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Ancestral and monophyletic presence of diplostigmaty in *Sebaea* (Gentianaceae) and its potential role as a morphological mixed mating strategy

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Summary

- Diplostigmaty, the presence of a primary (apical) stigma and secondary (mid-stylar) stigmas along the style, is only known from the genus *Sebaea* (Gentianaceae). Early work indicated that the secondary stigmas provide a mechanism of autogamy, suggesting that it might ensure reproductive assurance.
- Here, we test the monophyly of this unique morphological trait. Using Bayesian methods, we infer a nuclear DNA phylogeny for 96 accessions, including *c*. 50% of the species from the genus *Sebaea*. With this phylogeny, we infer the distribution of ancestral states on critical nodes using parsimony and likelihood methods.
- The inferred nrDNA phylogeny shows that the genus *Sebaea* is divided in two statistically well-supported clades, A and B, consistent with recent estimates. The most recent ancestor (MRCA) of clade A, except the most basal species (*Sebaea pusilla*), is resolved as diplostigmatic. No reversal to a single stigma is observed within this clade.
- We suggest that diplostigmaty is evolutionarily stable through time. We also discuss why this reproductive system is not found elsewhere than in Gentianaceae and the potential advantage of diplostigmaty as a stable mixed mating strategy.

Introduction

The majority of flowering plant species are hermaphroditic (i.e. flowers contain both male (stamens) and female (carpels) organs). Although most species fertilize their ovules with outcrossed pollen, self-pollination is widespread and the shift to self-pollination is a common transition in the evolutionary history of the angiosperms (Stebbins, 1974). Selfing relieves individuals from the requirement of having mating partners and allows rapid colonization, but it is associated with costs, including inbreeding depression, pollen discounting and in the long term a loss of genetic variability (Barrett, 2002). Mixed mating, where an individual reproduces both by self-fertilization (selfing) and mating with genetically different individuals (outcrossing) occurs in at least one-third of all angiosperm species, as shown by inferences of selfing rates based on genetic markers (Barrett, 2002).

Early theoretical models suggested that inbreeding depression, the main selective factor opposing the evolution of selfing, can be purged with self-fertilization, a process that favours pure strategies of either outcrossing or selfing (Lande & Schemske, 1985; Takebayashi & Morell, 2001; Goodwillie *et al.*, 2005; Johnston *et al.*, 2009). While further empirical and theoretical studies suggest that mixed mating strategies have evolved and can be evolutionarily stable (Charlesworth & Charlesworth, 1978; Gregorius, 1982; Holsinger, 1991; Haccou & Iwasa, 1995; Johnston *et al.*, 2009), the latter remains controversial (Lloyd, 1979; Lande & Schemske, 1985; Flaxman, 2000; Takebayashi & Morell, 2001; Herlihy & Eckert, 2002; Porcher & Lande, 2005; Schoen & Busch, 2008). Currently, the debate focuses primarily on factors that facilitate the maintenance of mixed mating systems, rather than on assessing whether, or which of the existing mixed mating systems are evolutionarily stable (Goodwillie *et al.*, 2005). It is therefore important to identify suitable study cases for such investigations that may provide examples of stable mixed mating systems.

Here, we investigate the ancestral and monophyletic presence of diplostigmaty, a unique morphological trait. We discuss why it is found in Gentianaceae and not in other angiosperm families, and suggest experimental tests of the potential role of diplostigmaty as a morphological mixed mating strategy.

Diplostigmaty, the presence of a primary (apical) stigma and secondary (mid-stylar) stigmas on the style (Marloth, 1909; Fig. 1) is only known from the genus *Sebaea* (Gentianaceae). *Sebaea* is composed of *c.* 100 species (Schinz, 1906; Kissling *et al.*, 2008) mainly from Africa, of which *c.* 65 are diplostigmatic. The flowers are radially symmetrical (actinomorphic), with five free sepals and five petals that are united basally into a long tube with five free lobes. As a rule, the anthers are exserted and attached by a long filament to the sinus of the corolla lobes (Fig. 1a). Before anther dehiscence, the style head of the gynoecium (with the apical stigma) is situated slightly below the level of the exserted stamens, while the secondary stigmas (when present) appear lower down on the style. When the anthers begin to open, the apical stigma is situated more or less at the same level as the anthers. The style continues its elongation and is mature when situated slightly above the anthers (avoiding self-pollen deposition on the apical stigma). The secondary stigmas are fully developed only much after the apical stigma reached its maturity, sometimes 2 or 3 d later, consistent with a potential role in reproductive assurance.

In 1913, Hill published a series of glasshouse experiments on several *Sebaea* species (i.e. *S. aurea*, *S. ambigua*, *S. confertiflora* and *S. imbricata*): he removed the apical stigma, the anthers or both. Hill also hand-pollinated the apical stigma, the secondary stigmas or both, and recorded seed set after these treatments. Removing the apical stigma led to significant enlargement of the secondary stigmas, and the plant produced viable seeds, while treatments involving removal of the anthers did not lead to any seed set, suggesting that cross-fertilization was excluded under the experimental conditions. Together with hand-pollination results, Hill's work demonstrated that (1) these species are self-compatible, (2) the secondary stigmas are functional as an organ allowing self-pollen receipt and germination, and (3) seed set after removal or not of the apical stigma must have resulted from self-pollen deposition on the secondary stigmas. Both Hill's (1913) and Marloth's (1909) studies on *Sebaea* suggest that secondary stigmas allow the production of selfed progeny when cross-pollination (mediated by the primary, apical stigma) fails to fertilize some or all of the available ovules. This idea is also consistent with the relatively basal position of the secondary stigmas on the style, where self-pollen naturally falls (Fig. 1).

The presence of diplostigmaty offers a unique opportunity to further test whether such a morphological feature allowing both self- and cross-fertilization as separate functions is stable through evolutionary time. If this mating system is not stable through time (i.e. over a very large number of generations) we should expect to observe reversals to a single stigma, assuming that secondary stigmas also entail some costs, and that owing to the unique stylar architecture (see the Discussion section) a reversal appears to be easy. Moreover, if, as suggested by theory, mixed mating could be advantageous, we should expect an individual selection advantage to diplostigmaty (this being reflected at a large time-scale by stability and by diversification of the group with this innovation).

Here, we discuss this prediction by inferring the nrDNA phylogeny for an extended taxonomic sampling of *Sebaea* species and assessing the monophyly of diplostigmaty within *Sebaea* by ancestral state inference.

Materials and Methods

Sampling, data collection and gene sequencing

Sebaea species were collected in Africa (from 2004 to 2007) and the sampling was maximized to cover the geographical, ecological and morphological variation of the genus. In addition, two representatives of the neighbouring tribe Chironieae were used as outgroups. The presence of secondary stigmas was recorded for all species sampled based on direct observation of living and herbarium materials.

When available, we used the previously published DNA sequence data of Exaceae (Yuan *et al.*, 2003, 2005). To these pre-existing data, we added 52 new sequences (GenBank Accession numbers FJ665987 to FJ666038) of *Sebaea* and *Exacum* species (Table S1). To obtain our new DNA sequences of nuclear ribosomal internal transcribed spacer (ITS1 and 2) and the 5.8S gene, we followed Yuan *et al.* (2003). To detect mistakes and correct uncertainties in the computer-generated sequence, we compared aligned tracefiles in CHROMASPRO version 1.33 (Technelysium Pty

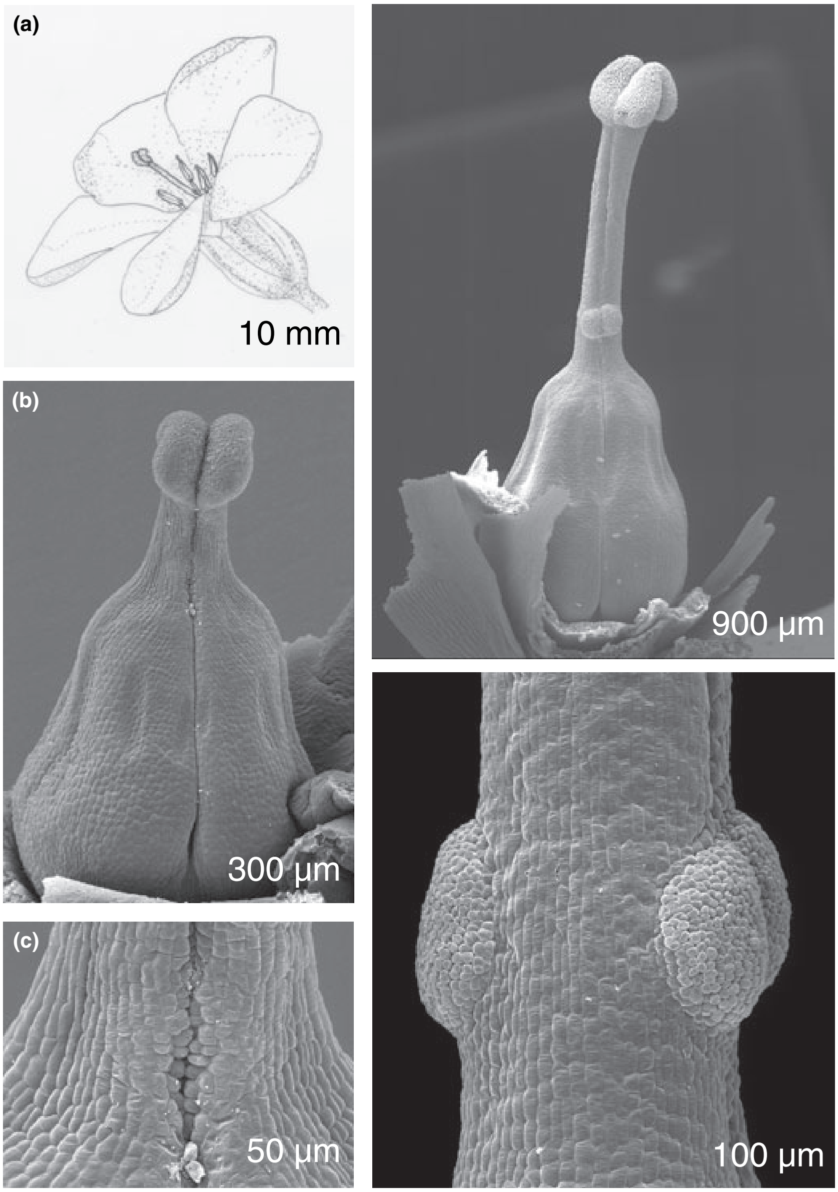

Fig. 1 *Sebaea macrophylla*. (a) Flower; (b) gynoecium at early stage, with carpels already postgenitally fused; (c) close up of part of (b) where the secondary stigmas will develop: the suture of postgenital fusion appears slightly expanded and epidermal papillae of the secondary stigmas are beginning to develop; (d) older gynoecium with secondary stigmas at the base of the style; (e) close up of part of (d, but in dorsal view of a carpel) with secondary stigmas on both sides.

Ltd). Alignment was performed using CLUSTAL W (Thompson *et al.*, 1994) as implemented in BIOEDIT 7.0.1 with subsequent manual improvement. The data matrix containing the aligned sequences is available on request from J.K or directly on TreeBase (preliminary number SN4561).

Phylogenetic analysis

Bayesian inference was conducted using MRBAYES version 3.1.2 (Huelsenbeck *et al.*, 2001; Ronquist & Huelsenbeck, 2003) and the data were partitioned by genes. The most appropriate model of sequence evolution for each partition was determined using MRMODELTEST version 2.2. (Posada & Crandall, 1998; Nylander, 2004; Posada & Buckley, 2004) to be the general time reversible model (GTR + G) for the ITS1, and the GTR + I + G model for the ITS2, and the K80 model for the 5.8S gene. Default priors were used for the base frequency parameters. Two independent analyses each with four Markov chains, three heated and one cold, starting from a random tree were run simultaneously for 5 million generations with trees sampled every 1000 generations. We used the online program AWTY (Wilgenbusch *et al.*, 2004) to check for stationarity.

Trees generated before the four Markov chains reaching stationarity (the burn-in) were discarded. The remaining trees were used to construct a 50% majority rule consensus tree. High posterior probabilities were recovered for all major lineages.

Morphological character state optimization

The character state evolution (using data on absence/presence of the secondary stigmas) was reconstructed with MESQUITE 1.12 (Maddison & Maddison, 2008) on to the obtained Bayesian trees by using two methods: maximum-likelihood-based discrete Markov k-state 1 parameter models (Lewis, 2001; Maddison & Maddison, 2008); and a maximum parsimony approach (Maddison & Maddison, 2000). The likelihood-based Markov k-state 1 model does not consider any particular plesiomorphic state at the root of the tree, and a character state can change to any other state on any branch of the tree with equal probability. We report proportional likelihood (P.L.) values of states scaled so that the sum of all states is 1. We used a decision threshold of 2.0 in MESQUITE (Maddison & Maddison, 2008) for statistical considerations, while parsimony analyses considered character state transformations unordered.

Results

With both parsimony and likelihood methods, diplostigmaty is unequivocally inferred to have arisen once in *Sebaea* (Fig. 2), in clade A. Moreover, no reversal to a single stigma has occurred within this clade.

The Bayesian topology with posterior probabilities is given in Fig. 2 (arithmetic mean of the –loglikelihood (L) of trees sampled after the burn-in = −7603.02; harmonic mean = −7683.94). The topology is in broad agreement with the relationships previously inferred in Exaceae using smaller taxonomic samplings (Yuan *et al.*, 2003, 2005; J. Kissling *et al.*, unpublished).

However, while the present phylogeny does not resolve the basal polytomy of the tribe, it confirms the recently described genus *Klackenbergia* (Kissling *et al.*, 2009) as distinct from *Sebaea*, and statistically supports the division of *Sebaea* into two main clades, hereafter called clade A and clade B, congruent with other estimates (J. Kissling *et al.*, unpublished) (Fig. 2).

Discussion

We used DNA sequence data from the nuclear genome to reconstruct the phylogeny of *Sebaea* and mapped the distribution of diplostigmaty character states, i.e. presence/absence of secondary stigmas, on the obtained phylogenetic tree. The data show that *Sebaea* is divided into two main, statistically well-supported, separate clades (clades A and B). All members of clade A are diplostigmatic (i.e. have secondary stigmas), except for the basalmost species (*Sebaea pusilla*), indicating that no reversals (loss of the secondary stigmas) have occurred. By contrast, none of the species in clade B is diplostigmatic. The age of *Sebaea* has been previously estimated by penalized likelihood method to be *c.* 20.3–54.3 Myr (Yuan *et al.*, 2003), and this age has been recently confirmed for the diplostigmatic *Sebaea* clade (Kissling, 2007). As *Sebaea* species are small short-lived annuals with probably more than one generation per flowering season, we could reasonably assume *c.* 2×10^6 generations since the origin of diplostigmaty. This provides support for the idea that diplostigmaty has been stable through evolutionary time. However, it should also be taken into account that a character state with a lower net diversification rate could persist for a long time, even if it is not a key innovation.

Clade A represents a large group of *c.* 65 species, which occur mainly in the Western Cape (South Africa). This species diversity, compared with the other genera of Exaceae (including clade B), suggests a greater diversification for the diplostigmatic *Sebaea*. Furthermore the basalmost species of clade A, *S. pusilla*, has both a similar distribution area and a similar dispersal strategy compared with the rest of the diplostigmatic *Sebaea* (Kissling, 2007). Therefore, climatic or ecological factors alone are unlikely to explain the diversification burst of the rest of that clade. *Sebaea pusilla* also shares with the rest of the clade several reproductive characters such as bilobed stigma, mode of anther dehiscence, and flower colour and size. Although more work is needed to substantiate the correlates of this diversification this is consistent with the idea that the secondary stigmas, which are absent only from this basal species, might constitute an innovation that has contributed the diversification of clade A.

Persistence through time of diplostigmaty and diversification of clade A suggest that diplostigmaty may convey an advantage under certain ecological conditions. A plausible hypothesis for this, which is consistent with Hill's (1913) results but still requires experimental verification in natural populations, is that the presence of a secondary stigmas with delayed maturation might serve to ensure seed set when outcross pollen or pollinators are scarce (reproductive assurance; Jain, 1976), while at the same time the apical stigma can serve to promote opportunities for outcrossing during early anthesis. Thus the persistence through time of diplostigmaty is consistent with the idea that the ability to self if outcrossing fails is always a selective advantage unless failure to outcross is so rare that the cost of the structures to enable selfing exceeds their benefit. An important advantage of diplostigmaty with delayed maturation of the basal stigmas, which may enable its evolutionary stability, is that with this morphological trait the structures that facilitate selfing do not interfere with and do not diminish the opportunity for

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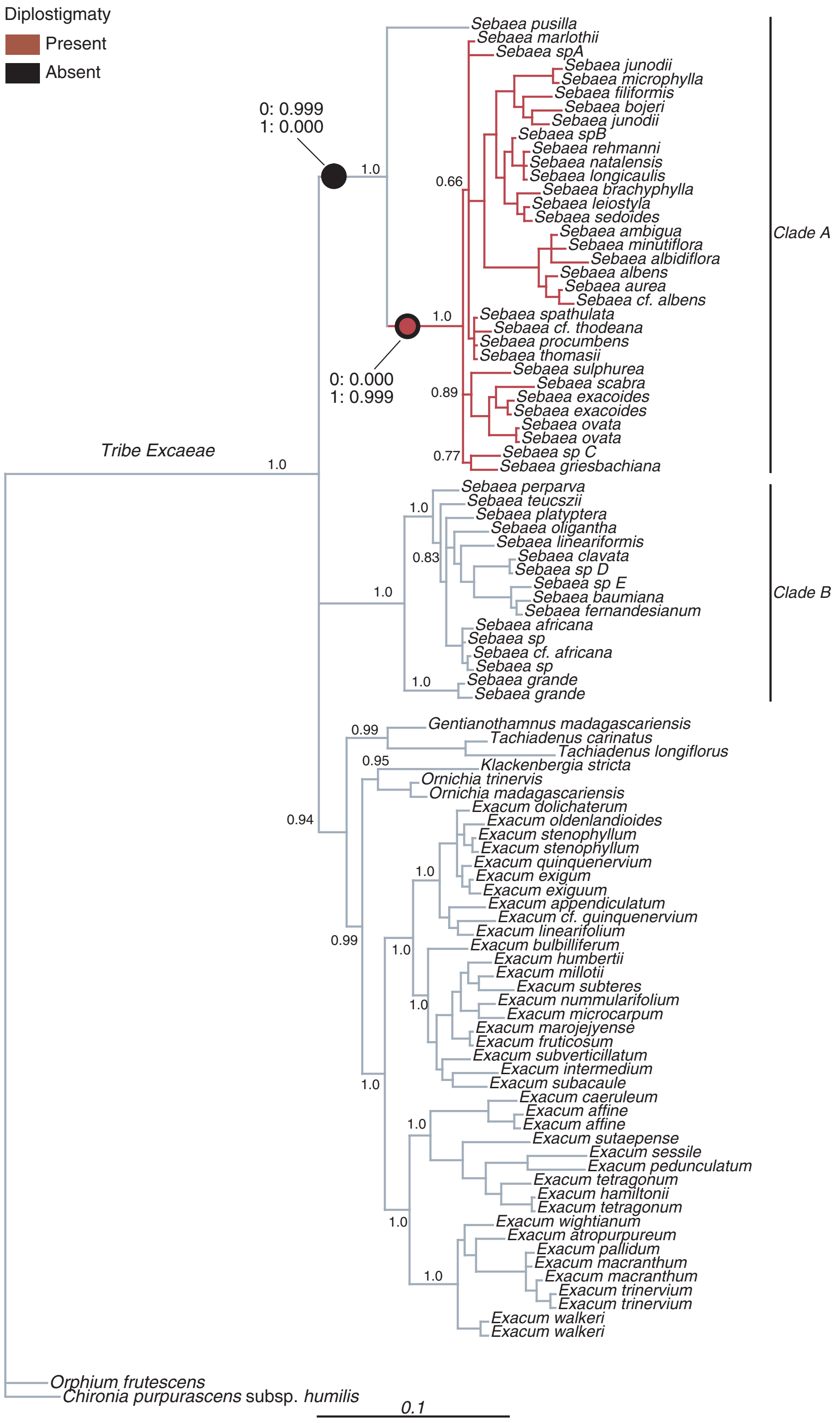

Fig. 2 Parsimony inference of ancestral states on the 50% majority rule consensus trees from Bayesian inference analyses of the combined data set. Likelihood inference of ancestral states is also reported for the nodes of interest. Branch support values are posterior probabilities from a 50% majority rule consensus tree derived from a Bayesian inference analysis.

outcrossing. Temporal and physical separation of primary and secondary stigmas may thus lower the risk of seed discounting (i.e. that production of selfed seed might use ovules that would have otherwise been outcrossed and more viable) (Barrett, 2002).

Because Hill's experiments indicate that removal of the apical stigma results in an enlargement of the secondary stigmas, and studies on other plants show that floral herbivory can influence the evolution of floral traits (e.g. Steets & Ashman, 2004; Ivey & Carr, 2005; Strauss & Whittall, 2006; Penet *et al.*, 2009), an alternative explanation for the putative ecological advantage of diplostigmaty in *Sebaea* could be that the secondary stigmas evolved because of high florivore pressure, rather than absence of pollinators. The 'surplus' stigma may function to replace the primary stigma lost to herbivores. In this scenario, mixed mating would be a byproduct of selection on diplostigmaty as an herbivore tolerance trait and not reproductive assurance *per se*. However, as we never found flowers with the primary stigmas destroyed neither in the field (> 200 populations) or in herbarium material (> 3000 collections), this scenario appears unlikely.

Whereas this may explain the persistence of this unique trait, it does not explain why secondary stigmas are not present in other groups. The answer may lie in unusual structural features in the development of the gynoecium (J. Kissling & P. K. Endress, unpublished). Although the gynoecium at anthesis appears as seemingly syncarpous (i.e. congenitally united) as in most angiosperms with a unified style, this is not really the case. In early development the two carpels are free at the level of the style and then fuse postgenitally (for the role of postgenital and congenital fusion, see Endress, 2006). Postgenital fusion of carpels by itself is unusual but not rare in angiosperms (Igersheim *et al.*, 2001; Matthews & Endress, 2005; Remizowa *et al.*, 2008). However, what makes it truly unique is that the free upper carpel parts that fuse postgenitally are not plicate (folded) as is usually the case, but are flat (Fig. 3) so that their margins are not hidden inside the style but come to lie at the surface of the style. Because, as a rule, the ventral side including the margins of a carpel develops stigmatic tissue, only a stylar architecture as in *Sebaea* has the potential to become stigmatic along the area of postgenital fusion (Fig. 1). Consistent with this, in *Sebaea* the secondary stigmas always develop along the postgenital fusion suture line between the carpels (Fig. 1c). The combination of these two features, postgenital fusion and flat carpels, is what makes this architecture unique. It is only known in Gentianaceae (McCoy, 1940; Baum, 1948; Leinfellner, 1951; Vijayaraghavan & Padmanaban, 1968) and the closely related Apocynaceae (Walker, 1975; Fallen, 1985). Among Gentianaceae, in *Bartonia* and *Lomatogonium* stigmatic tissue occurs all along the line of postgenital fusion of the two carpels. However, there is no separation into an upper and a lower stigma. In the majority of Apocynaceae the stigma is not apical but situated at the base of a 'style head'. However, their pollination apparatus is more complicated, and it is unlikely that the stigma is directly comparable with the secondary stigmas in *Sebaea*. In any case, the presence of two stigmas is only known in *Sebaea*.

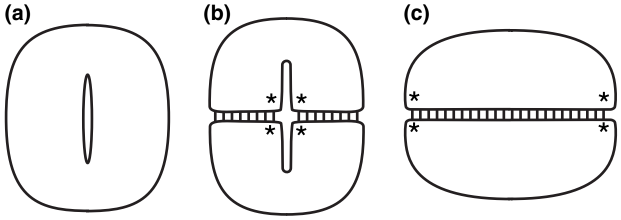

Fig. 3 Three different types of style of angiosperm gynoecia composed of two carpels (schematic transverse sections). (a) Type with the carpels congenitally united (the most common type in angiosperms); (b) type with plicate (folded) carpels postgenitally united (occurring in several families of eudicots and monocots); (c) type with flat carpels postgenitally united (occurring in *Sebaea*, some other Gentianaceae and a few Apocynaceae). Vertical lines between the two carpels, area of postgenital fusion; asterisks indicate carpel margins.

In conclusion, diplostigmaty has arisen once in *Sebaea* clade A, and no reversal to a single stigma has occurred, consistent with the idea that the presence of a secondary stigmas provides an ecological advantage. To test this idea, further work should explore the evolutionary stability of diplostigmaty and whether, and to what extent, the secondary stigmas serve for reproductive assurance in natural populations, whereby the presence of this trait across a set of related species allows for replication of experimental tests and comparative analyses.

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Table S1 Species of Exaceae and outgroups sampled for the present study (data are presented in the following order: Taxon, Voucher information, Origin, GenBank accession numbers)