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REVIEW

Stamen construction, development and evolution in *Salvia* s.l.

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Abstract

Lever-like stamens are among the most important floral traits in *Salvia* s.l. Their morphology, function and diversity were repeatedly studied to understand the lever mechanism and to reconstruct stamen evolution. The present paper reviews current knowledge on stamen diversity in *Salvia* species and combines morphogenetic and phylogenetic data. Five stamen types are distinguished based on absence vs. presence of versatile anthers, bithecate vs. monothecate anthers, curved vs. straight connective growth and presence vs. absence of the lower lever arm. Type I (no versatile anthers) is restricted to the four species of the Californian sect. Echinosphace. Versatile anthers evolved at least five times: Type II (bithectae) is the most common one in all old world *Salvia* s.l. clades, but also appears twice in America. Type III (monothectae, curved connective) is derived from type II (ontogenetic abbreviation). It is not restricted to the Eurasian-African clade I (*Salvia* s. str.) and the E-Asian clade IV-A, but also underlies some bee-pollinated American species. The new function of the lower lever arm to serve as a sterile barrier goes along with novel growth centres (ontogenetic addition). Type IV (monothecate, straight connective) characterises the majority of the American species (subgen. Calosphace). Type V (no lower lever arm) is derived by ontogenetic abbreviation and most likely homoplastic. It evolved at least four times independently, i.e. twice in *Salvia* s. str., once in the Californian clade II-B (sect. Audibertia) and once or several times in the East Asian clade. The study shows that stamen diversity results from few developmental changes. The latter appear in different combinations and support subclades rather than clades.

Keywords: Inhibition, flower force, novel growth centers, ontogenetic abbreviation, ontogenetic addition, parallel evolution, phylogeny, staminal lever mechanism, stamen diversity

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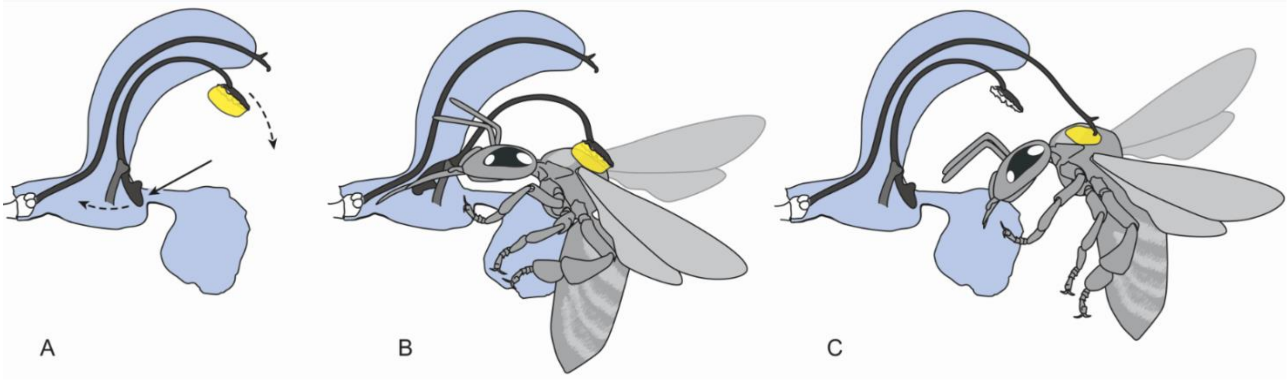
Introduction

More than 200 years ago, Christian Konrad Sprengel (1793) published his epochal work on pollination biology including the first description and illustration of the staminal lever mechanism in *Salvia.* Since that time, the peculiar pollination mechanism represents one of the classic examples of dorsal pollination (Fig. 1). For decades, it supported the assumed monophyly of the genus. However, today we know that *Salvia* is not monophyletic (Walker et al., 2004; Will & Claßen-Bockhoff, 2017) and that parallel evolution has played a significant role. Not surprisingly, also the staminal lever mechanism evolved several times in parallel (Walker & Sytsma, 2007, Will & Claßen-Bockhoff 2014) again raising the question of its evolutionary significance.

Interest in *Salvia* increased remarkably in the last 15 years. Due to the many species, the worldwide distribution and high ecological, structural and functional diversity (Figs. 2-5) the genus is an appropriate model system to study the function and evolution of floral traits (Claßen-Bockhoff & Speck, 2000; Claßen-Bockhoff et al., 2003; Speck et al., 2003). Among them, the lever-like stamens are of particular interest. Their reversible and precise movement contributes to pollen saving and pollen dispensing, their specific relative proportions to mechanical isolation and speciation (Claßen-Bockhoff et al., 2004b). Beyond, stamens served as morphological characters to group the diversity of *Salvia* species (Zalewska, 1928; Himmelbaur & Stibal, 1933; Hrubý, 1934) and are recently used to support phylogenetic clades (Walker & Sytsma, 2007; Walker et al., 2015; Will & Claßen-Bockhoff, 2014).

Figure 1. Pollen transfer in *Salvia pratensis.* **A**, longitudinal section showing the left lever-like stamen (from front view) and its movement (arrows). **B**, in the male flowering stage, a bee searching for nectar will be loaded with pollen (yellow) when releasing the lever mechanism. **C**, visiting a flower in the female flowering stage results in pollen transfer to the stigma, which is now in the position previously occupied by the anther.



The present paper reviews the recent knowledge on stamen diversity in *Salvia*. It uses developmental data to reconstruct the steps of diversification and illustrates the benefits of combining ontogenetic and phylogenetic data.

Materials and Methods

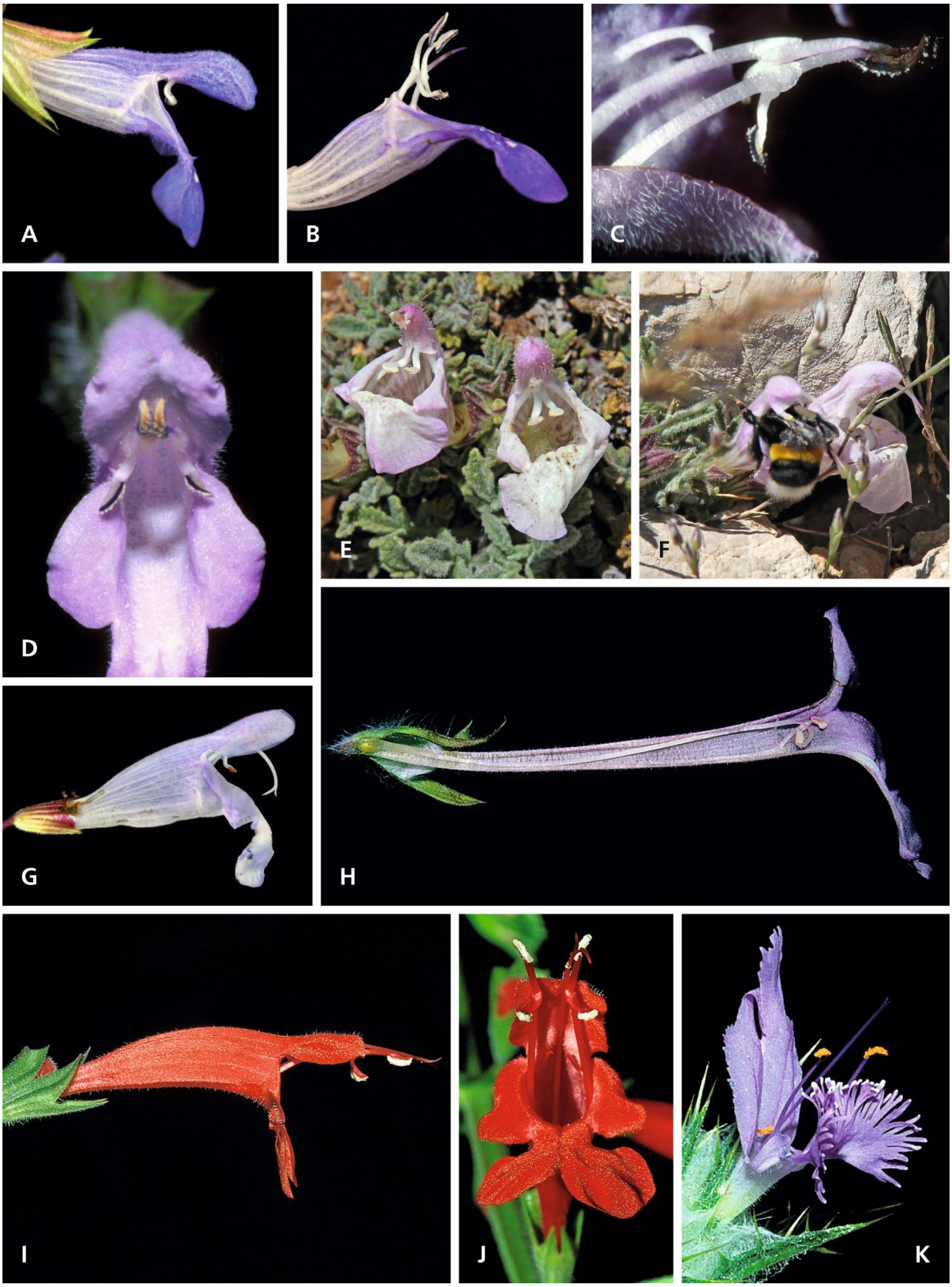
This present review is based on published and unpublished data collected during the last 15 years. Currently, there is a dispute about maintaining *Salvia* as a large genus including five genera into Salvia s.l. (Drew et al., 2017) or splitting the taxon into six genera (Will and Claßen-Bockhoff, 2017). The present paper is based on the second view, but (as a revision is still lacking) uses the traditional names to ease reading. The systematic position of the clades and the proposed generic names are given in Figure 10.

Results and Discussion

Stamen construction

The basal elements of the lever mechanism are the filaments, connectives and thecae of the two abaxial stamens. They vary in position, growth dynamics and size resulting in the well-known diversity of staminal forms.

Figure 2. Bithecate anthers (types I, II, Table 1, Fig. 10). **A-J**, type II. **A-B, *S. officinalis***. A, posterior thecae hidden below the upper lip; B, upper lip removed. **C, D, *S. scabra*.** C, lever-like conncetive exposed by a rather long filament; D, front view of the flower entrance showing theca arrangement for dorsal and lateral pollination. **E, F, *S. caespitosa***. E, flower entrance only half closed by the anterior lever arms allowing small insects to steal nectar and pollen; F, imprecise pollen deposition (`smear effect´) onto the thorax of a bumble bee due to the small lever proportions. **G. *S. brachyodon***. As in E, at the base a hole (arrow) bitten by bumblebees. **H, *S. whitehouseii***. Extremely long corolla tube with small connective levers (longitudinal section, only one connective shown). **I, J, *S. roemeriana***. Hummingbird-pollinated species. **K**: type I. ***S. carduacea***. No lever mechanism. Clade affiliation (Fig. 10): A, B, E, F: I-D; C, D: I- A; H-J: I-B; K: II-B. Photos D, H-K: P. Wester, Mainz/Düsseldorf.



Filaments

The filaments of the two stamens are adnate to the lateral flanks of the corolla. They lack any appendages or outgrowths, but vary considerably in length, exit angle from the lower lip and stability. When the lever mechanism is released, the filaments transfer the exerted force to the corolla (Reith et al., 2007). This can result in a severe deformation of the flower influencing pollination (Ott et al., 2016). Furthermore, the filaments support the levers and place them in their final position. Being essential floral traits, their diversity and functional significance are not carefully investigated so far.

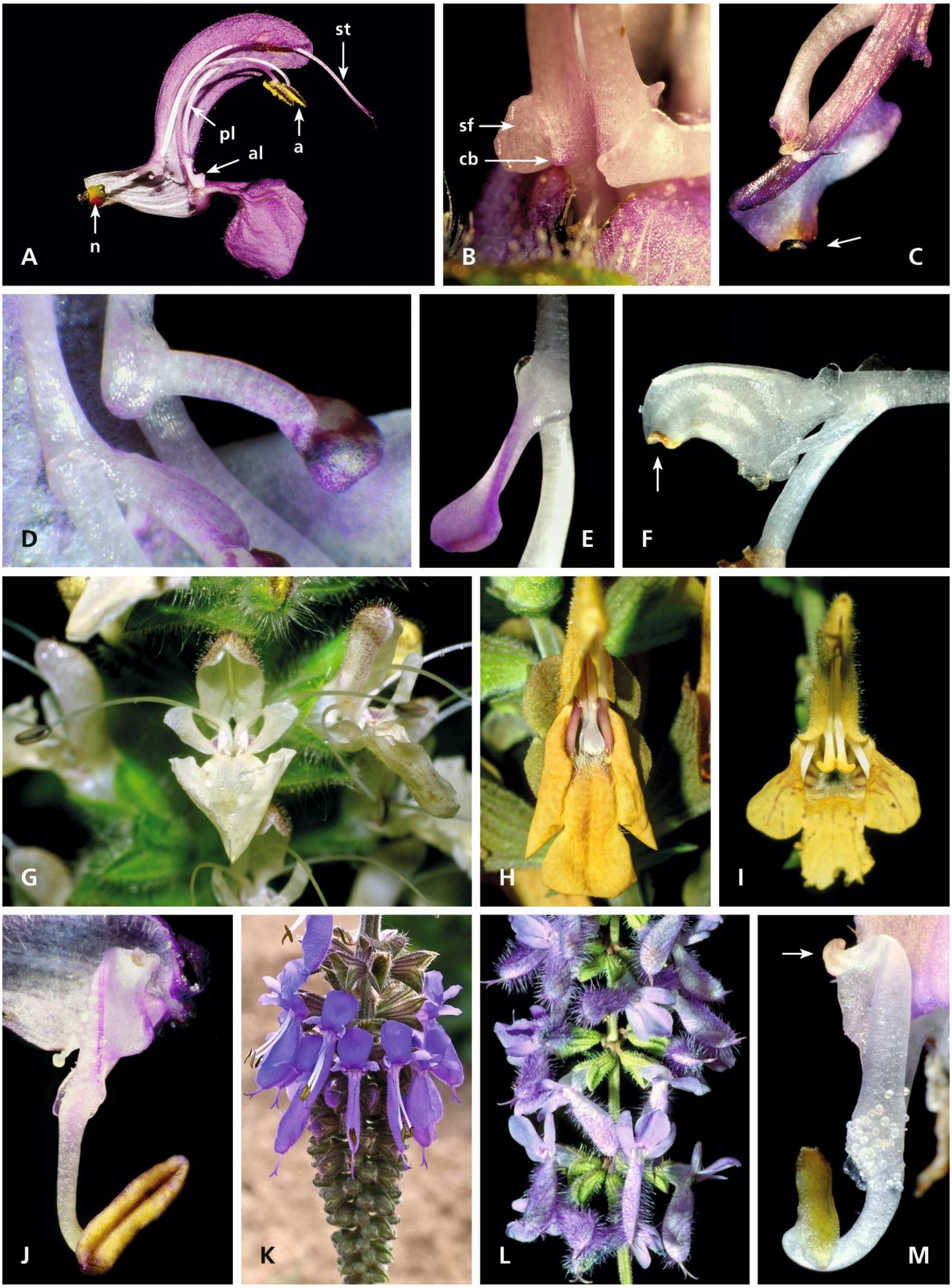
Filament tip and joint formation

Salvia species have versatile stamens (except type I, clade II-C, Fig. 10), i.e. the primary filament tip forms a very thin ligament allowing the connective to swing around the thus formed joint (Troll, 1929). In the majority of the species, tissue swellings in the shape of a secondary filament tip and a connective bulge cover the joint (Claßen-Bockhoff et al., 2004a; Fig. 6L-N). These structures closely interlock and restrict the free mobility of the anther like a hinge. Thereby, they increase and maintain precision during the reversible anther movement (Claßen-Bockhoff, 2004b; Thimm, 2008). The swellings, composed of large parenchymatic cells, may also play an essential role in the movement itself. Correns (1891) postulated torsion within the joint, but first histological studies (Thimm, 2008; unpubl. data) indicate that the joint remains stiff and gets passively twisted against the connective and filament protrusions. More histological and experimental investigations are needed to fully understand the biomechanics underlying the lever movement in *Salvia* s.l.

Connectives and lever formation

The remarkable extension of the connective separates the two thecae of each stamen and forms two lever arms. The upper (anterior) arm always ends in a fertile theca with two pollen-sacs, while the lower (posterior) arm is highly diverse producing pollen-sacs (**bithecate** anther; Fig. 2) or remaining sterile (**monothecate** anther; Figs. 3-4). Connectives are curved or straight and vary considerably in the proportions of their arms. The upper connective arm is rather uniform hiding the pollen-sacs below the upper lip (particularly in bee flowers; Fig. 3A) or exposing them out of the flower (many bird-pollinated species; Fig. 4B, G). The two neighbouring thecae are usually free, but may be fused increasing the precision of pollen deposition. The lower lever arms, in contrast, are highly diverse, often forming elaborate three-dimensional structures (e.g. Fig. 3C-F; Fig. 7E, F) or producing tooth-like outgrowths (e.g. Fig. 4J-M; Fig. 7A, B). They are free from each other (Fig. 2B, J), stick together at the rudimentary theca walls (Fig. 3C, F, M: arrows; Fig. 7F) or are connected with epidermal papillae or hairs along their whole length (Fig. 4B, D; Fig. 7B, D). They usually restrict access to nectar. Pollinators have to push back the barrier thereby releasing the lever mechanism (Fig. 1). As a consequence, the upper arm lowers down and transfers pollen onto the head or body of the visitor thus mediating dorsal (nototribic) pollen deposition (Müller, 1873; Correns, 1891; reviewed in Claßen-Bockhoff et al., 2003).

Figure 3. Monothecate anthers with curved connective (type III, Table 1, Fig. 10). **A-C**, ***S. pratensis***. A, longitudinal section: a, anther; al, anterior lever arm; n, nectary; pl, posterior lever arm; st, style. B, joint covered by secondary filament tip (sf) and connective bulge (cb). C, shovel-like lower lever formation. **D-F,** anterior lever formations. D, ***S. stenophylla***. E, ***S. forsskoalei.*** F, ***S. argentea.*** **G,** ***S. austriaca*****,** lateral pollination. **H,** ***S. africana-lutea,*** complete closure of flower entrance by paddle-like barrier. **I,** ***S. glutinosa,*** anterior lever arms with conspicuous rudimentary thecae. **J, K,** ***S. nutans*****.** J, resupinate lever-like anther. K, hanging inflorescence. **L, M,** ***S. jurisicii.*** L, erect inflorescence with resupinated flowers. M, resupinate lever-like anther. Arrows in C, F, M: rudimentary theca. Clade affiliation (Fig. 10): A- C, F, G J-M: I-C; D, H: I-A; I: IV-A. Photos B-F, J, M: M. Crone, Mainz.



Lever-movement

Salvia s.l. is characterised by the staminal lever-mechanism. The group is extremely successful as to species number and world-wide distribution. Thus, one might assume that the pollen transfer mechanism contributes to speciation by selecting pollinators by physical force (as shown for Fabaceae in Córdoba & Cocucci, 2011). Only strong bees and birds would then get access to nectar, while weak insects were excluded. However, force measurements in more than 30 species representing the diversity in *Salvia* s.l. (Thimm, 2008; unpubl. data) show the very reverse. Forces are generally low ranging between 0.5 mN and 10(-40) mN. They are lower than the average force honeybees (14 mN) and bumblebee workers (25 mN) can afford (Reith et al., 2006) and comparable to forces needed to penetrate a hairy ring in the corolla tube (*S. verticillata*: 3.6 mN, Thimm, 2008; *S. glutinosa*: 2.5 mN, Reith et al., 2006). Obviously, forces do not exclude weak insects from nectar (Stöbbe et al., 2016).

Forces needed to release the lever mechanism vary among species. Preliminary data indicate that the force depends more on the lever weight than on its size and that a higher force is demanded by bird- than by bee-pollinated species (Thimm, 2008). However, more data are needed to confirm the first results (in process).

Forces also vary within and among flowers of the same individual. The first release usually demands the highest force particularly in species with upper lips enclosing the fertile thecae (e.g. *S. pratensis*). When repeatedly used (e.g.10-20 repetitions), the lever shows symptoms of wilting. The fertile thecae do not return completely to their previous position and the needed forces decrease (Thimm, 2008). In bee-pollinated species, the different force demands of young and old flowers may play an important role. Flowers are protandrous in this species and open in an acropetal sequence. As bees usually visit their food plants bottom-up (Harder et al., 2004), they first transfer foreign pollen to the stigma of an old flower and then take pollen from a younger flower. As these flowers demand more force, the pollinator may decide to leave the inflorescence, a behaviour increasing outcrossing in the population. Experiments with bumblebees and honeybees on artificial flowers show that both species are able to discriminate between a barrier-free model and a lever-model demanding 2 mN (Stöbbe et al., 2016). It is thus very likely that weak forces influence the selection of a food plant, the foraging behaviour of a bee and the degree of outcrossing.

Lack and loss of lever function

The majority of the *Salvia* species follows the general picture having a bilabiate flower, a reversible lever mechanism and a dorsal pollination. However, there are remarkable exceptions concerning joint formation, flower construction and pollen deposition site.

No joint formation

In the Californian section Echinosphace (4 sp.; Fig. 10: type I, clade II-C), the anther is not versatile. Instead, the connective is inflexibly fixed to the filament (Fig. 6H). Consequently, the species lack the lever mechanism completely (Himmelbaur & Stibal, 1933; unpubl. data) and does not belong to *Salvia* if defined by mobile anthers. However, as in *Salvia* species, the connective widens and separates the thecae presenting the anterior one out of the flower and the posterior one in the flower entrance (Fig. 2K). Both thecae contribute to pollen transfer, depositing pollen on the front and lower side of the insect pollinator (own obs.).

Figure 4. Monothecate anthers with straight connective (type IV, Table 1, Fig. 10). **A, *S. involucrata***, anterior lever arm blocks access to nectar. **B**, ***S. tubiflora***, straight lever arms without any outgrowths. **C**, ***S. coccinea,*** joint covered by secondary filament tip (white), connective bulge small (not visible). **D, *S. splendens***, joint largely uncovered. **E, F, *S. elegans,*** longitudinal sections. E, no space for lever movement, F, rare appearance of a rudimentary posterior theca. **G, *S. haenkei*** with *Sappho sparganura.* Pollen transfer without lever mechanism. **H, *S. cacaliifolia***, no lever movement due to lever arm proportions. **I, *S. consobrina***, view onto the staminal levers, lower lip removed. **J-M,** species with ventral outgrowths. J, ***S. stachydifolia***, longitudinal section**.** K, L, ***S. rypara* ssp. *platystoma.*** K**,** frontal view showing the barrier in the flower entrance. L, longitudinal section. M, ***S. sophrona***, longitudinal section. **N-Q**, species with dorsal outgrowths, longitudinal sections (N-P) and detail of the anterior lever arm (Q). N, ***S. corrugata*.** O, ***S. cusidata* ssp. *bangii*.** P, Q, ***S. uliginosa*.** Clade affiliation (Fig. 10): all II-A. Photos: A, D-H, J-L, N, O: P. Wester, Mainz/Düsseldorf.

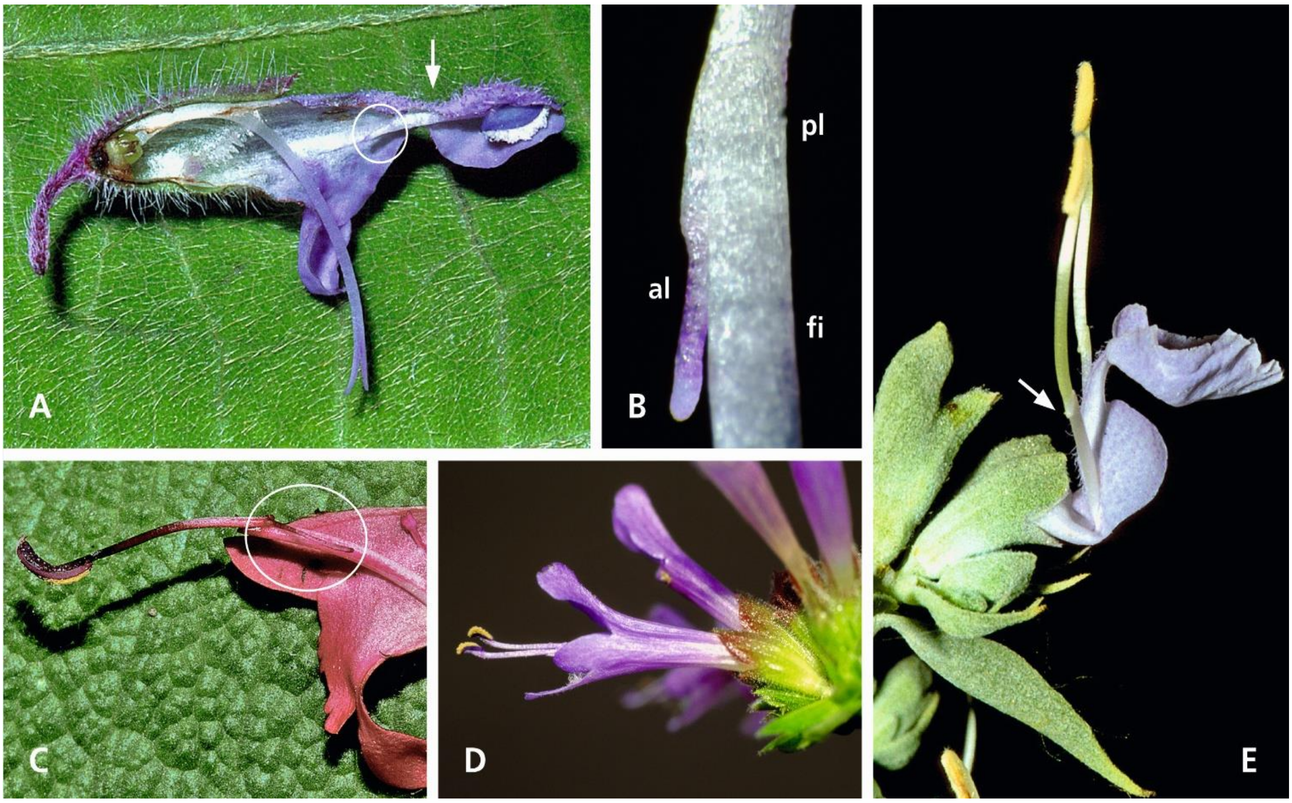
Reduction of lower lever arm

A second group of species lacks the lever mechanism due to the extreme reduction of the lower connective arm. This reduction is found at least four times independently (Fig. 10, type V: orange colour) and goes along with a change in flower construction and pollination mechanism.

In the *S. verticillata* group (4 spp., Will & Claßen-Bockhoff, 2017; Fig. 10: clade I), the lower connective arm forms a vermicular appendage (Fig. 5B). The fertile thecae hide the pollen-sacs below a movable upper lip which is pushed aside by the pollinator thus mediating frontal pollination (Fig. 5A; Correns, 1891; Claßen-Bockhoff et al., 2004b).

In the majority of the species placed in sect. Audibertia (19 ssp.; Walker et al., 2015; Fig. 10: clade II-B), the reduction of the lower connective arm (Fig. 5C) is so extreme that almost no rudiment is left in the adult flower (Fig. 5D, E:*, Fig. 6J). Rather, the upper connective arm continues the filament in a straight-line way looking like a `normal´ stamen, however, with only a single theca. In *S. apiana* (Fig. 5E), pollen is exposed out of the flower but not accessible for pollen collecting bees. It is deposited to the bee's body by corolla deformation passively lowering the fertile thecae (Ott et al., 2016).

Figure 5. Monothecate anthers with reduced anterior lever arm (type V, Table 1, Fig. 10). **A, B, *S. verticillata*. A,** longitudinal section showing the pollen-sacs hidden below the movable (arrow) upper lip, the reduced anterior lever arm (circle) and the style exposed out of the lower lip. B, detail with reduced anterior lever arm (al), part of the posterior lever arm (pl) and filament (fi). **C, *S. spathacea,*** with vermicular lower lever arm (circle), dorsal pollination. **D, *S. clevelandii***, ventral pollination. **E, *S. apiana***, lateral pollination. * in D and E: rudiment of anterior lever arm. Clade affiliation (Fig. 10): A, B: I *S. verticillata*-clade; C-E: II-C. Photos B: M. Crone, Mainz; C, E: P. Wester, Mainz/Düsseldorf.



Loss of the lever mechanism

The shift from bee to bird pollination is often associated with a dramatic change in flower construction. Wester & Claßen-Bockhoff (2007) listed 59 bird pollinated species (> 30% of all ornithophilous species) which transfer pollen without a lever mechanism. In these species, pollen and nectar are freely accessible as the thecae are exposed out of the flower and the lower lever arms do not block the flower entrance. Interestingly, some species still have a functional lever mechanism which is, however, not releasable due to the narrow flower tube (*S. haenkei*; Fig. 4G; Fig. 10: clade II-1). In other species, the non-use of the lever mechanism goes along with a reduction of the filament and connective protrusions around the joint (*S. longistyla*) or with a stiffening of the joint (*S. elegans*, Fig. 4E). In *S. exserta*, the lower lever arm blocks the flower entrance, but the joint is stiff. Here, the lever-mechanism is released because the force is transferred to the long and elastic filaments passively moving the lower lever arm (Wester & Claßen-Bockhoff, 2007).

Loss of dorsal pollination

While nototribic pollination is the common way of pollen deposition onto the head, thorax or bill of a pollinator, frontal pollination (*S. verticillata*; Fig. 5A), lateral pollination (*S. austriaca;* Fig. 3G) and abdominal pollination (*S. glutinosa*; Fig. 3I) appear dependent on the length and position of the upper lever arms (Claßen-Bockhoff et al., 2004b). In case of bithecate anthers, pollen from the anterior theca ensures dorsal and from the posterior theca lateral pollination (*S. scabra*, *S. roemeriana*; Fig. 2C, J). An exception is *S. carduacea* (Fig. 2K) with ventral and frontal pollination. In the Californian sect. *Audibertia* (Fig. 10: clade II-B), the monothecate anthers are in variable positions. The often-exposed fertile theca mediates dorsal pollination in *S. spathacea* (Fig. 5C), lateral pollination in *S. apiana* (Ott et al., 2016) and ventral pollination in *S. clevelandii* (Fig. 5D). It is likely that the different sites of pollen deposition on the pollinator contribute to mechanical isolation and speciation (Grant 1994).

As tested in *S. gravida* (Wester et al., 2012), hanging inflorescences often respond to gravity and turn their flowers to ensure nototribic pollination. However, in *S. nutans* (Fig. 10: clade I-C), this resupination does not happen resulting in ventral (sternotribic) pollination (Fig. 3K). Sternotriby also characterizes *S. jurisicii* (Fig. 3L; Fig. 10: clade I-C). This species has erect inflorescences whose flowers turn down individually, thus acting like *S. gravida* but in the opposite direction. Both sternotribic species have well-developed staminal levers (Fig. 3J, M). Experiments with bumble bees (*Bombus terrestris*; unpubl. data) have shown that the insects can only handle the flower and release the lever mechanism when the upper lip serves as landing place thus illustrating that the whole flower construction has adapted to ventral pollination.

Stamen development

The monosymmetric flowers in Lamiaceae lack the median-adaxial stamen. Consequently, the pentamerous flowers only develop four stamens. Their primordia appear in a cycle (Fig. 6A, B), but in the adult flower, the thecae are usually placed below or in front of the upper lip (Fig. 8A, B). This dislocation of the abaxial pollen-sacs takes place during flower development. A common process to move the thecae in the right position for dorsal pollination is connective widening (Fig. 8C). It is not only found in Lamiaceae, but also in Gesneriaceae (*Nemathathus*), Orobanchaceae (*Pseudosopubia*), Scrophulariaceae (*Calceolaria*) and other families of the Lamiales, most likely representing a plesiomorphioc condition in the lineage. While all four anthers are presented in a row in the adult flower of *Prunella vulgaris* (Fig. 8B), *Lamium* species show a didynamic anther position with two pairs of anthers located one upon the other (Fig. 8D, E). With respect to the central position of the style, this pollen-sac arrangement is more effective. Interestingly, the same pattern is repeated in *Salvia* based on only two stamens with spatially separated thecae (Fig. 2D, J).

Figure 6. Anther development and joint formation. **A-C**, flower development (*S. viridis*). Black stars: stamen primordial, white stars: staminode promordia. **D**, flower bud (*S. leucantha*): view onto the abaxial stamen. **E**, bithecate anther, type I (*S. funerea*). **F**, monothecate anther, type III (*S. leucantha*). **G**, monothecate anther, type V (*S. apiana*). **H**, anther not versatile, type I (*S. greatae*). **I**, anther versatile, type II (*S. columbaria*). **J**, joint stiff, type V (*S. mellifera*). **K-N**, *S. glutinosa*, type III. K, thin ligament between filament and connective (j: joint). L, coverage of the joint by filament and connective protrusions. M, secondary filament tip. N, connective bulge and outline of the joint (after removal). co, connective. f, filament. j, joint. lca, lower connective arm. lt, lower theca. uca, upper connective arm. ut, upper theca. Bars: A- C (same scale), 200µm. D, 40µm. E, G, K: 100µm. F: 50µm. H, I, J: 250µm. L-N: 200µm. Clade affiliation (Fig. 10): A, D, II-B. B, I-C. C, E, F, II-C. G-J, IV-A. Photos A-C: N. Daichent, Mainz. D, F, I, K-N. M. Crone, Mainz. E, G- J: S. Deobald, Mainz.

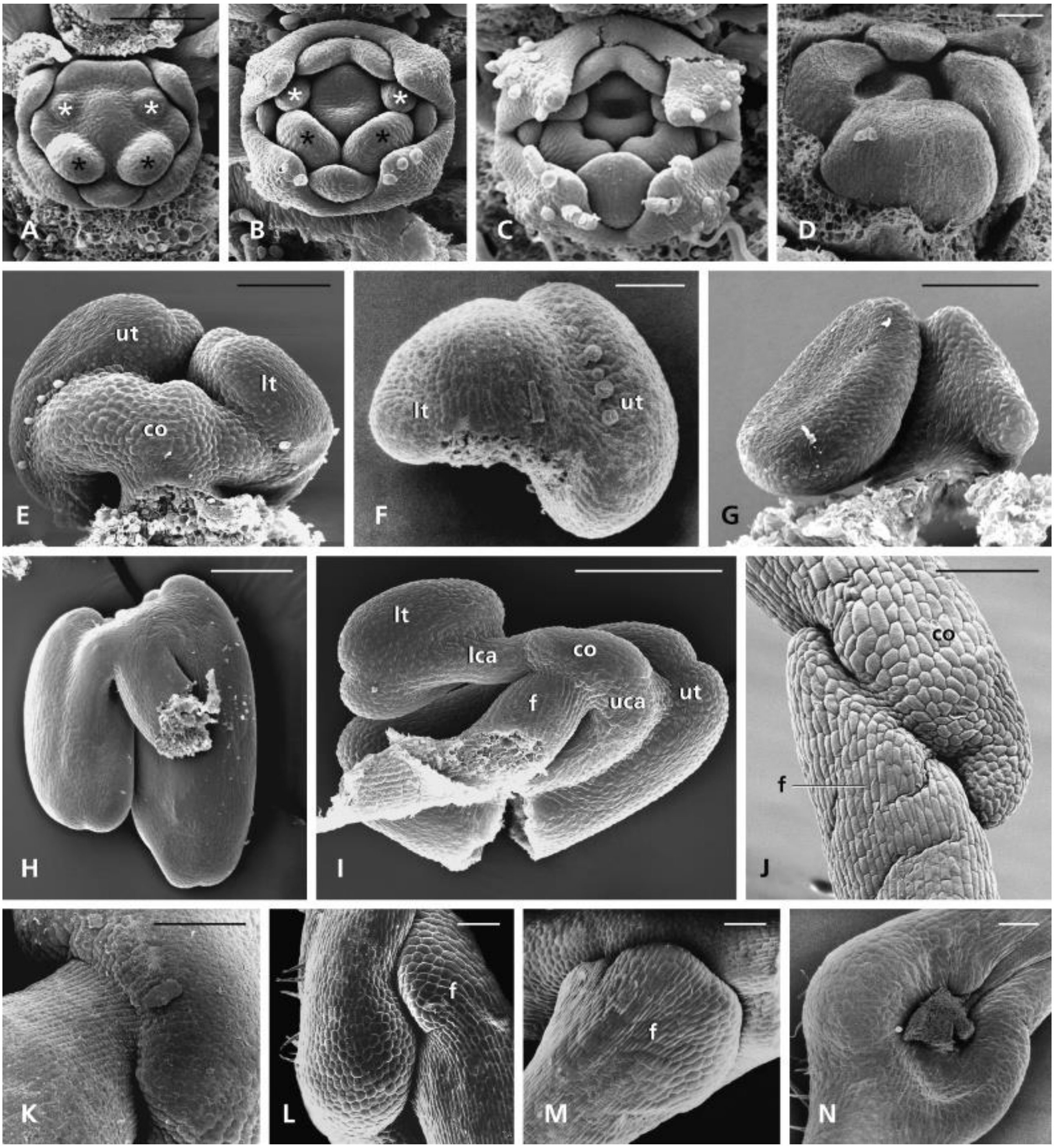
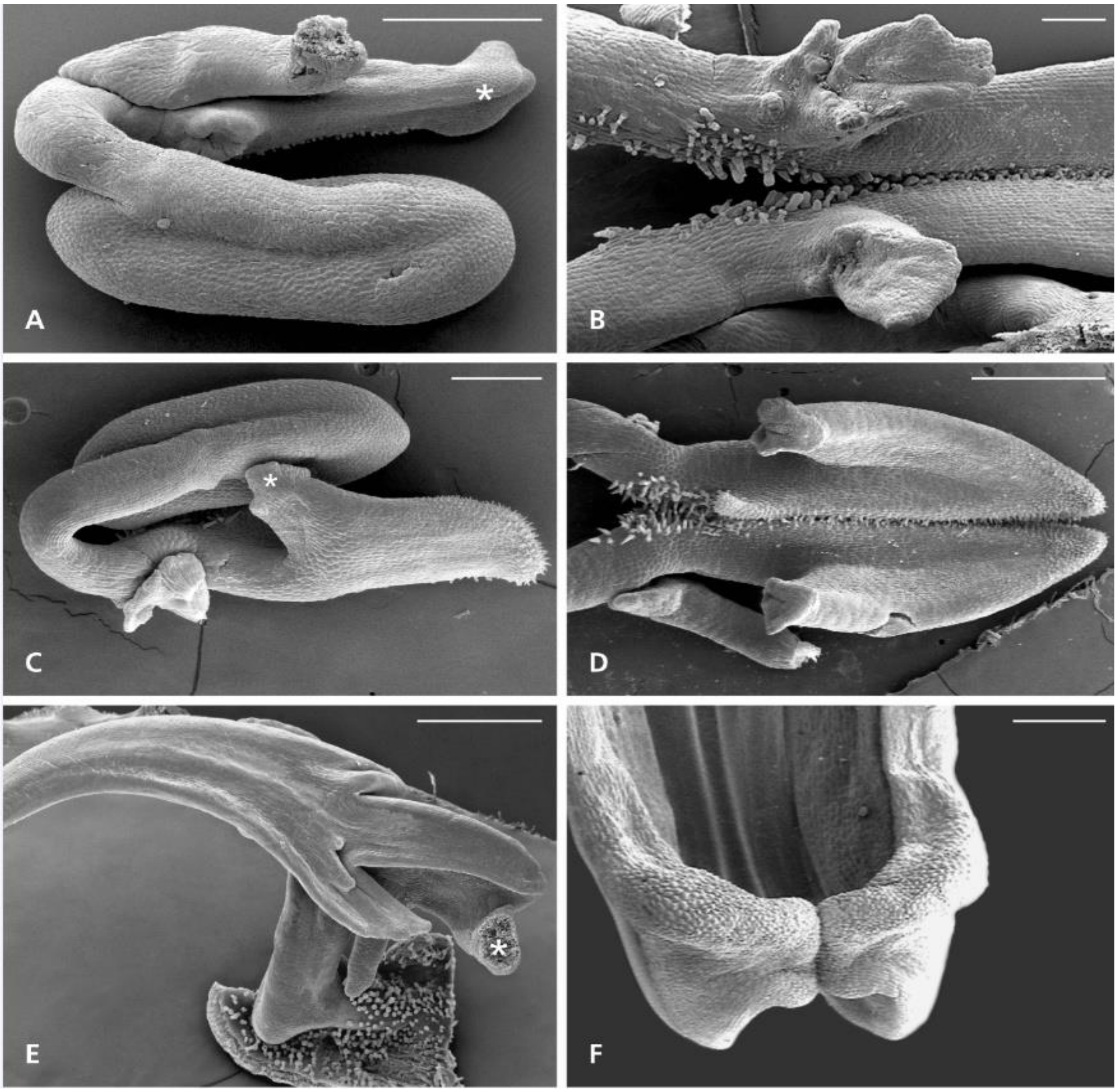
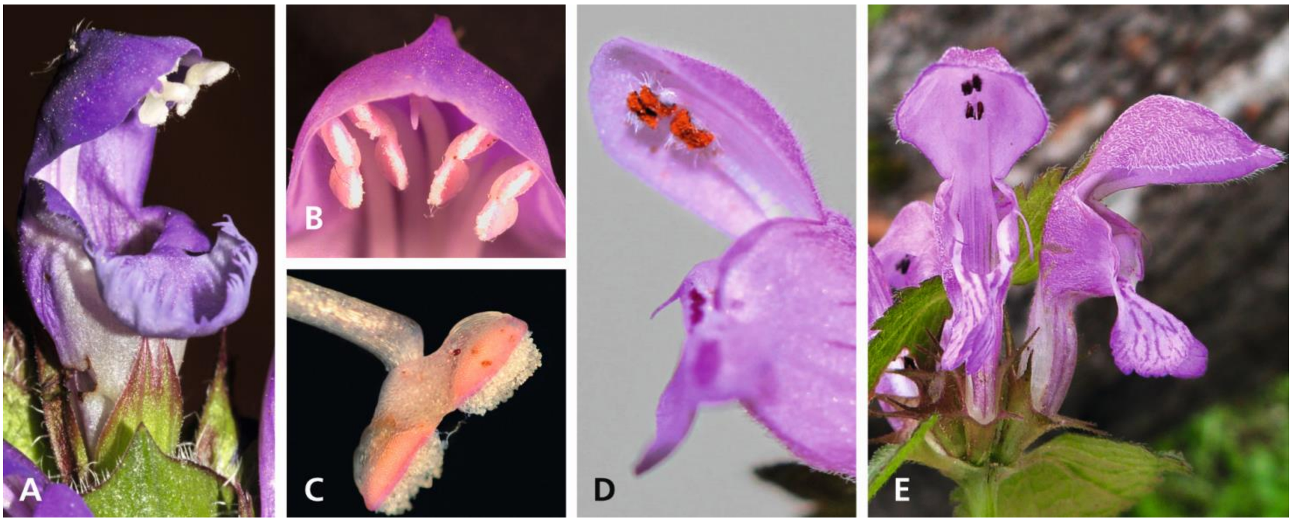


Figure 7. Diversity of anterior levers. **A, B, *S. rypara* ssp. *platystoma***. A, bud stage: ventral outgrowth close to the joint; *, position of reduced theca. B, adult stage: barrier formed by the outgrowths, straight lower lever arms connected by hairs. **C, D, *S. uliginosa***. C, bud stage: dorsal outgrowth; *, position of reduced theca. D, adult stage: barrier formed by the curved posterior levers arms. **E, *S. argentea***, adult stage: filament heel-like attached to the corolla, lower lever arm arched upwards with finger-like protrusions, connected with the neighboring lever at the position of the reduced theca (*). **F, *S. sclarea***, adult stage: two spoon-shaped lower lever arms forming a hole by being connected at the reduced thecae walls. Bars: A-C: 500µm; D, E: 1mm: F, 1.5mm. Clade affiliation (Fig. 10): A-D: II-A; E, F: I-C. Photos E, F: M. Crone, Mainz.



In *Salvia*, only the two abaxial stamens develop while the adaxial ones remain inhibited and form minute staminodes (Fig. 6A, B: white stars). The thecae are placed below the upper lip by filament and connective elongation (Fig. 2B, C). Extended connectives combined with versatile anthers result in the formation of the staminal lever mechanism. Developmental studies in more than 30 *Salvia* species indicate that the basic steps in stamen development are similar even among highly diverse species (Troll, 1929; Trapp, 1956a, b; Claßen-Bockhoff et al., 2004b; unpubl. data).

Figure 8. Anther position in Lamiaceae with four stamens. **A-C**, *Prunella grandiflora* (Lamiaceae). A, flower from the side, the four anthers located in a row below the upper lip. B, all anthers turned to enable dorsal pollination. C, anther with widened connective dislocating the theca for dorsal pollination. **D**, *Lamium purpureum* and, **E,** *L. villosifolium,* both species with didynamic anther position increasing precision of pollen transfer.



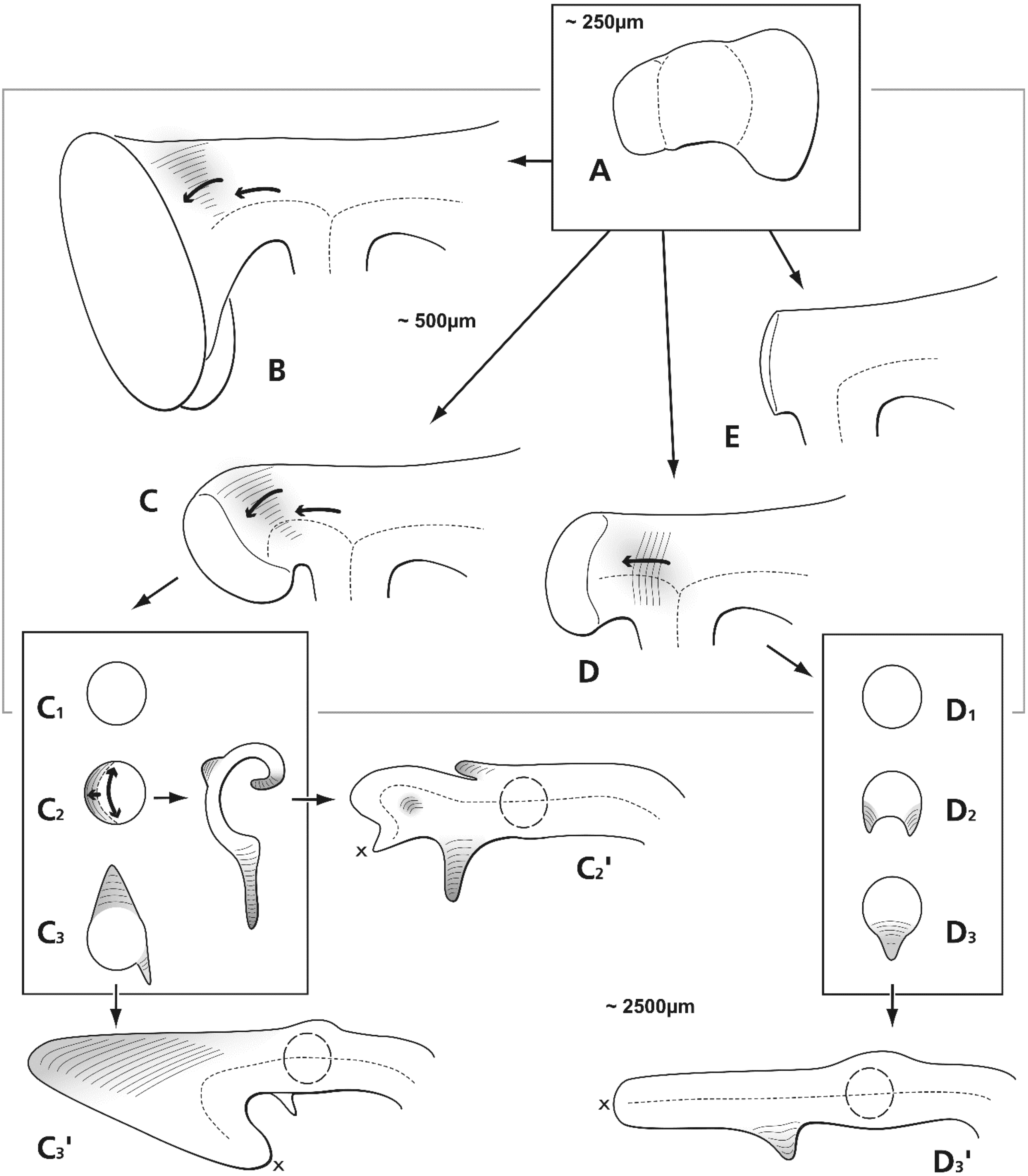
Thecae initiation and inhibition

The initial stage of stamen development in *Salvia* is the formation of an asymmetric anther (Fig. 6D-G; Fig. 9A). In bithecate anthers, both thecae are initiated early, but differ in size, the abaxial (anterior) theca being larger than the adaxial (posterior) one. In monothecate anthers, the posterior theca is inhibited from the beginning (*S. argentea*; Fig. 6F) or clearly initiated and inhibited later (*S. apiana*; Fig. 6G). Interestingly, the time of theca inhibition is independent on the lower lever arm formation as *S. argentea* forms a highly elaborate sterile structure (Fig. 7E), while *S. apiana* lacks this lever arm completely (Fig. 5E). Furthermore, even if the posterior theca is early inhibited, its surface may differ from the connective epidermis in having the characteristic papillae cells of theca walls (Claßen-Bockhoff et al., 2004a). At these papillae, the two adjoined anterior lever arms are often postgenitally fused (Fig. 3C, F, M: arrows; Fig. 7E, F). Occasional theca formation and pollen production in monothecate anthers (Fig. 4F) illustrate that the clear difference between bithecate and monothecate anthers in the adult stage is developmentally only based on a gradual inhibition process.

Connective widening

Young anthers have a relatively broad connective, which starts widening before the filament originates. The upper lever arm always curves in a rectangular way locating the fertile theca in the longitudinal direction of the developing flower (Fig. 6I). In the adult stage, it only varies in its length ranging from rather short (Fig. 2B) to extremely long connective arms (Fig. 4H). In contrast, development of the lower lever arm is delayed.

Figure 9. Stamen development in *Salvia*. **A,** asymmetric anther. **B-E**, developmental divergence resulting in bithecate (B) and monothecate anthers (C-E). **B (type II)**, the lower connective arm curves due to increased growth at its distal side and presents the lower theca in the flower entrance. **C (type III)**, as in B, but lower theca reduced and sterile lower connective arm forming a highly diverse barrier in the flower entrance. **C_1-$C_{3'}$**, cross sections and side views of sterile connective arms. **D (type IV)**, straight extension of the lower connective arm; lower theca extremely reduced. **D-$D_{3'}$**, cross sections and side view of sterile lever arm. **E (type V)**, lower lever arm reduced, resulting in a one-armed connective. Stamen type I (no versatile anther) not included. Hatched areas: novel centres of growth. Dotted circle: position of the joint (from Claßen-Bockhoff et al., 2004b).



Versatile anther formation

In the next developmental stage, the filament elongates. In *Salvia* clade II-C (sect. Echinosphace; Fig. 10; type I), the anther is immobile as the transition from the filament to the connective remains stiff. In all other *Salvia* species (as far as known, see Himmelbaur & Stibal, 1933), a joint is formed by differential growth of the filament and filament tip. While the filament gets thicker with further development, the filament tip remains thin and forms a ligament (Fig. 6I, K: j). Very soon, swellings at both sides of the joint develop and cover the ligament (Fig. 6I, L-M; Claßen-Bockhoff et al., 2004a). Species that lost the lever function often show a stiff joint characterized by a broad contact between filament and connective (Fig. 6J). Such a stiff joint easily results from a less distinct difference among filament thickening and filament tip formation.

Diversification of the lower lever arm

In bithectae anthers, the connective arm always grows more intensively on its dorsal side resulting in a curved shape (Fig. 9B; Fig. 10: type II). This bending allows both thecae to be placed in the flower entrance and contribute to pollination. In monothecate anthers, the process of theca inhibition is either continued by the complete inhibition of the posterior connective arm (Fig. 9E; Fig.10: type V) or followed by an immense promotion of the sterile connective part. In old world *Salvia* s.l. species, the lower lever arm still grows more on its dorsal side blocking the flower entrance (Fig. 10: type III). The formation of three-dimensional barriers (Fig. 3C-E; Fig. 7E, F) often goes along with novel growth centers at the connective (Fig. 9C-$C_{3'}$). In many new world species, the lower lever arms elongate equally and gets a straight shape (Fig. 4A, D, E; Fig. 9D; Fig. 10: type IV). Tooth-like protrusions on the ventral side (Fig. 4J-M; Fig. 7A, B), and dorsal outgrowths (Fig. 4N-Q; Fig. 7C, D) are based on novel cell division areas (Fig. 9 D-$D_{3'}$).

Evolution of stamen diversity: a functional phylogenetic view

Stamen development in *Salvia* s.l. only includes few developmental processes the most important ones being inhibition (joint formation, theca and lower lever arm reduction), differential growth (curved vs. straight shape) and late developmental formations (proportion, fusion, appendages). The combination of connective widening and versatile anther attachment constitutes the origin of the staminal lever mechanism. A similar construction evolved in some Australian species of the tribe Westringieae (Lamiaceae subfam. Prostantheroideae; unpubl. data) and in the Scrophulariaceae, e.g. *Pseudosopubia obtusifolia*, *Calceolaria pinnata* and *C. scabiosifolia* (Trapp, 1956a, b). However, protrusions covering the primary filament tip and guiding the lever movement are only known from *Salvia.* The thereby gained precision of pollen deposition may be one of the main forces driving the evolution of the staminal level mechanism and, in consequence, speciation.

Anther diversity was repeatedly used for systematic purposes (Correns, 1891; Zalewska, 1928: 206 sp.; Himmelbaur & Stibal, 1933: 430 sp.; Hrubý, 1934; Walker & Sytsma, 2007: 85 sp.; Table 1). While most authors used few \`types´ indicating basic differences, Walker & Sytsma (2007) aimed to support phylogenetic clades with stamen morphological characters. They added relative proportions and secondary fusion of anthers and lower lever arms thereby increasing the number of \`types´. The present situation is that the term \`stamen type´ refers to two different levels, i.e. the levels of characters (referring to early developmental events like presence, shape and fertility of the lower lever arm, Claßen-Bockhoff et al., 2004a) and character states (including late developmental events like variable proportions and secondary fusion modifying the underlying type). Though it is helpful to use stamen morphology to support clades, the increase of stamen types due to character combinations is questionable.

In the present paper, five stamen types are distinguished based on early developmental events (Claßen-Bockhoff et al., 2004a). Except the newly introduced type I, they largely correspond to the types introduced by Himmelbaur & Stibal (1933), Hrubý (1934) and Hedge (1974) (Table 1).

Table 1. Stamen types and distribution in *Salvia*. Himmelbaur & Stibal: types I-V, *Aud*, Audibertia. Walker & Sytsma: types A, B, E-I, M, N; free/fused refers to lower lever arms. Will & Claßen-Bockhoff 2017: clades I-IV (Fig. 10). NW, new world. OW, old world. *S, ver*., *S. verticillata*-clade.

Stamen types					**Clade affiliation**	
Present study	Himmelbaur & Stibal, 1933		Walker & Sytsma, 2007		Will & Claßen-Bockhoff 2017	
anther	OW	NW	OW	NW	OW	NW
anther not versatile						
bithecate Type I connective angled				I*		II-B
anther versatile						
bithecate Type II connective curved	I	I	A free M free N	G free	I-A I-D III	I-A I-B II-C
monothecate Type III connective **curved** anterior theca rudimentary	II, V	II	B fused N		I-A I-C IV	
Type IV connective **straight** anterior theca lacking		III		E fused F ± free		II-A
Type V connective straight anterior theca and lever arm **reduced**	IV	*Aud*		H	I-A I-*S. ver.*	II-C

* Walker & Sytsma (2007) did not consider the absence of the versatile anther.

Type I: Bithecate stamens lacking the lever mechanism

This stamen type only appears in *Salvia* clade II-C including the four species of sect. Echinosphace (Fig. 10: symbol with grey connective). The filament is extremely short. The two connective arms are angled and of different length. The upper thecae are exposed out of the flower, while the lower thecae are placed at the flower entrance (Fig. 2K).

Based on phylogenetic data (Walker et al., 2015; Fragoso-Martínez et al., 2017; Will & Claßen-Bockhoff, 2017), the American clades II-B/II-C (*Ramona*, i.e. subgen. *Audibertia*) and II-A (*Lasemia* (i.e. subgen. *Calosphace*) are sister to each other with the small SW-Asian genera *Dorystaechas* and *Meriandra* as closest relatives. It is thus reasonable to interpret the immobile stamen construction in clade II-C as a basal condition.

Lacking versatile anthers, the species are more similar to their Asian relatives than to *Salvia* species only differing from the first by connective widening and different filament and connective proportions (late developmental steps).

Type II: Bithecate stamens with lever mechanism

Versatile anthers with two fertile thecae represent the basal condition in the old world *Salvia* clades, i.e. clade I (*Salvia* s.str.; except clade I-B), III (*Pleudia, Polakia*) and IV *Glutinaria* and in the new world clades I-B and II-B (sect. *Audibertia*, Fig. 10: symbol with blue connective). Interestingly, also *S. axillaris* (Fig. 10^1), the basalmost species of clade II-A (subgen. Calosphace, Fragoso-Martínez et al., 2017), has bithecate anthers questioning the evolution of the straight stamens in this lineage. It is obvious that stamen construction II evolved several times in parallel. From the morphogenetic point of view this is not surprising as the two underlying components, i.e. connective widening and versatile anthers, are widely distributed among angiosperms. The peculiarity in *Salvia* is that both processes are combined resulting in a lever mechanism.

From the evolutionary point of view, connective widening is under strong natural selection to enable dorsal pollination. Based on this precondition, the staminal lever mechanism might have evolved in bee-pollinated species to protect pollen against mass collection (Claßen-Bockhoff et al., 2004b; Westerkamp & Claßen-Bockhoff, 2007; Wester & Claßen-Bockhoff, 2007). As long as the thecae are hidden below the upper lip, pollen is safe. However, in large flowers, e.g. in many E-Asian and Mediterranean species (Fig. 2 E-G), insects may visit the flowers and drink nectar without touching the pollen sacs and getting loaded with pollen. In these cases, the lever mechanism is a useful construction to lower the thecae down onto the pollinators increasing the degree of successful pollen transfer and decreasing the number of nectar thieves.

Except few bird-pollinated species in the American clade I-B (e.g. *S. roemeriana*, Fig. 2I, J) and the only known moth-pollinated species *S. arborescens* (Reith & Zona, 2016, clade II-A), *Salvia* species with bithecate anthers are bee-pollinated. The connective arms are usually short separating the thecae not too far from each other (Fig. 2). The functional constrain might be the same as in didynamic androecia (Fig. 8D, E), i.e., that a too large distance makes it impossible for the posterior pollen to get in contact with the stigma. This conflict limits the evolution of the staminal lever mechanism and may have triggered the evolution of monothecate anthers.

Type III: Monothecate stamens with inhibited thecae and curved mobile connectives

In old world *Salvia* species, monothecate anthers are derived from bithectae anthers (Fig 10: symbols with green connective). This is in accordance with the phylogenetic position of the species representing stamen type III (Walker & Sytsma, 2007; Will & Claßen-Bockhoff, 2014) and with stamen development-interpreting monothecate anthers as ontogenetic abbreviations (Gould, 1977) of bithecate anthers. Once sterile, the posterior lever arm is free to adapt to its new function as a barrier in the flower entrance. In particular, species of *Salvia* s. str., e.g., clade I-C (Fig. 3A-C, F, G, J-M; Fig. 7E, F) and clade I-A (Fig. 3D, H), have evolved elaborate structures guiding the pollinators (Reith et al., 2007). But also representatives of the E-Asian clade IV-A show large and often conspicuous barriers (e.g. Fig. 3I; Fig. 10; Himmelbaur & Stibal, 1933). Morphogenetically, the new structures formed by the lower connective represent ontogenetic additions (Gould, 1977).

Type IV: Monothecate stamens with reduced thecae and straight mobile connectives

The new world clade II-A (*Lasemia*, i.e. subgen. *Calosphace*; Fig. 10), with approx. 500 species the largest lineage in *Salvia* s.l., is characterised by monothecate anthers. The only exception is *S. axillaris* (own observation) placed at the base of the lineage (Fragoso-Martínez et al. 2017). In contrast to the curved shape of the old world species, the lower lever arm is straight blocking access to nectar in a diagonal way (Fig. 4A). Many species form one or two tooth-like structures of unknown function close to the joint (Himmelbaur & Stibal, 1933). The sterile end of the lower lever can be broad but does not form any additional appendages as in type III. The adjacent lever arms may be free from each other or postgentially fused, the latter being a late ontogenetic process.

While old world *Salvia* species are predominantly bee-pollinated, the American clade II-A includes more than 180 hummingbird pollinated species (Wester & Claßen-Bockhoff, 2011). The shift from bee to bird pollination has happened many times in parallel (Fragoso-Martínez et al., 2017) going along with multiple morphological changes in flower construction and loss of lever functions (Wester & Claßen-Bockhoff, 2007). Interestingly, the bee-pollinated species in this clade often (always?) form a ventral barrier compensating for the diagonal course of the posterior lever arm. Ontogenetic studies indicate that this barrier evolved differently (Baikova, 2002; Claßen-Bockhoff et al., 2004a). In *S. rypara* ssp. *platystoma* (Fig. 4K, L; Fig. 7A, B), the lower connective arm is straight and develops a ventral outgrowth blocking the flower entrance. In contrast, in *S. uliginosa* (Fig. 4P, Q; Fig. 7C, D) the lower lever arm is curved forming the barrier itself, while a dorsal outgrowth forms the straight part of the lever. This sword-like structure presses against the corolla when the lever mechanism moves. This increases the force, which is needed by the pollinator to get access to nectar, and the pressure by which pollen is loaded on the insect's body. Functionally, the two stamen constructions fulfil the same service but, morphologically, they are different.

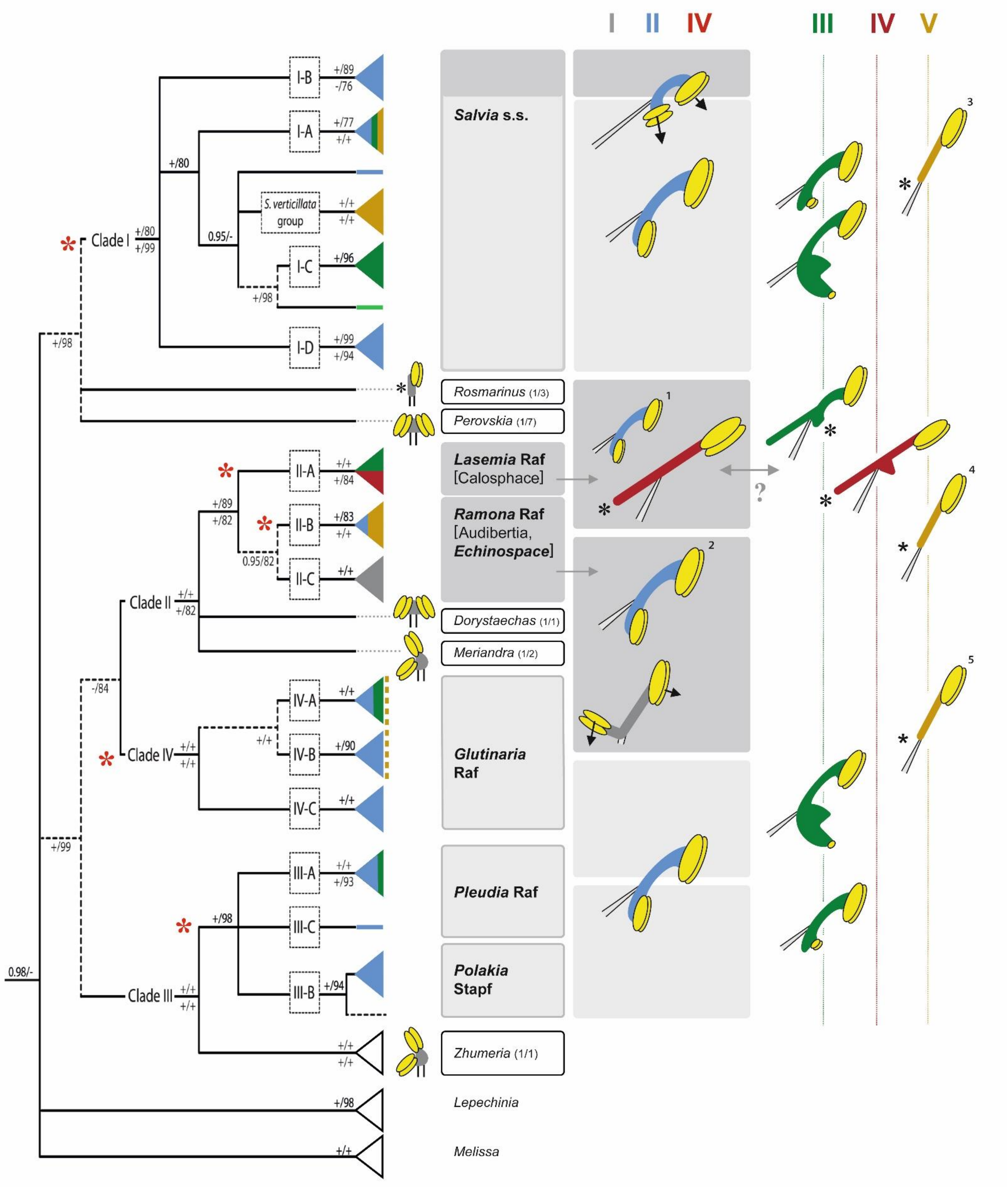
The phenomenon of partial analogy (Claßen-Bockhoff et al., 2004a) has been largely disregarded though Himmelbaur & Stibal (1933) already noticed that in some stamens with `straight levers´ (e.g. *S. uliginosa*), the vascular bundle ends in a `ventral tooth´ (type III°, III°°° sensu Himmelbaur & Stibal, 1933). Beyond its assumed, but not tested adaptive value, it is urgently needed to study stamen development in more bee-pollinated species of clade II-A to identify the homologous vs. analogous formation of ventral barriers and straight levers. As to the evolution of stamen diversity in *Salvia* s.l., it has to be shown which development represents the ancestral condition and which the more common one and whether the two forms evolved only once or several times in parallel in clade II-A.

Type V: Monothecate stamens with one-armed immobile connectives

The early inhibition of the posterior theca is coupled with an almost complete inhibition of the lower connective arm in stamen type V resulting in monothecate anthers with minute appendages (*S. verticillata*, *S. apiana*; Fig. 5B, E; Fig. 9E). This stamen type is derived from stamen type II again through ontogenetic abbreviation. It has evolved at least four times in parallel in the clades I-A, I-*S. verticllata*-group, II-B and IV-A and/or IV-B. If stamen development is the same in all clades, stamen type V would be an excellent example for homoplastic similarity, meaning morphological and developmental identity (morphological homology) originating in different phylogenetic clades.

Interestingly, monothecate anthers with an extremely reduced posterior theca also appear in *Rosmarinus officinalis*, which stands in a polytomy with *Perovskia* and *Salvia* s.s. (Fig. 10). However, despite its outer similarity, a joint is lacking in the anther of this species (Trapp, 1956a, b; Naghiloo et al., 2014; unpubl. data), thus representing just a monothecate anther as also found in some Australian Westringieae (unpubl. data) or *Crossandra* species and *Chaetothylax umbrosus* (both Acanthaceae; Trapp, 1956a, b).

Figure 10. Stamen evolution in *Salvia*. *Salvia* s.l. includes five non-*Salvia* genera and six *Salvia* clades (shaded). Stamens are bithecate in *Zhumeria, Meriandra, Dorystaechas* and *Perovskia* and monothecate in *Rosmarinus*. They all lack versatile anthers as the stamens in *Salvia* sect. Echinosphace (clade II-C, type I) do (connectives grey). Versatile anthers evolved at least five times (red *) resulting in four mobile stamen types (Table 1). Stamen type III (green) and V (orange) derived several times independently from stamen type II (blue), stamen type IV (red) only appears in clade II-A with unclear relation to type III (grey query). Arrows: direction of pollen shedding. *, position of rudimentary/lacking theca. [1] *S. axillaris*. [2] *S. columbariae*. [3] *S.namaensis* and *S. verticllata*-group (4 ssp.). [4] *S.* subgen. *Audibertia* (14 ssp.). [5] *S. scapiforme* (possibly more ssp.). Light grey, old world clades: *Salvia* s.str. (I-A): Eurasia, Africa; *Glutinaria* (IV): E-Asia; *Pleudia* and *Polakia* (III): SW Asia to N-Africa. Dark grey, new world clades: Sect. Heterospace (I-B): N-/C-America; *Lasemia* (i.e. subgen. *Calosphace*, II-A): C-/S-America; *Ramona* (i.e. subgen. *Audibertia*, II-B, II-C): California. Phylogeny and clade names after Will and Claßen-Bockhoff, 2017.



Conclusions

Walker & Sytsma (2007) for the first time mapped stamen types on a phylogenetic tree allowing an evolutionary interpretation of stamen diversity. They characterised each of their nine *Salvia* clades and subclades by a specific `type´. However, considering the description of their types, it is evident that they used character state combinations of the four types introduced by Himmelbaur & Stibal (1933) and, in part, by Hedge (1974). These types, supported by morphogenetic processes, are also used in the present paper and added by a fifth, non-versatile stamen type (Table 1). Walker & Sytsma's (2007) stamen types A, G, M and N correspond to bithecate anthers (type II), B and N to monothecate anthers with curved connectives (type III) and E and F to those with straight connectives (IV). Stamen type H is characterised by a reduced lower lever arm (type V) and stamen type I (capital, no number) is immobile corresponding to the newly introduced type I (number, no capital; Table 1).

The present study clearly illustrates that the diversification of stamens in *Salvia* s.l. is higher than expected by Walker & Sytsma (2007). *Ramona* (i.e., subgen. *Audibertia*; Fig. 10: clades II-B/II-C) has three instead of two stamen types. The same is true for *Salvia* s. str. (Fig. 10: clade I). *Lasemia* (i.e., subgen. *Calosphace*; Fig. 10: clade II-A) has two types based on different development. Little is known about their evolution and thus it is presently not possible to map them on individual subclades. Whether they correspond to the two types distinguished by Walker & Sytsma (2007) based on free (type F) or fused lower lever arms (type E) remains to be tested. Stamen type H (here type V) has evolved not only once but at least four times.

The distribution of stamen types within *Salvia* s.l. clearly shows that the staminal lever mechanism has evolved several times in parallel. Drew & Sytsma (2012) expected at least three independent origins, but in fact, versatile anthers with widened connectives originated at least five times (Fig. 10: red *), while one group traditionally grouped into *Salvia* (sect. Echinosphace; clade II-C) has no lever mechanism at all. Interestingly, all lineages with a staminal lever mechanism are richer in species than their relatives. This indicates that precise pollen transfer by mobile stamens may have triggered speciation and adaptive radiation.

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