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Staminal Evolution in the Genus *Salvia* (Lamiaceae): Molecular Phylogenetic Evidence for Multiple Origins of the Staminal Lever

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• *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, the staminal morphology of the various lineages of *Salvia* and related genera is characterized and an evolutionary interpretation of staminal variation within the tribe Mentheae is presented.
• *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. A monophyletic lineage within the Mentheae is characterized consisting of the genera *Lepechinia, Melissa, Salvia, Dorystaechas, Meriandra, Zhumeria, Perovskia* and *Rosmarinus*.
• *Conclusions* Based on these results the following are characterized: (1) the independent origin of the staminal lever mechanism on at least three different occasions in *Salvia*, (2) that *Salvia* is clearly polyphyletic, with five other genera intercalated within it, 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.

INTRODUCTION

The genus *Salvia* (Lamiaceae: tribe Mentheae) represents a cosmopolitan assemblage of nearly 1000 species displaying a remarkable diversity in growth forms, secondary compounds, floral morphology 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.) (Alziar, 1988–1993). All these species display the unusual morphological character that has led to the long-standing assumption that *Salvia* is monophyletic: the significant elongation of the connective tissue of the two expressed anthers (Figs 1 and 2). The demonstration of the non-monophyly of the genus (Walker *et al.*, 2004) has led to a reinvestigation of the defining character of the genus, the elongation of the connective tissue of the stamen, within *Salvia* and closely related genera in the Mentheae. This paper presents a molecular phylogeny of *Salvia* and related genera, characterizes the stamen morphology in the different clades of the genus *Salvia* and closely related genera, and interprets that stamen morphology in a phylogenetic context.

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). *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 allows the formation of the lever mechanism of pollination for which *Salvia* is best known (Fig. 1) (for thorough reviews, see Claßen-Bockhoff *et al.*, 2003, 2004*a*). The significant species radiations that are correlated with the presence of the lever mechanism in *Salvia* (e.g. subgen. *Calosphace* – 500 spp.) suggest it may be the lever mechanism in a selective regime of pollination that is driving evolution in the group (Claßen-Bockhoff *et al.*, 2004*b*). The significance of this lever mechanism to the reproductive biology in *Salvia*, first described by Sprengel (1793), has received considerable attention (Müller, 1873; Zalewska, 1928; Hruby, 1934; Werth, 1956; Baikova, 2002, 2004; Claßen-Bockhoff *et al.*, 2003, 2004*a*; Reith *et al.*, 2006; Wester and Claßen-Bockhoff, 2006). 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. This

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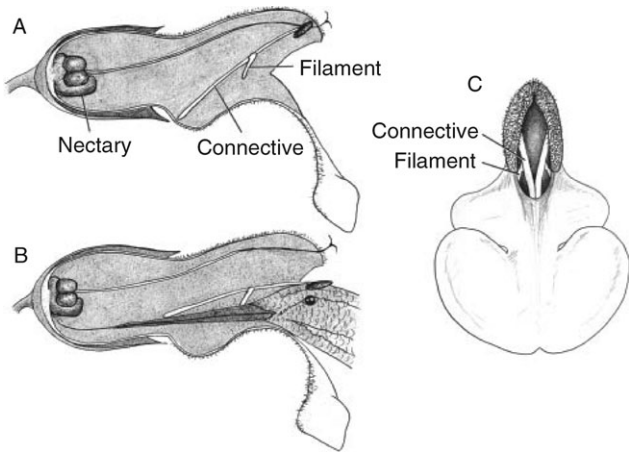

FIG. 1. Stylized representation of the flower and lever mechanism of pollination of a hypothetical member of *Salvia* subgen. *Calosphace* (*Salvia* clade II). A flower prior to the activation of the lever mechanism (A). The pollinator enters the flower and activates the lever mechanism (B), depositing pollen on the head of the pollinator. (C) A 'birds-eye' view of the flower, with the fused posterior branches of the connective blocking access to the nectar at the base of the corolla (sketch by Cody Williams).

papers presents the first, robust, *Salvia*-wide molecular phylogeny with sampling across the tribe Mentheae directly to evaluate Himmelbaur and Stibal's (1932–1934) hypothesis of independent origins of the lever mechanism in *Salvia*. 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*?

MATERIALS AND METHODS

Taxa sampling

Sampling within the genus *Salvia* attempted to include as wide a morphological and biogeographical diversity as possible. Within the New World, there is a high level of confidence that the sampling represents every major clade of *Salvia*. In addition to the monophyly of the 500 species in the subgenus *Calosphace* being supported by morphology (Bentham, 1876; Epling, 1939; Claßen-Bockhoff *et al.*, 2004*a*), the monophyly is supported by molecular data collected as part of this project and by a continuing project sampling 200 species in the subgenus (our unpubl. data). Sampling included 20 of the remaining 28 non-subgenus *Calosphace* species of *Salvia* in the New World. The Old World represents a larger challenge for sampling within *Salvia*, as the subgeneric groups are less well established. Sampling was attempted from each of the informal subgeneric groups suggested by Hedge (1974*a*, *b*, 1982*a*, *b*) based on morphology. The 26 Old World *Salvia* sampled certainly do not represent every major clade of *Salvia* present. However, the sampling includes southern African, northern African, Mediterranean, European, west Asian, central Asian and east Asian species of *Salvia*.

Nomenclature for *Salvia* follows that suggested by Alziar (1988–1993). One hundred and forty-four *trnL-F* sequences, 139 nuclear rDNA internal transcribed spacer (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 also given 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 comprised 93 taxa. Studies have demonstrated the monophyly of the tribe Mentheae, as well as its close relation to the tribe Ocimeae (Paton *et al.*, 2004; Walker *et al.*, 2004; Bräuchler *et al.*, 2005). 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 all subtribes of 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'.

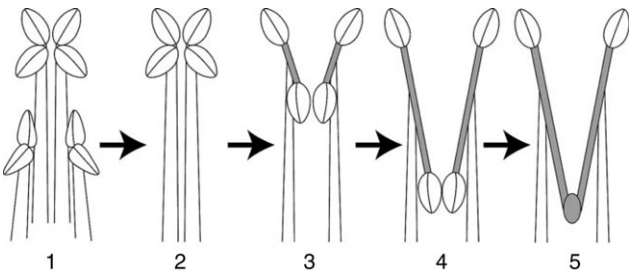

FIG. 2. The generalized trend in stamen morphology seen within tribe Mentheae leading to that seen in *Salvia*. Shaded areas represent connective tissue. Step 2 (the functional loss of two of the four stamens) has apparently happened only once in the *Salvia* clade. The progression from step 2 to step 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.

Extractions, amplification and sequencing

Total genomic DNA was extracted using DNeasy Plant Mini kits (Qiagen, Valencia, CA, USA). Leaves used for DNA extractions were fresh, frozen, silica dried or obtained from herbarium specimens (see Table 1). 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 the QIAquick PCR purification kit (Qiagen) or with the AmPure PCR purification kit (Agencourt, Beverly, MA, USA). Sequenced products were precipitated in ethanol and sodium acetate to remove excess dye terminators or cleaned with the CleanSEQ Sequencing Reaction Clean-up system (Agencourt). Contiguous alignments were edited using Sequencher v. 3·0 (Gene Codes, Ann Arbor, MI, USA).

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TABLE 1. *Plant materials included in this study*

Men.	Sal.	Taxon	Locality	Voucher	*psbA-trnH* sequence	ITS sequence	*trnL-trnF* sequence
Yes		*Acanthomintha lanceolata* Curran	herb-MO 3133280	Crosby&Morin 14383	DQ667418	DQ667333	DQ667522
Yes		*Agastache urticifolia* Kunth	wild-USA (WIS)	JBW 815	DQ667357	DQ667247	AY570452
Yes		*Cleonia lusitanica* L.	herb-F	D. Sanches & R. Garilan 20-VI-8?	DQ667395	DQ667309	DQ667495
Yes		*Clinopodium ashei* Small	wild-USA (WIS)	JBW 742	DQ667348	DQ667237	DQ667437
Yes		*Clinopodium coccineum* Kuntze	wild-USA (WIS)	JBW 741	DQ667344	DQ667233	DQ667433
Yes		*Clinopodium vulgare* L.	wild-USA (WIS)	JBW 3227	DQ667409	DQ667324	DQ667513
Yes		*Collinsonia canadensis* L.	wild-USA (WIS)	JBW 958	DQ667358	DQ667248	AY570453
Yes		*Conradina canescens* A. Gray	wild-USA (WIS)	JBW 604	DQ667349	DQ667238	DQ667438
Yes		*Cunila galioides* Benth.	wild-Argentina (WIS)	Sytsma 7247	DQ667391	DQ667305	DQ667491
Yes		*Cunila incana* Benth.	wild-Argentina (WIS)	Sytsma 7224	DQ667403	DQ667316	DQ667504
Yes		*Dicerandra oderatissima* R.M. Harper	wild-USA (WIS)	JBW 1063	DQ667345	DQ667234	DQ667434
Yes	Yes	*Dorystaechas hastata* Boiss. & Heldr. Ex Benth.	cult-RBG-Edinburgh	1972–0177D	DQ667360	DQ667252	AY570454
Yes		*Drepanocaryum sewerzowskii* (Regel) Pojark.	herb-MO 5201825	Rinziraeva 7540	DQ667413	DQ667328	DQ667517
Yes		*Glechoma hederacea* L.	cult-USA (WIS)	JBW 2579	DQ667355	DQ667245	AY570455
Yes		*Glechon marifolia* Benth.	wild-Argentina (WIS)	Sytsma 7214	DQ667390	DQ667303	DQ667489
Yes		*Glechon thymoides* Spreng.	herb-F	CA Mondin 1421	DQ667396	DQ667310	DQ667496
Yes		*Hedeoma costatum* (Greene) Irving	wild-USA (WIS)	JBW 2143	DQ667347	DQ667236	DQ667436
Yes		*Hoehnea epilobioides* (Epl.) Epl.	herb-F	G. Hatschbach 8/3/1984	DQ667397		DQ667497
Yes	Yes	*Horminum pyrenaicum* L.	cult-RBG-Edinburgh	1997–2109a	DQ667365	DQ667257	AY570456
Yes		*Hyptis alata* (Raf.) Shinners	wild-USA (WIS)	JBW 1019	DQ667346	DQ667235	DQ667435
Yes		*Lepechinia calycina* Epl.	wild-USA (WIS)	JBW 3186	DQ667394	DQ667308	DQ667494
Yes	Yes	*Lepechinia chamaedryoides* Epl.	cult-USA (WIS)	JBW 2537	DQ667343	DQ667231	AY570459
Yes	Yes	*Lepechinia conferta* Epl.	herb-F	Alonso 8376	DQ667393	DQ667307	DQ667493
Yes	Yes	*Lepechinia lancifolia* Epl.	herb-F	Smith 444	DQ667392	DQ667306	DQ667492
Yes		*Lycopus uniflorus* Michx.	wild-USA (WIS)	JBW 2586	DQ667389	DQ667302	DQ667488
Yes	Yes	*Melissa officinalis* L.	cult-USA (WIS)	JBW 2575	DQ667387	DQ667291	DQ667477
Yes		*Mentha arvensis* L.	wild-USA (WIS)	JBW 3228	DQ667410	DQ667325	DQ667514
Yes		*Mentha spicata* L.	cult-USA (WIS)	JBW 2566	DQ667354	DQ667244	AY570461
Yes	Yes	*Meriandra bengalensis* (Roxb.) Benth	herb-MO 2633828	Lavranus & Newton 15796	DQ667414	DQ667329	DQ667518
Yes		*Monarda fistulosa* L.	wild-USA (WIS)	JBW 3223	DQ667405	DQ667318	DQ667506
Yes		*Nepeta cataria* L.	wild-USA (WIS)	JBW 3054	DQ667388	DQ667301	DQ667487
Yes		*Ocimum basilium* L.	cult-USA (WIS)	JBW 2557	DQ667350	DQ667240	AY570462
Yes		*Origanum vulgare* L.	cult-USA (WIS)	JBW 2567	DQ667353	DQ667243	AY570463
Yes		*Perilla frutescens* (L.) Britton	cult-USA (WIS)	JBW 1078	DQ667356	DQ667246	DQ667439
Yes	Yes	*Perovskia atriplicifolia* Benth.	cult-USA (WIS)	JBW 2524	DQ667341	DQ667223	AY570464
Yes	Yes	*Perovskia scrophulariaefolia* Bunge	herb-MO 5201778	Kinziraeva 6751	DQ667415	DQ667330	DQ667519
Yes		*Pogogyne floribunda* Jokerst	herb-MO 4282587	Bartholemew 6021	DQ667416	DQ667331	DQ667520
Yes		*Poliomintha palmeri* Hemsl	herb-F	Diggs Nee 2531	DQ667398	DQ667311	DQ667498
Yes		*Prunella vulgaris* L.	wild-USA (WIS)	JBW 3225	DQ667407		DQ667508
Yes		*Pycnanthemum virginianum* (L.) Durand & Jacks ex Rob & Fernald	wild-USA (WIS)	JBW 3224	DQ667406	DQ667319	DQ667507
Yes		*Rhododon ciliatus* (Benth.) Epl.	herb-F	W.C. Holmes 8215	DQ667399	DQ667312	DQ667499
Yes	Yes	*Rosmarinus officinalis* L.	cult-USA (WIS)	JBW 2558	DQ667351	DQ667241	AY570465

Continued

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Table 1. *Continued*

Men.	Sal.	Taxon	Locality	Voucher	*psbA-trnH* sequence	ITS sequence	*trnL-trnF* sequence
Yes	Yes	*Salvia aegyptiaca* L.	herb-E	McLeish 3728	DQ667380	DQ667285	DQ667470
Yes	Yes	*Salvia aethiopis* L.	wild-Armenia (MJG)	Hellwig 26/6/02	DQ667370	DQ667272	AY570466
Yes	Yes	*Salvia apiana* Jepson	wild-USA (WIS)	JBW 2509	DQ667338	DQ667214	DQ667425
Yes	Yes	*Salvia aristata* Aucher	herb-E	Wedelbo & Assadi s.n.	DQ667375	DQ667280	DQ667465
	Yes	*Salvia atrocyanea* Epl.	wild-Bolivia (MJG)	P. Wester 3		DQ667270	DQ667456
Yes	Yes	*Salvia aucheri* var. *canescens* Benth.	herb-E	Archibald 7670	DQ667381	DQ667286	DQ667471
Yes	Yes	*Salvia austriaca* Jacq.	cult-Mainz. Bot. Gar.	Claßen-Bockhoff – 2004	DQ667408	DQ667323	DQ667512
	Yes	*Salvia axillaris* Moc. et Sesse ex Benth.	Wild-Mex (WIS)	JBW 3038		DQ667294	DQ667480
Yes	Yes	*Salvia azurea* Michx. ex Lam.	wild-USA (WIS)	JBW 3222	DQ667404	DQ667317	DQ667505
	Yes	*Salvia bangii* Rusby	wild-Bolivia (MJG)	P. Wester 10		DQ667263	DQ667449
Yes	Yes	*Salvia cabulica* Benth.	herb-E	Ghafoor & Goodman 5148	DQ667382	DQ667287	DQ667472
Yes	Yes	*Salvia cacaliifolia* Benth.	cult-RBG-Edinburgh	1959–9358A	DQ667367	DQ667259	DQ667445
	Yes	*Salvia californica* Brandegee	cult-USA (WIS)	JBW 2520		DQ667213	DQ667424
Yes	Yes	*Salvia canariensis* L.	cult-RBG-Edinburgh	1986–0478	DQ667364	DQ667256	AY570469
	Yes	*Salvia candicans* Mart. & Gal.	Wild-Mex (WIS)	JBW 3001		DQ667299	DQ667485
Yes	Yes	*Salvia candidissima* Vahl.	cult-RBG-Edinburgh	1999–2202A	DQ667368	DQ667261	DQ667447
	Yes	*Salvia cedrosensis* Greene	cult-USA (WIS)	JBW 2539		DQ667228	AY570470
	Yes	*Salvia chionopeplica* Epl.	cult-USA (WIS)	JBW 2545		DQ667227	AY570472
	Yes	*Salvia clevelandii* (Gray) Greene	wild-USA (WIS)	JBW 2508		DQ667219	AY570473
Yes	Yes	*Salvia cynica* Dunn	herb-MO 4026698	Boufford&Bartholemew 24763	DQ667417	DQ667332	DQ667521
Yes	Yes	*Salvia daghestanica* Sosn.	cult-RBG-Edinburgh	1988–2283A	DQ667366	DQ667258	DQ667444
Yes	Yes	*Salvia digitaloides* Diels.	cult-RBG-Edinburgh	1999–2200A	DQ667363	DQ667255	AY570477
Yes	Yes	*Salvia disermas* L.	herb-E	Goldblatt 7500	DQ667385	DQ667290	DQ667475
	Yes	*Salvia divinorum* Epl. et Jativa	cult-USA (WIS)	JBW 3230		DQ667249	DQ667440
	Yes	*Salvia dolomitica* Codd	cult-USA (WIS)	JBW 3200		DQ667322	DQ667511
	Yes	*Salvia dorrii* (Kell.) Abrams	cult-USA (WIS)	JBW 2541		DQ667229	DQ667430
	Yes	*Salvia eremostachya* Jeps.	cult-USA (WIS)	JBW 2533		DQ667232	DQ667432
	Yes	*Salvia fulgens* Cav.	herb-WIS	1967–1496A		DQ667251	DQ667441
Yes	Yes	*Salvia garipensis* E. Meyer ex Benth.	herb-E	Strohbach 149	DQ667376	DQ667281	DQ667466
Yes	Yes	*Salvia glutinosa* L.	cult-USA (WIS)	JBW 2568	DQ667359	DQ667250	AY570480
Yes	Yes	*Salvia graciliramulosa* Epl. et Jativa	wild-Bolivia (MJG)	P. Wester 14	DQ667372	DQ667276	DQ667461
Yes		*Salvia greatai* Brandegee	wild-USA (WIS)	JBW 2511	DQ667339	DQ667215	AY570481
	Yes	*Salvia haenkei* Benth.	wild-Bolivia (MJG)	P. Wester 71		DQ667271	DQ667457
	Yes	*Salvia henryi* Gray	wild-USA (WIS)	JBW 2516		DQ667216	AY570482
	Yes	*Salvia hians* Royle	cult-USA (WIS)	JBW 2577		DQ667239	AY570483
Yes	Yes	*Salvia hirtella* Vahl.	wild-Peru (MJG)	Schmidt-Lebuhn 395	DQ667411	DQ667326	DQ667515
Yes	Yes	*Salvia hydrangea* Benth.	herb-E	Rechinger 47123	DQ667383	DQ667288	DQ667473
	Yes	*Salvia hydrangea* Benth.	wild-Armenia (MJG)	Hellwig 6/18/02		DQ667265	DQ667451
	Yes	*Salvia inconspicua* Benth.	Wild-Mex (WIS)	JBW 3045		DQ667298	DQ667484
	Yes	*Salvia lasiantha* Benth.	Wild-Mex (WIS)	JBW 3009		DQ667300	DQ667486
	Yes	*Salvia lavanduloides* Kunth	Wild-Mex (WIS)	JBW 3044		DQ667297	DQ667483
	Yes	*Salvia leucophylla* Greene	Cult.-USA	JBW s.n.		DQ667210	DQ667422
	Yes	*Salvia mellifera* Greene	wild-USA (WIS)	JBW 2550		DQ667220	DQ667427
Yes		*Salvia miltiorrhiza* Bunge	herb-MO 04702028	Wang Shilong s.n.	DQ667419	DQ667334	DQ667523
	Yes	*Salvia miltiorrhiza* Bunge	herb-MO	Boufford *et al.* 26067	DQ667379	DQ667284	DQ667469

	Yes	*Salvia mocinoi* Benth.	wild-Mexico (MJG)	Crone 15/9/00		DQ667274	DQ667459
	Yes	*Salvia mohavensis* Greene	Cult.-USA	JBW s.n.		DQ667212	DQ667423
	Yes	*Salvia munzii* Epl.	wild-USA (WIS)	JBW 2507		DQ667224	DQ667428
Yes	Yes	*Salvia officinalis* L.	cult-USA (WIS)	JBW 2580	DQ667342	DQ667225	AY570488
Yes	Yes	*Salvia orbignaei* Benth.	wild-Bolivia (MJG)	P. Wester 43	DQ667374	DQ667279	DQ667464
	Yes	*Salvia ovalifolia* St.-Hil. ex Benth	wild-Argentina (WIS)	Sytsma 7226		DQ667315	DQ667502
	Yes	*Salvia oxyphora* Briq.	wild-Bolivia (MJG)	P. Wester 16		DQ667262	DQ667448
	Yes	*Salvia pachyphylla* Epl. ex Munz	cult-USA (WIS)	JBW 2535		DQ667230	DQ667431
Yes	Yes	*Salvia patens* Cav.	cult-RBG-Edinburgh	1973–9197	DQ667361	DQ667253	DQ667442
Yes	Yes	*Salvia penstemonoides* Kunth et Bouche	cult-USA (WIS)	JBW 2578	DQ667340	DQ667221	AY570489
	Yes	*Salvia personata* Epl.	wild-Bolivia (MJG)	P. Wester 17		DQ667269	DQ667455
Yes	Yes	*Salvia platystoma* Epl.	wild-Bolivia (MJG)	P. Wester 18	DQ667373	DQ667277	DQ667462
	Yes	*Salvia polystachya* Epl.	Wild-Mex (WIS)	JBW 3035		DQ667292	DQ667478
	Yes	*Salvia procurrens* Benth.	wild-Argentina (WIS)	Bonif 941		DQ667304	DQ667490
Yes	Yes	*Salvia prunelloides* Kunth	wild-Mexico (MJG)	Crone 15/9/00	DQ667371	DQ667275	DQ667460
Yes	Yes	*Salvia przewalskii* Maxim.	cult-RBG-Edinburgh	1993–2067A	DQ667362	DQ667254	DQ667443
	Yes	*Salvia pubescens* Benth.	Wild-Mex (WIS)	JBW 3043		DQ667296	DQ667482
Yes		*Salvia regla* Cav.	Wild-Mex (WIS)	JBW 3019	DQ667402		DQ667503
Yes	Yes	*Salvia roborowskii* Max.	herb-E	SBQ 852	DQ667384	DQ667289	DQ667474
	Yes	*Salvia roemeriana* Scheele	wild-USA (WIS)	JBW 2515		DQ667211	AY570491
	Yes	*Salvia rusbyi* Britton ex Rusby	wild-Bolivia (MJG)	P. Wester 31		DQ667278	DQ667463
	Yes	*Salvia rypara* Briq.	wild-Bolivia (MJG)	P. Wester 32		DQ667266	DQ667452
	Yes	*Salvia sagittata* Ruiz et Pav.	herb-WIS	Weigend & Dostert 97/ s.n.		DQ667260	DQ667446
Yes		*Salvia santolinifolia* Boiss.	herb-E	Runemark *et al.* 22255	DQ667386		DQ667476
	Yes	*Salvia sclarea* L.	cult-USA (WIS)	JBW 2527		DQ667222	AY570492
Yes	Yes	*Salvia scutellarioides* Kunth.	wild-Peru (MJG)	Schmidt-Lebuhn 469	DQ667412	DQ667327	DQ667516
	Yes	*Salvia semiatrata* Zucc.	herb-WIS	JBW 3041		DQ667295	DQ667481
Yes	Yes	*Salvia sessilifolia* Baker	herb-E	Jongkind & Rapanarivo 929	DQ667377	DQ667282	DQ667467
	Yes	*Salvia sonomensis* Greene	wild-USA (WIS)	JBW 2519		DQ667218	DQ667426
	Yes	*Salvia sophrona* Briq.	wild-Bolivia (MJG)	P. Wester 34		DQ667268	DQ667454
	Yes	*Salvia stachydifolia* Benth.	wild-Bolivia (MJG)	P. Wester 35		DQ667267	DQ667453
	Yes	*Salvia summa* A. Nelson	wild-USA (WIS)	JBW 1972		DQ667217	AY570496
Yes	Yes	*Salvia taraxacifolia* Hook. fil.	cult-USA (WIS)	JBW 2521	DQ667337	DQ667209	AY570497
Yes	Yes	*Salvia tetrodonta* Hedge	herb-V 12403	Podlech 18906	DQ667421		DQ667526
	Yes	*Salvia texana* (Scheele) Torrey	wild-USA	P. Wester 362		DQ667321	DQ667510
	Yes	*Salvia thymoides* Benth.	wild-Mexico (MJG)	Crone 10/8/00		DQ667273	DQ667458
Yes	Yes	*Salvia trichocalycina* Benth.	herb-E	Breckle 4963	DQ667378	DQ667283	DQ667468
	Yes	*Salvia tricuspidata* Mart. & Gal.	wild-Mexico (WIS)	JBW 3037		DQ667293	DQ667479
	Yes	*Salvia vaseyi* (Porter) Parish	wild-USA (WIS)	JBW 2530		DQ667226	DQ667429
Yes	Yes	*Salvia verbascifolia* M. Bieb.	wild-Armenia (MJG)	Hellwig 6/13/02	DQ667369	DQ667264	DQ667450
	Yes	*Salvia whitehousei* Alziar	wild-USA (MJG)	P. Wester 352		DQ667320	DQ667509
Yes		*Schizonepeta multifida* Huang, Feng & Wang	herb-F	Boyd 4805	DQ667400	DQ667313	DQ667500
Yes		*Thymus serpyllum* L.	cult-USA (WIS)	JBW 2564	DQ667352	DQ667242	AY570502
Yes		*Zhumeria majudae* Rech. F. & Wendelbo	herb-V 01176	Ghazi s.n.		DQ667335	DQ667524
	Yes	*Zhumeria majudae* Rech. F. & Wendelbo	herb-V 21730	Wendelbo 15793	DQ667420	DQ667336	DQ667525
Yes		*Ziziphora taurica* M. Bieb.	herb-F	I. Kapetariidis s.n.	DQ667401	DQ667314	DQ667501

Men., included in the Mentheae-wide analysis; Sal., included in the *Salvia* clade analysis. In the locality column: herb., herbarium material — herbarium code; wild, wild collected; cult., cultivated material.

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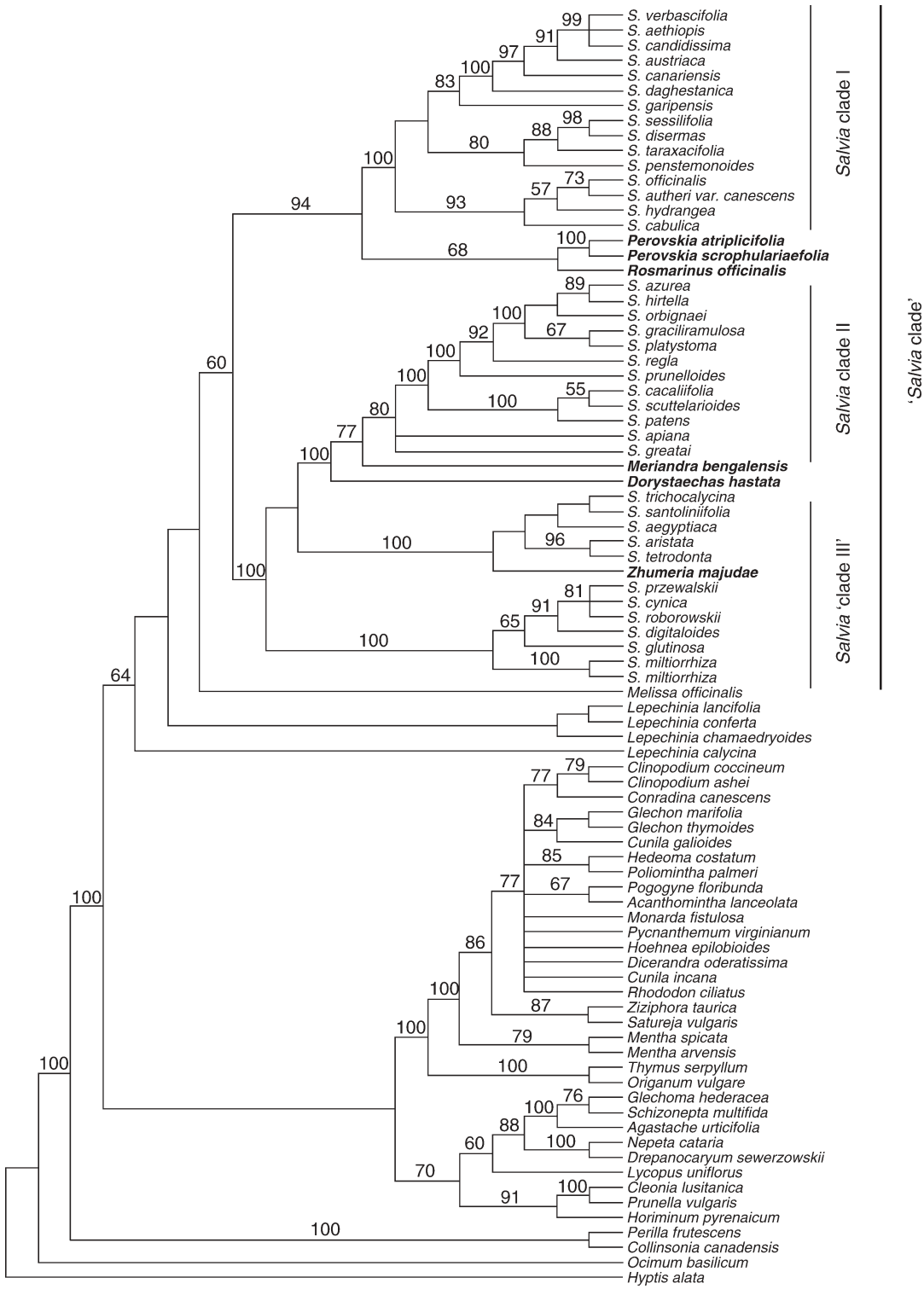

FIG. 3. The 'Mentheae-wide' analysis. A three-region DNA, combined parsimony analysis of the chloroplast regions *trnL-F*, *psbA-trnH* and the nuclear rDNA ITS. Strict consensus of 2094 equally parsimonious trees of length 1737 steps. Bootstrap values above 50 % are shown above the branches. In addition to all *Salvia*, the '*Salvia* clade' includes the genera highlighted in bold.

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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 analysis. The first involved an 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 analysed using maximum parsimony (MP). The heuristic MP analysis (Fitch, 1971) in PAUP* 4·0b10 (Swofford, 2002) used 100 random addition sequences, with ten 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 ten random addition sequences with TBR replicates with no more than 5000 trees saved per replicate.

The second analysis (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 v. 3·06 (Posada and Crandall, 1998). Heuristic ML searches with TBR branch-swapping were conducted.

Staminal morphological investigations

Staminal features investigated by this project are difficult to observe in herbarium specimens. Where fresh material was not available, literature that included detailed information regarding staminal morphology was used to determine the staminal form in each species (see Table 2). General stamen types were characterized for each major clade suggested by the molecular results and mapped onto the terminals in the cladograms (see Figs 4 and 5).

RESULTS

Analysis of Mentheae-wide data set

The aligned length of the *trnL-F* data set was 1137 base pairs (bp). With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 1062 bp. Twenty indel events were scored for the *trnL-F* data set, of which 18 were parsimony- informative and were 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 bp. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 382 bp. 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. tetrodonta*, *S. regla*, *Hoehnea epilobioides* or *Prunella vulgaris*. The aligned length of the nuclear ITS data set was 811 bp. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 659 bp. 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 with random partitions of the same size ($P < 0·01$). Further analyses of the specific topological differences found between individual data sets indicate that none of the incongruent clades has bootstrap support above 50 % in the individual region analyses. The partition homogeneity test has been demonstrated to be overly sensitive in large data sets such as this (Hipp *et al.*, 2004). Thus, the incongruence suggested by the partition homogeneity test may in fact not reflect genealogical discordance, but artefacts of the overly sensitive nature of the incongruence length difference (ILD) test in large datasets. Despite the incongruence of the data sets, all three data sets independently support the integrity of the '*Salvia* clade' as discussed below, and the three specific clades of *Salvia* discussed in this paper. That is to say, each of the three data sets independently support *Rosmarinus* and *Perovskia* sister to *Salvia* clade I,

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TABLE 2. *Stamen types of* Salvia *included in study. Types were determined by direct observation or through literature references that describe stamen form in detail*

Taxon	Stamen type	Reference*	Taxon	Stamen type	Reference
Salvia aegyptiaca L.	M	6, 9	*Salvia mellifera* Greene	H	1, 2, 3
Salvia aethiopis L.	B	1, 8, 9	*Salvia miltiorrhiza* Bunge	N	11
Salvia apiana Jepson	H	1, 2, 3	*Salvia mocinoi* Benth.	E	13
Salvia aristata Aucher	M	9	*Salvia mohavensis* Greene	H	1, 2, 3
Salvia atrocyanea Epl.	E	13	*Salvia munzii* Epl.	H	1, 2, 3
Salvia aucheri var. canescens Benth.	A	14	*Salvia officinalis* L.	A	1
Salvia austriaca Jacq.	B	10	*Salvia orbignaei* Benth.	E	13
Salvia axillaris Moc. et Sesse ex Benth.	G	1, 13	*Salvia ovalifolia* St.-Hil. ex Benth	E	13
Salvia azurea Michx. ex Lam.	E	1, 13	*Salvia oxyphora* Briq.	E	13
Salvia bangii Rusby	E	13	*Salvia pachyphylla* Epl. ex Munz	H	1, 2, 3
Salvia cabulica Benth.	A	9	*Salvia patens* Cav.	E	1, 13
Salvia cacaliifolia Benth.	E	1, 13	*Salvia penstemonoides* Kunth et Bouche	A	1
Salvia californica Brandegee	I	1, 2, 3	*Salvia personata* Epl.	E	13
Salvia canariensis L.	B	6	*Salvia platystoma* Epl.	E	13
Salvia candicans Mart. & Gal.	E	1, 13	*Salvia polystachya* Epl.	E	1, 13
Salvia candidissima Vahl.	B	9	*Salvia procurrens* Benth.	E	13
Salvia cedrosensis Greene	E	1, 13	*Salvia prunelloides* Kunth	E	1, 13
Salvia chionopeplica Epl.	H	1, 2, 3	*Salvia przewalskii* Maxim.	N	7, 11
Salvia clevelandii (Gray) Greene	H	1, 2, 3	*Salvia pubescens* Benth.	E	1, 13
Salvia cynica Dunn	N	11	*Salvia regla* Cav.	E	1, 13
Salvia daghestanica Sosn.	B	10	*Salvia roborowskii* Max.	N	11
Salvia digitaloides Diels.	N	11	*Salvia roemeriana* Scheele	A	1, 4
Salvia disermas L.	A (?)	6	*Salvia rusbyi* Britton ex Rusby	E	13
Salvia divinorum Epl. et Jativa	E	1, 13	*Salvia rypara* Briq.	E	13
Salvia dolomitica Codd	A	1, 6	*Salvia sagittata* Ruiz et Pav.	E	1, 13
Salvia dorrii (Kell.) Abrams	H	1, 2, 3	*Salvia santolinifolia* Boiss.	M	9
Salvia eremostachya Jeps.	H	1, 2, 3	*Salvia sclarea* L.	B	1
Salvia fulgens Cav.	E	1, 13	*Salvia scutellarioides* Kunth.	E	13
Salvia garipensis E. Meyer ex Benth.	B	6	*Salvia semiatrata* Zucc.	E	1, 13
Salvia glutinosa L.	N	1, 7	*Salvia sessilifolia* Baker	A	6
Salvia graciliramulosa Epl. et Jativa	E	13	*Salvia sonomensis* Greene	H	1, 2, 3
Salvia greatai Brandegee	I	1, 2, 3	*Salvia sophrona* Briq.	E	13
Salvia haenkei Benth.	E	13	*Salvia stachydifolia* Benth.	E	13
Salvia henryi Gray	A	1, 4	*Salvia summa* A. Nelson	A	1, 4
Salvia hians Royle	N	1, 11	*Salvia taraxacifolia* Hook. fil.	A	1,6
Salvia hirtella Vahl.	E	13	*Salvia tetrodonta* Hedge	M	9, 12
Salvia hydrangea Benth.	A	9, 10	*Salvia texana* (Scheele) Torrey	A	5
Salvia hydrangea Benth.	A	9, 10	*Salvia thymoides* Benth.	E	1, 13
Salvia inconspicua Benth.	E	1, 13	*Salvia trichocalycina* Benth.	M	9
Salvia lasiantha Benth.	E	1, 13	*Salvia tricuspidata* Mart. & Gal.	E	1, 13
Salvia lavanduloides Kunth	E	1, 13	*Salvia vaseyi* (Porter) Parish	H	1, 2, 3
Salvia leucophylla Greene	H	1, 2, 3	*Salvia verbascifolia* M. Bieb.	B	10
			Salvia whitehousei Alziar	A	5

*Reference: 1, personal observation by the first author; 2, Epling (1938); 3, Neissess (1983); 4, Walker and Elisens (2001); 5, Whitehouse (1949); 6, Hedge (1974*a*); 7, Claßen-Bockhoff *et al.* (2004*b*); 8, Hedge (1985); 9, Hedge (1982*b*); 10, Pobedimova (1954); 11, Xi-wen and Hedge (1994); 12, Hedge (1974*b*); 13, Epling (1939); 14, Hedge (1982*a*).

Meriandra and *Dorystaechas* sister to *Salvia* clade II, and *Zhumeria* embedded in *Salvia* clade III (i.e. the source of the incongruence between the data sets lies elsewhere than the clades discussed herein). These facts combined with the high bootstrap support associated with each of the clades discussed in this paper in the combined analysis suggests that a 'total evidence', 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 %) with the genera *Lepechinia* and *Melissa* appearing as likely sister genera (Fig. 3). For the purposes of this discussion, the term '*Salvia* clade' is used to refer to the least inclusive clade which contains all members of *Salvia*. In addition to all *Salvia*, the '*Salvia* clade' includes the genera *Dorystaechas*, *Meriandra*, *Perovskia*, *Rosmarinus* and *Zhumeria* (see Fig. 3). Three clades of *Salvia* are identified more closely related to one or more of these other genera than to the other major clades 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 (bootstrap = 94 %). *Salvia* clade II, likewise, forms a well-supported monophyletic lineage including two other genera, *Meriandra* and *Dorystaechas* (bootstrap = 100 %). Two remaining, well-supported lineages of *Salvia*, one of

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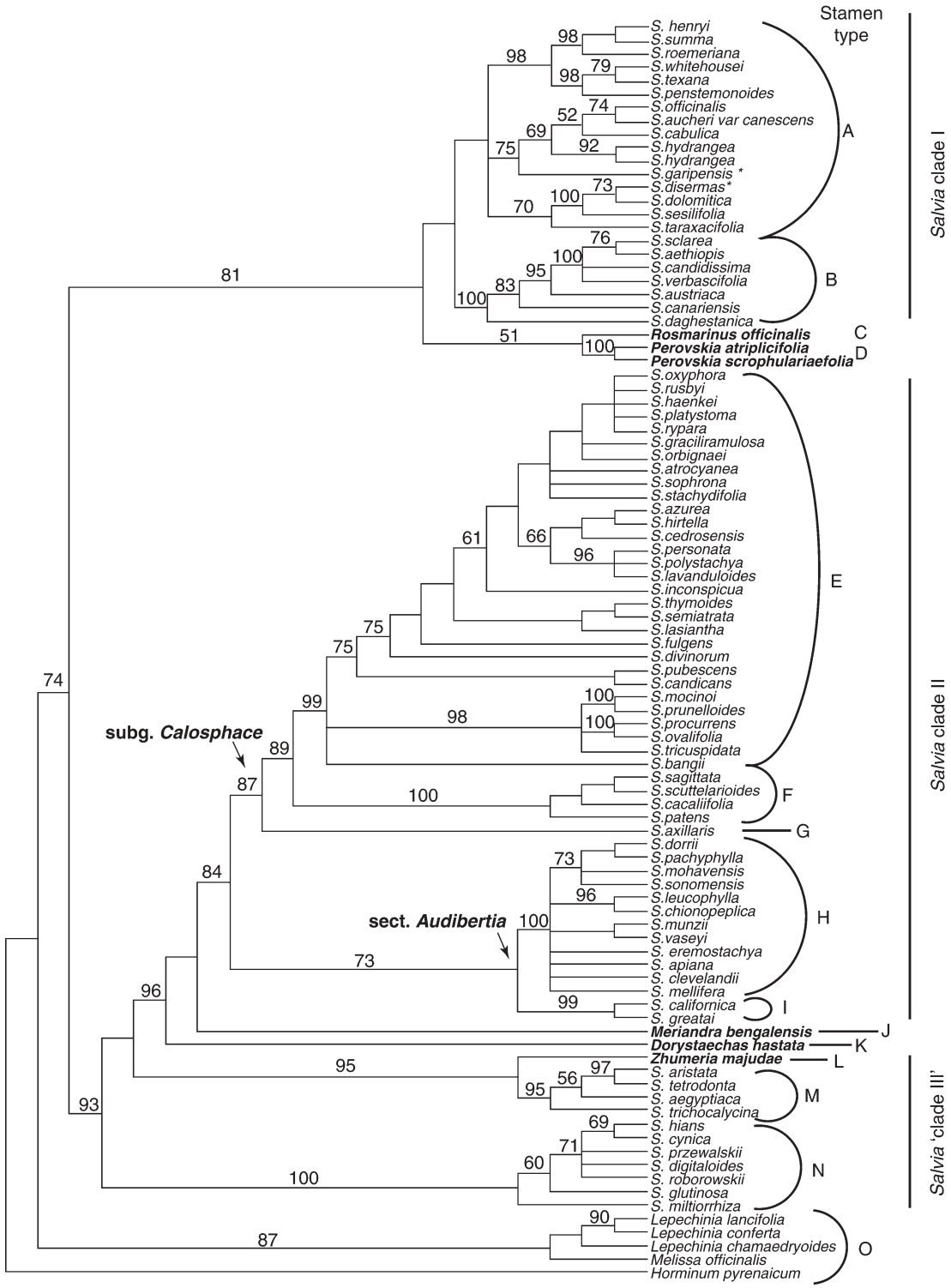

FIG. 4. The '*Salvia* clade' analysis. A two-region DNA, combined parsimony analysis of the chloroplast region *trnL-F* and the nuclear rDNA ITS. Strict consensus of over 100 000 equally parsimonious trees of 1489 steps. Bootstrap values above 50 % are shown above the branches. Stamen types corresponding to those in Fig. 5 and Table 2 are shown. Non-*Salvia* genera are highlighted in bold.

which includes the genus *Zhumeria*, occupy one of the few unresolved areas within the 'backbone' of 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).

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Analysis of the 'Salvia *clade' data set*

The aligned length of the *trnL-F* data set was 1019 bp. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 923 bp. 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 26 007 equally parsimonious trees of 163 steps (CI = 0·748, RI = 0·971, RC = 0·727).

The aligned length of the nuclear ITS data set (for the 93 included taxa) was 807 bp. With regions of ambiguous alignment or ambiguous sequences excluded, the total length of included characters was 762 bp. 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 with random partitions of the same size ($P < 0{\cdot}01$). 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 regard 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 has bootstrap support above 50 % in the ITS analysis.

ML produced a single tree with a log likelihood score of −11 859·60033. The ML analyses were performed under the K80(K2*P*) + G + I model of evolution: ti/tv ratio = 1·683386; proportion of 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 subgen. *Calosphace*.

Staminal morphology

Two distinct stamen types were identified in the species sampled from *Salvia* clade I (stamen types A and B, Fig. 5; Table 2). The two posterior thecae are expressed and not fused in stamen type A. In stamen type B, the two posterior thecae are not expressed, and the distal posterior ends of the adjacent connectives are fused into a complex structure blocking access to nectar. 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 *Standleyana*, *Blakea* and *Hastatae* (stamen type F, Figs 4 and 5), both posterior thecae are aborted, and the adjacent posterior thecae are not or only little fused. The remaining members of *S*. subgen. *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* (Figs 4 and 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, Figs 4 and 5) has both posterior thecae expressed and not fused to one another. The second type of stamen found in *Salvia* 'clade III' (stamen type N, Figs 4 and 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, 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 nectar is not necessarily blocked, a lever mechanism has been observed in this stamen type in at least some of these species (*S. glutinosa*, *S. hians*).

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 and 4). And third, the staminal lever mechanism has evolved three times independently, each time with a distinct morphology (Figs 5 and 6).

Relationships within the Mentheae

This project has sampled all putative *Salvia* relatives, as well as representatives of all other major lineages within the

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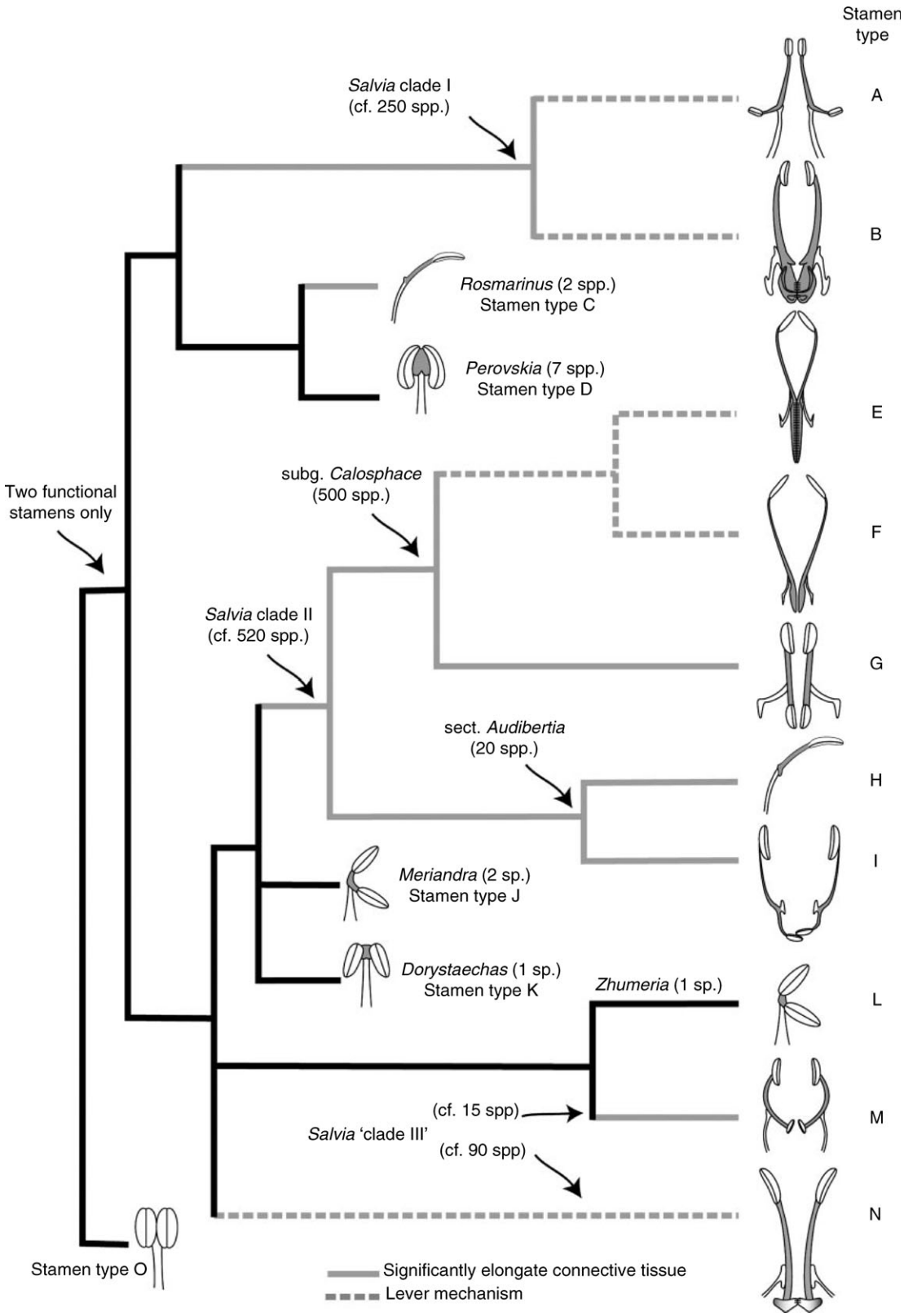

FIG. 5. A summary of the cladogram shown in Fig. 4, with representations of the stamen types found in each clade. Shaded areas of the sketches represent connective tissue. Grey lines in the cladogram represent branches in which significantly elongate connectives are seen. Dashed lines in the cladogram 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. Species numbers were hypothesized based on subgeneric groups suggested in the literature (Epling, 1938, 1939; Hedge 1974, 1982*a*, *b*). The two taxa with asterisks represent taxa not possessing the 'typical' stamen type A, and both possessing stamens with no expressed posterior thecae.

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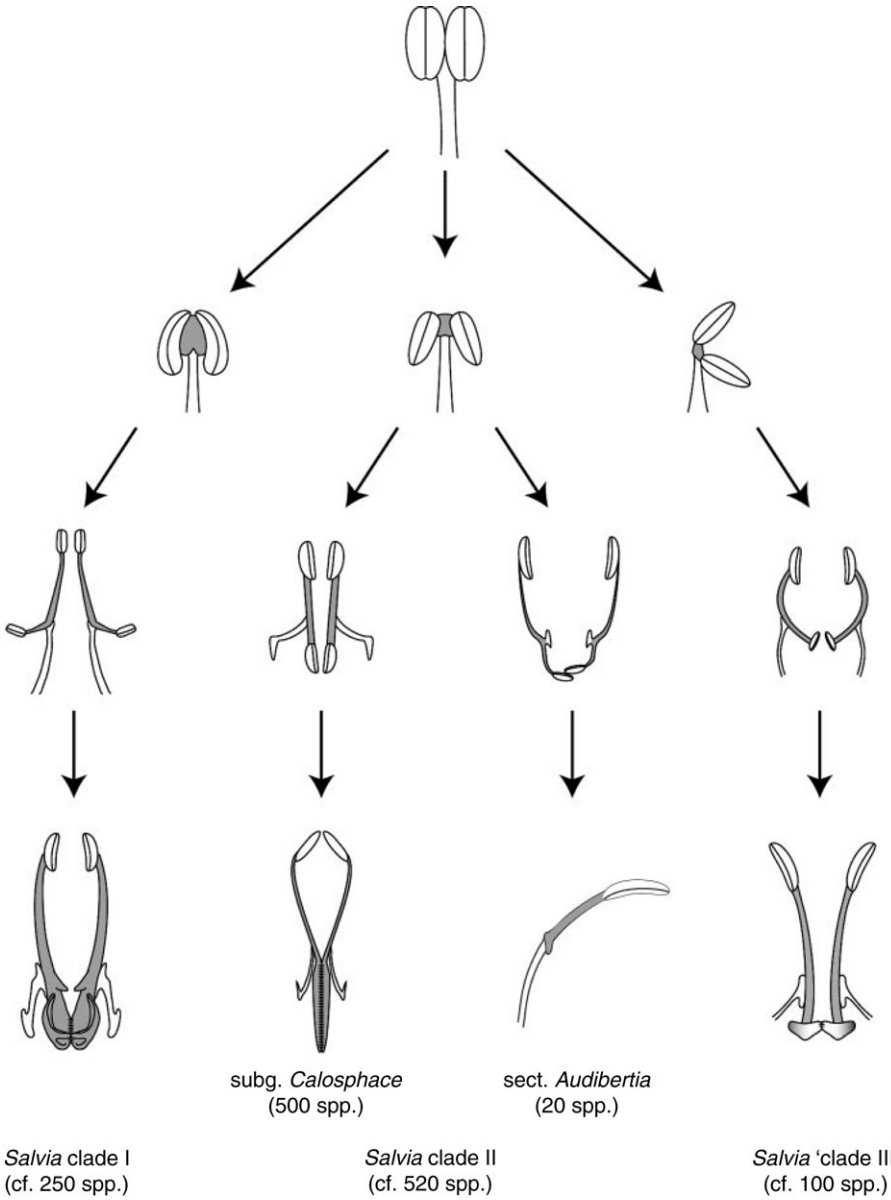

FIG. 6. Hypothesis of evolutionary progression in the independent origin of the three different staminal lever mechanisms found in the tribe Mentheae. This figure represents a modification and revision of Himmelbaur and Stibal's (1934) original interpretation of staminal evolution in *Salvia*. The three 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 in this figure), but have been independently derived and are morphologically distinct from one another.

tribe Mentheae. The 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 to 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 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 adaxial stamens. Our sampling is insufficient in the genus *Lepechinia* to address the relationship between *Lepechinia* and *Melissa*; however, in all analyses, '*Salvia* clade', *Lepechinia* and *Melissa* form a monophyletic group (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). Both

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Lepechinia and *Melissa* have four expressed stamens, each with two parallel thecae and a connective that is not 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*. 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 and 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 (stamen type C, Fig. 5). The resulting appearance results in the stamen appearing essentially 'normal' (i.e. with no elongate connective), albeit with only one theca at the end, and a notch half way up the 'filament' representing where the filament ends and the connective begins (Trapp, 1956). Thus, unlike the other four genera intercalated in the genus *Salvia*, *Rosmarinus* exhibits the defining character of *Salvia*, a significantly elongate connective. Furthermore, this is the same staminal morphology found in *Salvia* sect. *Audibertia* from western North America, and thus, independent of phylogeny, there is no *morphological* basis for why *Rosmarinus* should not be included in the genus *Salvia*.

Within *Salvia* clade I, two 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 all display the staminal character of total fusion of the posterior thecae into what Bentham (1876) termed a glutinatorium, and what Claßen-Bockhoff *et al.* (2004*a*) 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 thecal tissue and activate the lever in order to access the nectar. Using the species groups established by Hedge (1974*a*, *b*, 1982*a*, *b*) and the alliances suggested by Pobedimova (1954), it can be assumed that this clade probably contains an additional 50 European and western Asian species.

The other taxa sampled from *Salvia* clade I produce a wide diversity of stamen types, generally including rudimentary posterior thecae, sometimes with pollen produced, and not entirely fused to the adjacent posterior theca or connective arm. Exceptions to this generality can be noted in *S. disermas* and *S. garipensis*, both of which have aborted posterior theca which are fused (Hedge, 1974*a*). The variations in staminal morphology present in this group is best appreciated by noting the diversity of stamens in the sketches included in Hedge's (1974*a*) treatment of the *Salvia* of Africa. Field observations by the first author suggest that a lever mechanism is employed in some of these taxa (e.g. *S. taraxacifolia*, *S. texana*) but not in others (e.g. *S. summa*, *S. roemeriana*). Using the species groups established by Hedge (1974*a*, *b*, 1982*a*, *b*) based on morphological characters and the alliances suggested by Pobedimova (1954), it can be hypothesized 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 eight 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 subgen. *Calosphace*. *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 south-west 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*, 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, shrubby habit with strongly lignified stems (although not present in all species), and, most importantly, on the structure of its stamens (Neissess, 1983). Sect. *Audibertia* is unusual 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

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as would a simple filament, albeit with only a single theca at its end (Bentham, 1876; Epling, 1938; Neissess, 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 (Neissess, 1983), our preliminary data suggest that sect. *Audibertia* (*sensu* Bentham) is a monophyletic lineage (Figs 3 and 4), and the species included in Neissess' (1983) sect. *Echinosphace* probably represent a grade toward a monophyletic sect. *Audibertia* (*sensu* Neissess, 1983). The staminal morphology of sect. *Echinosphace* (four spp.) is distinct from sect. *Audibertia* (*sensu* Neissess) in that sect. *Echinosphace* displays the plesiomorphic character of the posterior branch of the connective and the posterior theca always being expressed, albeit reduced (stamen type I, Fig. 5). Section *Audibertia* (*sensu* Neissess) displays the derived character of no expressed posterior theca, and thus it is possible to define a progression from both thecae being expressed to the entire abortion of the posterior theca in this clade as well.

Salvia subgen. *Calosphace* consists of nearly 500 species and occurs throughout the New World, with centres 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). Stumbling blocks to past and future work in subgen. *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 (eight 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 subgen. *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, among others, Claßen-Bockhoff *et al.* (2004*a*), Baikova (2002, 2004), Epling (1939), a tooth is often present on the lower side of the posterior connective branch. Claßen-Bockhoff *et al.* (2004*a*) clearly demonstrated ontogenetically that the aborted posterior theca may be either located at the distal end of the connective arm, or in some cases represented by a dorsal outgrowth of the connective. Their finding suggests that the formations of the connective arm found within subgen. *Calosphace* that form the basis of the lever mechanism may not all be homologous. Despite that important difference, staminal morphology within the subgenus is uniform with respect to no posterior thecae being expressed and the two posterior connective arms, or dorsal outgrowths of the connective being fused. This uniformity is true across the entirety of subgen. *Calosphace* except for four of Epling's sections (sections *Hastatae*, *Blakea*, *Standleyana* and *Axillares)*. Sections *Hastatae* (seven spp.), *Blakea* (four spp.) and *Standleyana* (one 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 and 5), and form a monophyletic group. *Salvia axillaris*, of monotypic section *Axillares*, is the only member of *Salvia* subgen. *Calosphace* to have expressed posterior thecae (stamen type G, Fig. 5). The molecular phylogeny suggests that *S. axillaris* is sister to the remainder of subgen. *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 subgen. *Calosphace* of abortion of posterior thecae and fusion of connective branches (see Figs 5 and 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 (Figs 3 and 4).

One of the two lineages consists of a group of western Asian and northern African species including *S. aristata*, *S. aegyptiaca*, *S. tetrodonta*, *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 (1974*a*, *b*, 1982*a*, *b*), based on morphological characters in addition to the species sampled here, this first lineage of *Salvia* 'clade III' probably also includes *Salvia bazmanica*, *S. santolinifolia*, *S. macilenta*, *S. tebesana*,

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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. In our sampling, this clade consists of *S. glutinosa*, *S. miltiorrhiza*, *S. hians*, *S. cynica*, *S. przewalskii*, *S. digitaloides* and *S. roborowskii. Salvia glutinosa* and *S. miltiorrhiza* are probably the best known members of this group, and each expresses 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 (e.g. *S. glutinosa*, *S. przewalskii*; Claßen-Bockhoff *et al.*, 2004*a*). 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 can be employed whether or not the posterior thecae fuse. Although this group of species probably includes nearly 100 species with a likely centre of diversity in China, it is currently impossible to define the exact extent of this clade owing to lack of familiarity 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

The inferred progression in staminal evolution within the *Salvia* clade is depicted in Fig. 6 based on the tree-mapping of the stamen types defined in this project 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 and 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 staminal lever has thus independently originated three times, each time following the progression described above, and each time resulting in the functionally convergent feature of a staminal lever (Figs 5 and 6).

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 that presented 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* sections *Hastatae*, *Blakea* and *Standleyana* being intermediate between the plesiomorphic state and derived state seen in core *S.* subgen. *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.

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.* (2004*a*) 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.* (2004*a*) concerning homology of the staminal lever contradict the findings here 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.*, 2004*a*, 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.*, 2004*a*, 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 herein, Zalewska (1928), Himmelbaur and Stibal (1932–1934), Hedge (1974*a*, *b*, 1982*a*, *b*) and Claßen-Bockhoff *et al.* (2004*a*) have noted the uniformity of staminal morphology. Second, further support for three independent origins of the staminal lever mechanism comes from the molecular phylogeny, which 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 and 5), but itself often has the posterior branch of the connective aborted (Himmelbaur and Stibal, 1932–1934; Claßen-Bockhoff *et al.*, 2004*a*, *b*; Walker *et al.*, 2004). In each of these three examples, the stamens have gone through a complicated evolutionary progression only

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to end up with a stamen that in superficial appearance 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') there is 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 (Figs 5 and 6).

Issues in cases of parallel evolution

That all *Salvia* belong to a single, well-defined lineage within the tribe Mentheae begs the question of whether *Salvia* is truly polyphyletic or simply paraphyletic. To make the nearly 1000 species of *Salvia* monophyletic would require only the inclusion of 13 species from the genera *Perovskia* (seven spp.), *Rosmarinus* (two spp.), *Meriandra* (two spp.), *Dorystaechas* (one sp.) and *Zhumeria* (one 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 polyphyletic 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 of staminal evolution in *Salvia* presented here 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 seen 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.* (2004*a*), Thimm *et al.* (2005), Wester and Claßen-Bockhoff (2006) and Reith *et al.* (2006), 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, will, it is hoped, ultimately afford the opportunity to address the suggestion of Claßen-Bockhoff *et al.* (2004*b*) 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).

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