvia*

- Old World
 - corolla with annulus
 - 2 posterior anther thecae sterile, rudimentary, connivent
- Hymenospace*, *Euspace*, *Drynospace*

Subgenus *Sclarea*

- Old World
 - corolla without annulus
 - 2 posterior anther thecae sterile, forming glutinatorium
- Horminum*, *Aethiopsis*, *Plethiospace*

Subgenus *Calospace*

- New World
 - corolla without annulus
 - 2 posterior anther thecae sterile, connate, forming gubernaculum
- Calospace*

Subgenus *Leonia*

- Old and New World
 - corolla with annulus
 - 2 posterior anther thecae fertile, separate
- Echinospace*, *Pycnospace*, *Heterospace*, *Notiospace*, *Hemisphere*

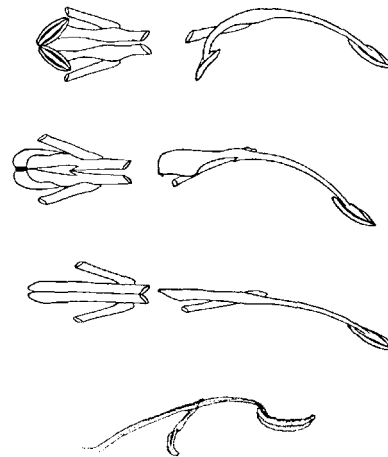


Fig. 3. Bentham's organization of *Salvia*. The staminal morphology shown for Bentham's subg. *Salvia* corresponds to that seen in *Salvia* clade III of this project. Stamen types shown for subg. *Sclarea* and subg. *Leonia* correspond to that found in *Salvia* clade I in the molecular analysis.

oghue et al., 1992) were used to evaluate support for relationships within the resulting trees. Bootstrap values were obtained through an 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. Bremer support values were obtained using reverse topological constraints, with 100 random addition replicates for each search, as suggested by Swofford (1993), and implemented by Baum et al. (1994). Maximum likelihood analyses were conducted on the individual and combined data sets 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. Bayesian analyses in MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001) were done with 1 000 000 cycles, running four Markov chain Monte Carlo (mcmc) chains, a temperature of 0.2 with branch lengths saved, all substitution rates equal, and no among-site variation. Four separate Bayesian chains were conducted starting with a different random seed each time.

The second approach involved an expanded *rbcL* data set with 127 taxa (71 species of *Salvia*) using only MP. Because a full heuristic MP search was not feasible with the expanded *rbcL* data, an iterative approach was used to develop a strict consensus tree following methods of Catalán et al. (1997). An initial heuristic search was performed in the same manner as in the 55-taxa analysis, with each replicate limited to 1 min and the number of saved trees limited to 2000. A strict consensus tree was created from this analysis and loaded as a constraint in a second heuristic search and performed in the same way as the original (with no limit placed on number of trees saved), searching for equally or more parsimonious trees not compatible with the constraint. If trees of equal or lesser length than the previous maximum parsimony trees were found, a new strict consensus tree was created from those trees and the previous consensus tree and was then loaded as a constraint in the next iteration. This process was continued until no equally parsimonious trees incompatible with the previous strict consensus tree were found in 1000 random addition sequences, each limited to 2 min.

RESULTS

Analysis of *rbcL* in 55-taxon data set—The aligned length of the *rbcL* data set, including some 3' spacer sequence, was 1500 base pairs. Because of differences in length of sequences

obtained between taxa and ambiguous sequence data, the first 26 base pairs were excluded from the analysis, as were the last 103 base pairs. Of the remaining 1371 characters in the 55 taxa included in the combined analysis, 1179 were constant, 192 characters were variable, and 98 characters (7.1%) were potentially parsimony-informative. Fitch parsimony analysis of the *rbcL* region of the 55 taxa included in the combined analysis found 1481 equally parsimonious trees of 350 steps (consistency index [CI] = 0.59, retention index [RI] = 0.78, rescaled consistency index [RC] = 0.46).

Analysis of *trnL-trnF* in the 55-taxon data set—The aligned length of the *trnL-F* data set was 1271 base pairs. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 914 base pairs. Of the 914 characters in the 55 taxa included in the combined analysis, 689 were constant, 109 variable characters were parsimony-uninformative, and 116 characters (12.7%) were potentially parsimony-informative. Fitch parsimony analysis of the *trnL-F* region of the 55 taxa included in the combined analysis found 1728 equally parsimonious trees of 319 steps (CI = 0.83, RI = 0.92, RC = 0.77). Twenty-two indel events were scored for the *trnL-F* data set, of which 15 were potentially parsimony-informative. In the combined analysis, indel events were not included, but are noted on Fig. 4. Analyses were also performed with the indel events included (Fig. 5). The analysis of *trnL-F* with indels included contained 936 characters of which 642 were constant, 147 variable characters were parsimony-uninformative, and 147 characters were potentially parsimony-informative. Fitch parsimony analysis of the *trnL-F* region of the 55 taxa found 180 equally parsimonious trees of 352 steps (CI = 0.83, RI = 0.92, RC = 0.76).

In the *trnL-F* analysis of 55 taxa, constraining *Salvia* to be monophyletic resulted in a shortest tree 12 steps longer than unconstrained trees. Constraining *Salvia* + *Dorystaechas* as

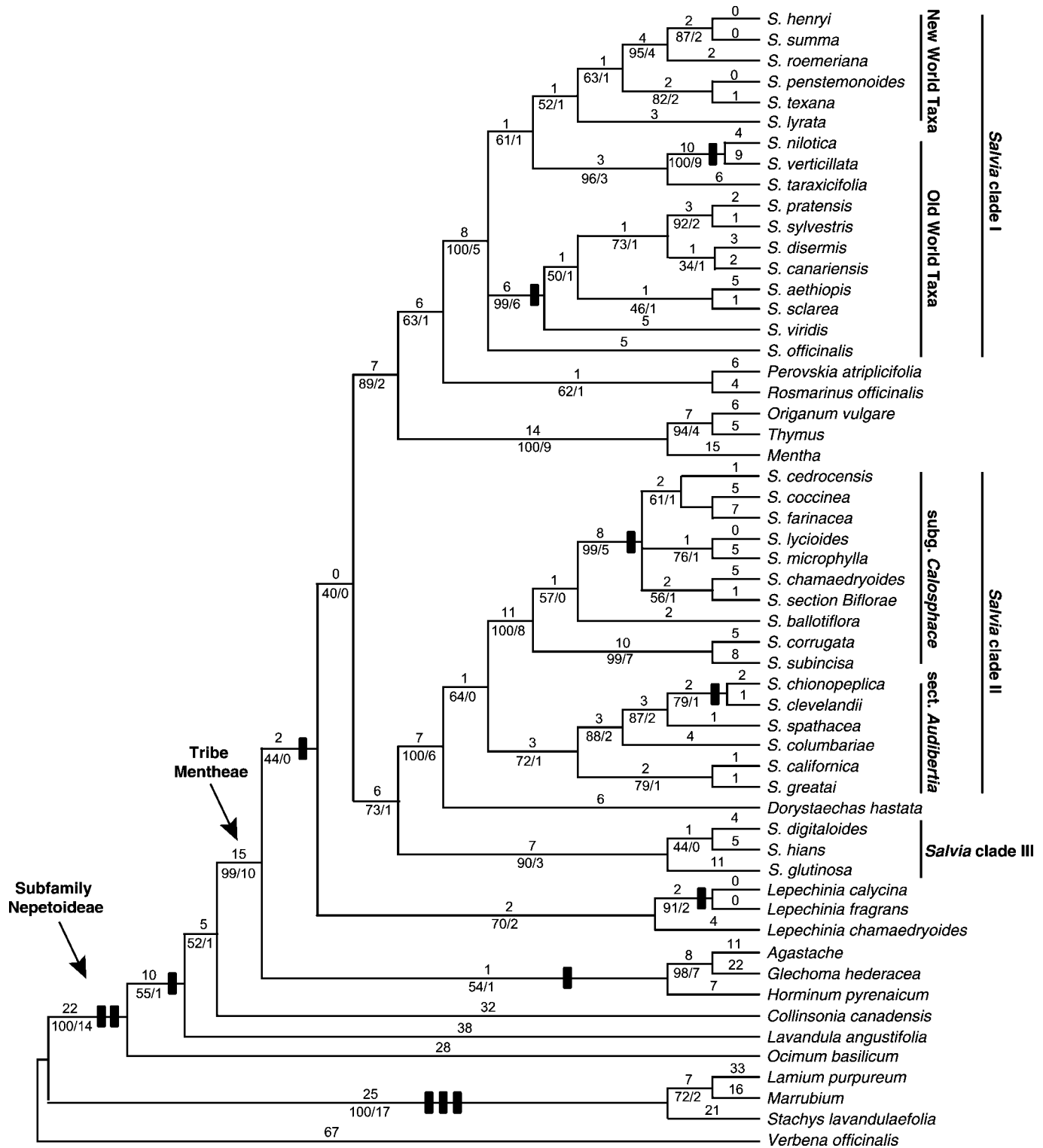


Fig. 4. One of 180 most parsimonious trees of length 686 from combined analysis of *rbcL* and *trnL-F* data set of 54 species of Lamiaceae and one Verbenaceae (outgroup). Branch lengths are given above each line and bootstrap values/decay indices below. Thick lines represent indel events with a CI = 1.0 (not included in the analysis). *Salvia* clade I contains members included in Bentham's (1876) subg. *Salvia*, *Leonia*, and *Sclarea*. *Salvia* clade II corresponds to subg. *Audibertia* and subg. *Calosphace*. *Salvia* clade III consists of a group of East Asian species loosely corresponding to Bentham's sect. *Drymosphace*.

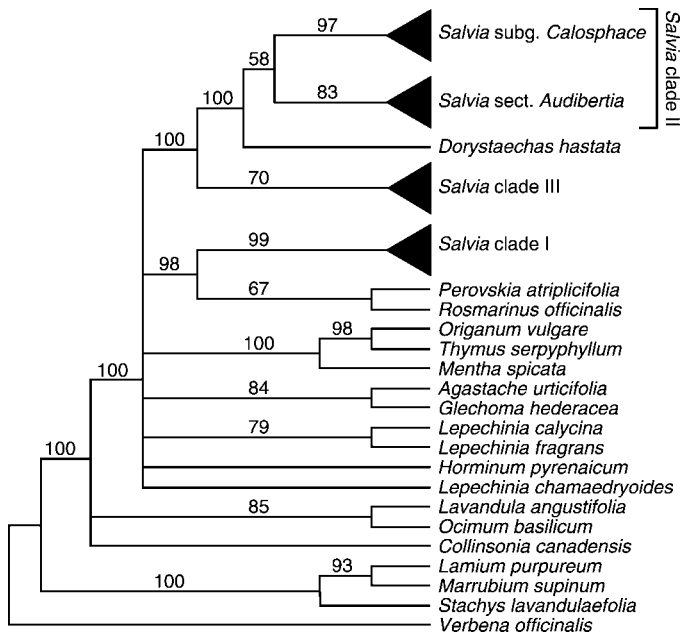


Fig. 5. Strict consensus of most parsimonious trees of length 352 steps based on *trnL-F* sequences of 55 taxa of Lamiaceae and one species of Verbenaceae (outgroup) with indels included in the analysis. Bootstrap values are shown above the branches. Although included in the analysis, members of *Salvia* clade I (17 species), *Salvia* clade II (16 species), and *Salvia* clade III (3 species) are not included in this figure.

monophyletic resulted in a shortest tree four steps longer. Templeton tests rejected congruence of trees produced constraining all *Salvia* together, but did not reject congruence of trees produced from the other constraints mentioned above ($P > 0.05$).

Combined *rbcL* and *trnL-trnF* analysis in the 55-taxon data set—The aligned length of the combined data set was 2771 base pairs. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 2285. Of those 2285 characters, 1868 were constant, 417 were variable, and 214 (9.4%) were potentially parsimony-informative. Fitch parsimony analysis of the combined *trnL-F* and *rbcL* data set of the 55 taxa included in the combined analysis found 180 equally parsimonious trees of 686 steps (CI = 0.69, RI = 0.84, RC = 0.58). Constraining *Salvia* to be monophyletic resulted in a shortest tree 19 steps longer than unconstrained trees. Constraining *Salvia* + *Dorystaechas* as monophyletic resulted in a shortest tree seven steps longer. Constraining *Salvia* + *Dorystaechas* + *Rosmarinus* + *Perovskia* to be monophyletic resulted in a tree two steps longer. Templeton tests rejected the trees produced from the search constraining all *Salvia* together, but did not reject trees produced from the other constraints ($P > 0.05$).

The topology of trees produced with indels included did not differ from trees produced from sequence data alone. The tree shown (Fig. 4) was produced without indels included. Indel events with a CI = 1.0 were mapped onto the combined phylogeny (Fig. 4).

The partition homogeneity test of the two data sets suggests significant incongruity between the *rbcL* and *trnL-F* partitions compared to random partitions of the same size ($P < 0.01$). The most significant source of incongruence between the two data sets is the placement of a *Mentha/Origanum/Thymus*

clade (hereafter referred to as the *Mentha* clade). The different positions of the *Mentha* clade are well-supported by each data set, respectively (*trnL-F* bootstrap = 100%; *rbcL* bootstrap = 75%). In the *trnL-F* analysis, this clade is placed in an unresolved polytomy including *Lepechinia*, *Horminum*, *Rosmarinus*, *Perovskia*, *Salvia*, *Dorystaechas*, *Agastache*, and *Glechoma* (Fig. 5). In the 55-taxon data set, the *rbcL* analysis places the *Mentha* clade sister to *Salvia* clade I. However, in the *rbcL* analysis *Mentha* itself has a relatively long branch length (10 steps under ACCTRAN), and long branch attraction may well be responsible for the peculiar and incongruent placement of the *Mentha* clade in the *rbcL* analysis. Despite data set incongruence, well-supported clades are generally supported by both regions (Fig. 4): *Salvia* clade I (bootstrap = 100%), *Dorystaechas* + *Salvia* clade II (bootstrap = 100%), *Salvia* subg. *Calosphace* (bootstrap = 100%), *Salvia* clade III (bootstrap = 90%), tribe Menthae (bootstrap = 99%), and subfamily Nepetoideae (bootstrap = 100%). Each of the above clades are also maintained in the expanded *rbcL* analysis (Fig. 6).

Maximum likelihood produced a single tree with a log likelihood score -7606.4747853 . The ML analyses were performed under the GTR + G model of evolution: rate substitution matrix, AC = 1.039275, AG = 2.472209, AT = 0.243713, CG = 1.25665, CT = 2.300892, GT = 1.00000; nucleotide frequencies, A = 0.28056, C = 0.18434, G = 0.21961, T = 0.32549; gamma shape parameter = 0.0344716 with two rate categories. All clades pertinent to this paper mentioned above were present in the ML tree. Bayesian analysis performed on the combined data set produced a posterior probability 50% tree compatible with the strict consensus tree from the maximum parsimony analysis. The log likelihood of the best state for the “cold” chain was -7934.21 . Posterior probabilities of the clades pertinent to this paper mentioned earlier were all 100% in each of the four Bayesian analyses. The Bayesian and ML trees were essentially identical to the maximum parsimony consensus tree and are therefore not shown.

Analysis of *rbcL* in 127-taxon data set—In the expanded *rbcL* analysis including 127 taxa, 1063 characters were constant, 308 were variable, and 185 (13.5%) were potentially parsimony-informative. Fitch parsimony analysis of the *rbcL* region for the 127 taxa included in the expanded *rbcL* analysis found trees of 705 steps (CI = 0.50, RI = 0.83, RC = 0.41). Although, as expected with *rbcL*, resolution within the *Salvia* clades is quite limited, the integrity of the major lineages (*Salvia* clades I, II, III) suggested by the combined analysis and by *trnL-F* alone are maintained in this expanded sampling (Fig. 6). Constraining *Salvia* to be monophyletic resulted in a shortest tree five steps longer than the most parsimonious unconstrained tree. Constraining *Salvia* + *Dorystaechas* as monophyletic resulted in a most parsimonious tree three steps longer. Constraining *Salvia* + *Dorystaechas* + *Rosmarinus* + *Perovskia* to be monophyletic resulted in a tree four steps longer. Templeton tests failed to significantly reject the constraint trees ($P > 0.05$).

DISCUSSION

The molecular results presented here highlight a number of important systematic and evolutionary issues pertaining to *Salvia* and related mints. First, *Salvia* is not monophyletic but comprises at least two and possibly three distinct lineages each



Fig. 6. Strict consensus of most parsimonious trees of length 705 steps based on *rbcL* sequences of 127 species of Lamiaceae and one species of Verbenaceae (outgroup).

related to other genera of the tribe Mentheae: *Salvia* clade I—largely Old World but with one New World lineage; *Salvia* clade II—New World lineage comprising subg. *Calosphace* and sect. *Audibertia*; and *Salvia* clade III—an independent Asian lineage. Second, the unique two-staminal lever mechanism for pollination in *Salvia* has evolved at least two times independently (or evolved once and was lost at least twice). Third, much of the diversification of *Salvia* fits along bioge-

graphical lines. And fourth, the taxonomic implications of these results are significant.

Relationships within Mentheae—In view of the polyphyletic nature of *Salvia* as evidenced by these molecular results, understanding the evolution of *Salvia* requires a broader perspective within the family. Although considerable molecular phylogenetic work has examined the relationships within Lam-

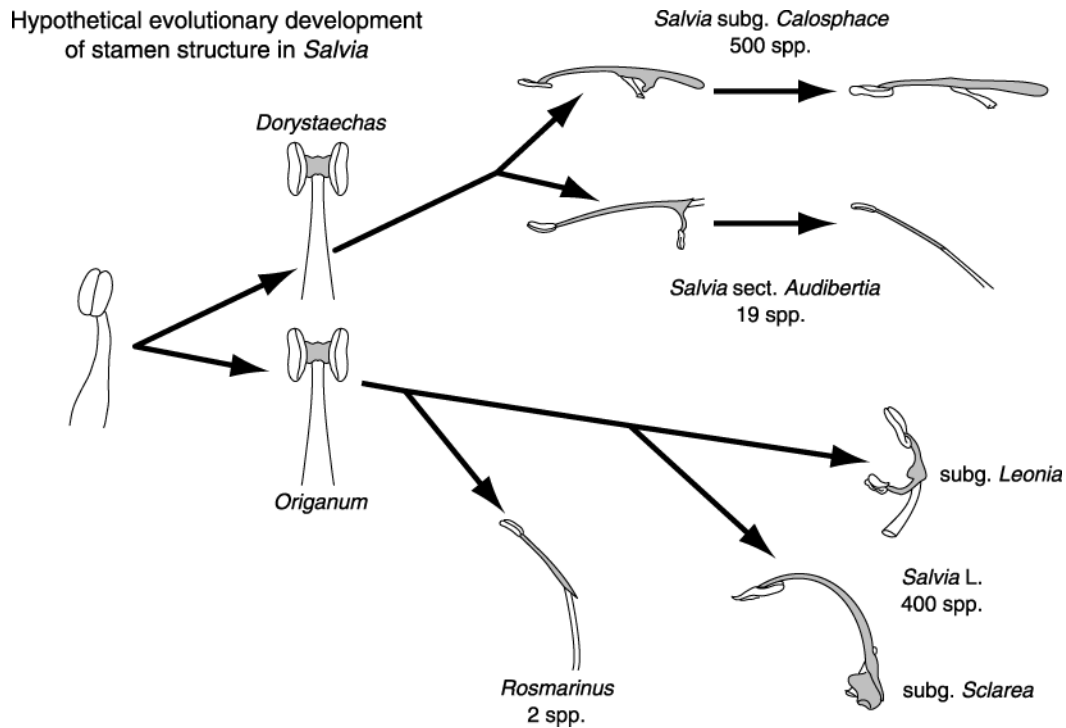


Fig. 7. Hypothetical stamen development in the genus *Salvia*. Results of this project suggest at least two independent origins of an elongate connective (shown in gray) and of the lever mechanism in the tribe Menthaeae. Each origin of the lever mechanism has resulted in large species radiations, while the sister groups not employing the lever mechanism (represented in this sketch by *Rosmarinus* and *Salvia* sect. *Audibertia*) have remained relatively species poor.

iales and the placement of Lamiaceae (e.g., Olmstead et al., 1992, 1993, 2000, 2001; Wagstaff and Olmstead, 1997) and relationships within some mint genera (*Clerodendrum*, Steane et al., 1997, 1999; *Monarda*, Prather et al., 2002; *Satureja*, Cantino and Wagstaff, 1998; *Sideritis*, Barber et al., 2002; *Teucrium*, El Oualidi et al., 1999), comparatively little has been done to examine relationships among genera in the mint family (Cantino and Sanders, 1986; Wagstaff et al., 1995, 1998; Wink and Kaufmann, 1996). *Salvia* and 122 other genera form the subfamily Nepetoideae, the best supported of the eight subfamilies of the Lamiaceae (Wagstaff et al., 1998). The Nepetoideae is defined by hexacolpate pollen, an investing embryo, myxospermy, presence of rosmarinic acid, cpDNA restriction sites, and DNA sequence data (Cole, 1992; Wagstaff, 1992; Fig. 4).

The treatments of relationships within the subfamily Nepetoideae have varied considerably between researchers, ranging from 18 tribes (Wunderlich, 1967) to four (Cantino et al., 1992; Wagstaff et al., 1995). Based on cpDNA restriction site analyses, Wagstaff et al. (1995) recognized four monophyletic lineages within the Nepetoideae: tribe Lavanduloideae (consisting of *Lavandula*), tribe Ocimeae (defined by declinate stamens and consisting of 52 genera), tribe Elsholtzieae (defined by spreading stamens and consisting of six genera), and the tribe Menthaeae, consisting of *Salvia* and 72 other genera. A monophyletic Menthaeae, as suggested by Wagstaff et al. (1995), is supported by our analyses with *trnL-F* and *rbcL*.

In his treatment of Nepetoideae for *Genera Plantarum*, Bentham (1876) placed the genus *Salvia* within his tribe Monardeae, defined by two ascending stamens. He recognized three ad hoc groups within Monardeae based on staminal structure: Meriandreae (*Perovskia*, *Dorystaechas*, and *Meriandra*), Sal-

viae (*Salvia*, *Salviastrum* [now included within *Salvia*], *Audibertia* [now included within *Salvia*], and *Rosmarinus*), and Monardeae (*Monarda*, *Blephilia*, and *Ziziphora*). The two thecae of each stamen of Meriandreae are parallel, those of Monardeae are divergent, and those of Salviae are separated by an elongate connective. It should be noted that the unsampled *Zhumeria* has been suggested as a fourth genus in Meriandreae based on its staminal structure, pubescence, and corolla (Bokhari and Hedge, 1976). Recent molecular investigations (Wagstaff et al., 1995; Prather et al., 2002) suggest that Bentham's subtribe Monardeae is not closely related to the subtribes Meriandreae and Salviae. However, the relationships of the genera in the Meriandreae and Salviae have been a source of debate in the literature. Although numerical analyses support an expanded Meriandreae to include *Rosmarinus* and *Salvia* (El Gazzar and Watson, 1970), anatomical and morphological investigations (Bokhari and Hedge, 1971) call into question the monophyly of an expanded Meriandreae (sensu Bentham, 1876). Because of the conflict surrounding tribal relationships within the Nepetoideae and the non-monophyly of *Salvia* suggested by our molecular data (Figs. 4–6), any attempt to address the phylogenetics of and evolution within *Salvia* must be done at the next higher but clearly monophyletic level—the tribe Menthaeae (sensu Cantino et al., 1992). An important aspect will be a thorough reevaluation of subtribal relationships within the Menthaeae (sensu Cantino et al., 1992) in light of a well-supported molecular phylogeny.

The nature of *rbcL* makes it a suboptimal choice at the subgeneric and of limited use at the subfamilial level in the Lamiaceae. The slowly evolving nature of the region, in concert with the large sample size, imparts little chance of high support being obtained for clades. However, owing to the large

number of sequences previously obtained for the *rbcl* in the Lamiaceae, we felt compelled to complete the sampling to represent all putative lineages of *Salvia* to investigate whether, even with a suboptimal and slowly evolving region, multiple lineages of *Salvia* were supported. The *rbcl* consensus tree (Fig. 6) supports the existence of the same three clades of *Salvia* identified by *trnL-F* (Fig. 5).

Staminal structure in *Salvia*—Central to any investigation into the phylogenetics, species radiation, or pollination biology of *Salvia* is the structure, ontogeny, and function of its peculiar stamens. Staminal morphology is the major defining character of *Salvia*, as well as being integral to the current subgeneric organization of the genus. Our preliminary research investigating the chloroplast regions *rbcl* and *trnL-trnF* demonstrates a polyphyletic *Salvia* and thus multiple origins of this staminal structure (Fig. 7).

Our results support the hypothesis that the evolution of the stamen connective and unique lever mechanism has occurred in parallel or convergent fashion (Figs. 4, 7). At least two species radiations in *Salvia* are correlated to the acquisition of this trait and suggest that the origin of the lever mechanism may be responsible for major species radiations (Fig. 7). Section *Audibertia* (19 species) in the New World, although expressing an elongate connective, does not employ a lever mechanism in pollination. Subgenus *Calosphace*, likely sister to sect. *Audibertia* (Figs. 4, 5), does employ this lever mechanism and comprises nearly 500 species. This trend is also seen in the Old World with *Salvia* clade I. *Salvia* clade I has the lever mechanism and has radiated to include over 200 species, whereas its sister group *Rosmarinus* and *Perovskia* does not employ the lever mechanism and includes fewer than 10 species.

Although the elongation of the connective into a lever mechanism may seem an exceedingly unlikely event to have occurred independently at least twice and probably three times in the Menthaeae, initial morphological investigations suggest the lever mechanism found in *Salvia* clade I is in fact not homologous to the lever mechanism found in *Salvia* clade II (Fig. 7). In all of *Salvia* clade I, the aborted (or expressed) posterior theca is at the distal end of the connective. In *Salvia* clade II (particularly subg. *Calosphace*), it seems likely that the aborted posterior theca is expressed as a small projection next to the attachment with the filament. The actual lever in *Salvia* clade II would then consist of an extension of tissue on top of the connective, rather than an extension of the connective that is seen in *Salvia* clade I. Issues of pollination biology and biomechanics of the *Salvia* flower are currently being investigated in the laboratory of Regine Claßen-Bockhoff (Claßen-Bockhoff et al., 2003), and future integration of this work with our phylogenetic results will be instrumental in understanding the ecological and functional aspects of staminal morphology in *Salvia*.

Phylogenetics of *Salvia* clade I—*Salvia* clade I appears to include all members of Bentham's subg. *Sclarea* and *Leonia* and those members of subg. *Salvia* not included in *Salvia* clade III. The eight species of New World *Salvia* belonging to *Salvia* clade I include four species of sect. *Heterosphace* (Walker and Elisens, 2001), three species in subg. *Salviastrum*, and *Salvia penstemonoides* Kunth et Bouche. The three species of subg. *Salviastrum*, all native to Texas and northern Mexico, were originally placed by Scheele (1849) in a separate genus.

Although the three species of *Salviastrum* express the elongate connective diagnostic of *Salvia*, Scheele placed them in their own genus based on a dense annulus in the calyx. Torrey (1859) soon thereafter noted the close affinities of *Salviastrum* and *Salvia* sect. *Heterosphace* and included *Salviastrum* as a section within *Salvia*. Bentham (1876), however, agreed with Scheele and maintained *Salviastrum* as its own genus. The most recent treatment of *Salviastrum* (Whitehouse, 1949, p. 153) agrees with Torrey's treatment and suggests, "except for the dense ring of hairs in the calyx throat . . . there are no common differences which will separate them from other species of *Salvia*. If the species included in Bentham's section *Heterosphace* are included in *Salvia*, then undoubtedly the *Salviastrum* section should be included, for they are closely linked by their similar calices which are alike in form and accrescence." The molecular data, identifying a monophyletic lineage consisting of New World *Heterosphace* + *Salviastrum*, support the suggestions of Whitehouse.

Salvia penstemonoides is a narrowly endemic species native to south-central Texas, thought to be extinct until its rediscovery in 1987 (Clebsch, 1997). Originally described as belonging to Bentham's sect. *Eusphace* (Kunth and Bouche, 1848), its *rbcl* sequence is exactly the same as *S. texana* (but differs at one site with *trnL-trnF*) and indicates its affinities with sect. *Salviastrum*. Its calyx (although lacking an annulus) and leaf morphology are more similar to *Salviastrum* than any other New World *Salvia*. Mitotic chromosome counts by the senior author (data not shown) totaled $2n = 28$ for *S. penstemonoides*. The only other New World species with known counts of $n = 14$ or $2n = 28$ in *Salvia* are the New World *Heterosphace* (Walker and Elisens, 2001). If *Salviastrum* is maintained as its own section (albeit a paraphyletic taxon), *S. penstemonoides* should be included within it.

***Salvia* clade II**—*Salvia* clade II (Figs. 4–6) is exclusively New World, is composed of the subg. *Calosphace* and the sect. *Audibertia*, and is likely sister to *Dorystaechas*. *Dorystaechas* is a monotypic genus restricted to southwest Anatolia placed by Bentham (1876) in his "subtribe" Meriandreae along with *Perovskia* and *Meriandra* based on its two expressed stamens and parallel anther thecae. *Dorystaechas* is closely enough related to *Salvia* clade II that the molecular data cannot rule out its inclusion in clade II. Bentham suggested that *Dorystaechas* was most closely allied to the Indian and Himalayan genus *Meriandra*. Bokhari and Hedge (1976) suggested the recently described Asian genus *Zhumeria* as a fourth genus assignable to the Meriandreae based on its staminal structure, pubescence, and corolla. The anthers of *Dorystaechas* and *Meriandra* are separated by slightly elongated connective (Bokhari and Hedge, 1971). The connective in *Perovskia* is somewhat swollen, but not elongated. Following an analysis of the tribe Meriandreae based on anatomical and morphological characters, Bokhari and Hedge (1971) concluded that the genera *Perovskia*, *Meriandra*, and *Dorystaechas* were in fact not likely to be closely related. Although we have yet to sample *Meriandra*, our molecular analysis supports this assertion as it relates to the genera *Perovskia* and *Dorystaechas*. Molecular evidence suggests *Dorystaechas* is likely sister to *Salvia* clade II, and *Perovskia*, together with *Rosmarinus*, sister to *Salvia* clade I (Fig. 4). The placement of *Dorystaechas*, *Meriandra*, and the members of *Salvia* clade III are currently being further investigated and will no doubt be central to fully understanding phylogenetics within the Menthaeae, evolution of staminal

structure within the Mentheae, and the origins of New World species of *Salvia*.

The New World section *Audibertia* and subg. *Calosphace* appear to be monophyletic sister groups in the combined analysis. *Salvia* sect. *Audibertia* is restricted to the California Floristic Province and adjacent deserts. The section contains 19 species, four of which (sect. *Echinosphace* Benth.) are distinct enough to have been separated into a section separate from sect. *Audibertia* by various authors (Bentham, 1832; Neissess, 1983). Section *Echinosphace* is characterized as having expressed and fertile posterior anther thecae, arachnoid pubescence, and a base chromosome number of 16. Section *Audibertia* is separated from sect. *Echinosphace* by phytochemical characters, by sterile or entirely aborted posterior anther thecae, and a base chromosome number of 15 (Neissess, 1983). Our results suggest Neissess' sect. *Echinosphace* (in our data represented by *S. greatai* and *S. californica*) is sister to all other members of sect. *Audibertia* sensu lato. This relationship is consistent with previous work suggesting the isolated taxonomic nature of the latter two taxa (Epling, 1938; Emboden, 1971; Neissess, 1983).

Subgenus *Calosphace* is a large assemblage of nearly 500 species separated from sections *Audibertia* and *Echinosphace* on the basis of sterile, fused posterior anther thecae forming a lever mechanism important in pollination (Fig. 3). Whereas Bentham (1876) included *Calosphace* as part of *Salvia*, he treated *Audibertia* as a separate genus. Epling, whose revision of *Calosphace* remains the most complete treatment of the subgenus (Epling, 1939), considered *Audibertia* and *Calosphace* as closely related (Epling, 1938). Neissess (1983), who investigated relationships within sect. *Audibertia* based on morphological, chemical, and pollen characters, suggested sect. *Audibertia* was not allied with subg. *Calosphace* and more closely related to *Rosmarinus* and Old World groups of *Salvia* included in Bentham's subg. *Leonia*. Analyses of megagametophyte types (Carlson and Stuart, 1936) and chromosome data (Epling et al., 1962) in *Salvia* also suggested Old-World affinities of sect. *Audibertia*. Our results support a monophyletic subg. *Calosphace* sister to sect. *Audibertia*.

***Salvia* clade III**—One of the more surprising results is the existence of a potential third clade of *Salvia*, clade III (Figs. 4–6). All species of this clade are East Asian (Hedge, 1998) and correspond to Bentham's sect. *Drymosphace*. Section *Drymosphace* is defined by being large, herbaceous, glutinose plants with hastate leaves. The flowers are unique with the upper lip of the calyx short tridentate, a falcate and often compressed corolla, and connivent posterior anther thecae stretched forward. The *rbcL* analysis suggests this clade likely includes additional species outside sect. *Drymosphace* (e.g., *S. barrelieri*, *S. fruticosa* [both Mediterranean], *S. digitaloides* [Asian]) (Fig. 6). Staminal structure of *S. glutinosa* and *S. hians* is unique among the species of *Salvia* so far examined, with two connivent club-shaped posterior thecae producing no pollen and elongate anterior thecae producing pollen (Fig. 3). This unusual staminal structure is also found in *S. koyamae*, a Japanese species (J. B. Walker, personal observation). Future inclusion of more East Asian taxa in a combined analysis may elucidate the circumscription of this clade and help to define the morphological characters defining the clade as well as the broader relationships of *Salvia* clade III to the remainder of *Salvia*.

Biogeographic issues—A number of interesting biogeographical issues are raised with these molecular systematic results. Although the larger biogeographic history of *Salvia* and relatives in the Mentheae will need additional taxon sampling in the tribe for clarification, several finer scale biogeographic issues are clarified. Two of these issues involving New World taxa, the relationship of sect. *Audibertia* and subg. *Calosphace* (*Salvia* clade II) and the relationships within sect. *Heterosphace* (*Salvia* clade I), are briefly addressed here.

The putative sister relationship between sect. *Audibertia* and subg. *Calosphace* has interesting biogeographic implications. Although the California Floristic Province (CFP) is home to innumerable examples of distinct lineages, few groups respect the "boundaries" of the CFP as clearly as this group of *Salvia*. With the exception of adjacent desert regions, the 19 species of sect. *Audibertia* occur only in the CFP. More surprisingly, of the 500 species of subg. *Calosphace* (275 of which occur in Mexico [Ramamoorthy and Lorence, 1987] and 18 in the Sonoran desert [Shreve and Wiggins, 1964]) not one species occurs in California, Oregon, or Washington. Those that occur in Baja California (eight species) are all restricted to central and southern parts of the peninsula (Wiggins, 1980). Why a group that has undergone explosive species radiations in the New World and is represented in nearly every region from southern South America to Canada should not have a single representative in the CFP is a question of considerable interest.

The second biogeographic issue revolves around sect. *Heterosphace* in *Salvia* clade I. Bentham (1848) was the first to recognize the close affinity of a group of southern African species to a southwestern group of North American species of *Salvia* that he placed in sect. *Heterosphace*. Our results further support the close relationship of the New World members of sect. *Heterosphace* (*S. henryi*, *S. summa*, *S. roemeriana*, *S. lyrata*) with Old World species. The fact that the New World members of sect. *Heterosphace* (together with sect. *Salviastrum* and *S. penstemonoides*) form a weak monophyletic group suggests a single dispersal from the Old World to the New, with subsequent diversification to give rise to the four species of sect. *Heterosphace* and three species of sect. *Salviastrum* in the New World. Defining a sister group to this lineage awaits further sampling of African species of *Salvia*.

Taxonomic implications for *Salvia* and relatives in Mentheae—A number of alternative taxonomic approaches are possible in dealing with the non-monophyly of *Salvia*. One approach would involve a nomenclatural change at the generic level of over 500 members of the genus *Salvia*. As the type species for the genus is *Salvia officinalis* L. (Britton and Brown, 1913) belonging to *Salvia* clade I, all but eight of the 500 species of New World *Salvia* would transfer to new genera if *Salvia* were to be split. For example, *Salvia* subg. *Calosphace* would become *Calosphace* (Bentham) Rafinesque and *Salvia* sect. *Audibertia* would become *Audibertia* Bentham. A second approach would be the "sinking" of *Dorystaechas*, *Perovskia*, *Rosmarinus*, and possibly other genera into *Salvia*. A third approach would be to employ phylogenetic nomenclature, retaining current species binomials while naming clades to which they belong. This approach has been advocated for mints and their relatives (Cantino et al., 1997, 1998).

Our research continues to investigate relationships within this important group of plants, and we believe it is premature to present any taxonomic rearrangements before we have completed sampling across the diversity of *Salvia* and related gen-

era. Regardless of which approach is embraced, one would be hard-pressed to find a group of plants containing more species of horticultural, ethnobotanical, and culinary import than the Nepetoideae which includes sage (*Salvia*), savory (*Satureja*), balm (*Melissa*), rosemary (*Rosmarinus*), thyme (*Thymus*), bee balm (*Monarda*), catnip (*Nepeta*), oregano (*Origanum*), basil (*Ocimum*), and mint (*Mentha*). This popularity leads one to hope that as many of these genera are demonstrated to be non-monophyletic (e.g., *Salvia*, *Satureja* [Cantino and Wagstaff, 1998], *Lepechinia* [Hart, 1983]), any modifications to nomenclature at the generic level will take into account the significant broader impact of any changes (Sytsma and Pires, 2001).

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