American Journal of Botany 89(1): 37–49. 2002.

COMPARATIVE ANALYSIS OF LATE FLORAL DEVELOPMENT AND MATING-SYSTEM EVOLUTION IN TRIBE COLLINSIEAE (SCROPHULARIACEAE S.L.)[1]

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Species of *Collinsia* and *Tonella*, the two sister genera of self-compatible annuals that constitute tribe Collinsieae, show extensive variation in floral size and morphology and in patterns of stamen and style elongation during the life of the flower (anthesis). We used a nuclear ribosomal ITS phylogeny, independent contrasts, and phylogenetically corrected path analysis to explore the patterns of covariance of the developmental and morphological traits potentially influencing mating system. Large-flowered taxa maintain herkogamy (spatial separation of anthers and stigmas) early in anthesis by differential elongation of staminal filaments, which positions each of the four anthers at the tip of the ''keel'' upon dehiscence. Small-flowered taxa do not show this pattern of filament elongation. The styles of large-flowered taxa elongate late in the 2–5 d of anthesis, resulting in late anther-stigma contact and delayed self-pollination. Anther-stigma contact and self-pollination occur early in anthesis in small-flowered species/populations. Thus, we found complex covariation of morphological and developmental traits that can be interpreted as the result of multitrait adaptation for early selfing and high levels of autogamy, delayed selfing and higher levels of outcrossing, or intermediate levels of outcrossing. Continuous variation in these traits suggests the operation of continuous variation in selective optima or the combined effects of divergent selection and phylogenetic inertia.

Key words: autogamy; correlated evolution; Collinsieae; cross pollination; flower development; herkogamy; mating system; pollination; Scrophulariaceae; self-pollination.

A major feature of flowering plant evolution is repeated, parallel, evolutionary changes in mating system (Stebbins, 1950, 1974; Barrett, Harder, and Worley, 1996). Associated with transition from cross-pollination to self-pollination are changes in various other floral traits, including loss of self-incompatibility and heterostyly, reduction in flower size (Grant, 1958; Jain, 1976) and pollen-ovule ratio (Cruden, 1977, 2000), and developmental adjustments affecting the spatial separation of pollen and stigma (herkogamy) and the timing of self-pollination (Ritland and Ritland, 1989; Fenster et al., 1995; Barrett, Harder, and Worley, 1996; Schoen, Morgan, and Bataillon, 1996; Fishman and Wyatt, 1999; Motten and Stone, 2000). However, little is known about the developmental processes that underlie differences in morphology, herkogamy, and timing of self-pollination (but see Fenster et al., 1995; Stewart, Stewart, and Canne-Hilliker, 1996; Stewart and Canne-Hilliker, 1998) or how various morphological and developmental traits are functionally and evolutionarily interrelated (Fenster et al., 1995).

We have identified sister genera in the Scrophulariaceae, sensu lato (s.l.), *Collinsia* Nutt. (18–20 species) and *Tonella* Nutt. ex Gray (two species), both comprising self-compatible North American annuals, as a candidate ''model system'' for integrative study of the evolution of floral morphology, later stages of floral development, herkogamy, pollination ecology, and timing of self-pollination, using a phylogenetic framework. Results from traditional systematic (Newsom, 1929; Munz, 1959; Neese, 1993), molecular-phylogenetic (B. G. Baldwin, W. S. Armbruster, and B. Wessa, unpublished data), and genetic (Garber, 1958a; Grant, 1958) investigations lead us to hypothesize that repeated transitions in mating system have occurred in *Collinsia*. Changes in flower size (Grant, 1958), degree of herkogamy, and timing of self-pollination (Rust and Clement, 1977; Armbruster, 1980; Kalisz et al., 1999) appear to have accompanied these putative changes in mating system.

Here, we combine comparative morphological and developmental measurements with molecular phylogenetic information to examine evolutionary patterns and functional consequences of stamen and style elongation during anthesis. We test the following predictions: (1) Large-flowered taxa have delayed self-pollination, and small-flowered taxa have early self-pollination. (2) Selection for prolonged and consistent anther-stigma separation (herkogamy) has occurred in large-flowered, cross-pollinating species. This selection has led to more precise positioning of dehiscing anthers (''relative developmental precision'') and hence to a proportionately smaller region within the lower corolla lobe in which pollen is shed (the ''pollen zone''). (3) Small-flowered, self-pollinating populations should have proportionally smaller anthers than out-

[1] Manuscript received 30 January 2001; revision accepted 3 July 2001.

The authors thank Elizabeth Chase Neese, Marla Knight, Ann Herzig, Mary Edwards, and Susan Bainbridge for help in the field, Ann Herzig for the line drawing, A. Mergenthaler and K. Hanley for help with pollen-tube counts, and Charles Fenster and two reviewers for comments on earlier versions of the manuscript. This research was funded in part by NSF grant DEB-9708333 to WSA and by the University of California Intercampus Travel fund (WSA), NSF grants DEB-9421781 and DEB-9726980 to SK, and grants from the Lawrence R. Heckard Endowment Fund and the Committee on Research at U.C. Berkeley to BGB.

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crossing populations, because self-pollinators have lower optimal pollen-ovule ratios, smaller pollen, or both (Lloyd, 1965; Baker, 1967; Cruden, 1973, 1977, 2000). (4) Flower size, anther size, developmental precision, and time of anther-stigma contact vary continuously instead of falling into discrete inbreeding and outcrossing ''syndromes'' (Vogler and Kalisz, 2001; but see Lande and Schemske, 1985, and Schemske and Lande, 1985). Further, these traits should covary because of their functional relationships (e.g., Armbruster and Schwaegerle, 1996).

MATERIALS AND METHODS

Study system—*Collinsia* and *Tonella* (Collinsieae, Scrophulariaceae s.l.) are sister genera of self-compatible annuals, with a center of diversity in western North America. One species of *Collinsia* (*C. parviflora*) is found as far north as Alaska and at least as far east as Manitoba, and two species (*C. verna* and *C. violacea*) are restricted to eastern and midwestern North America. Plants grow and bloom from late winter to early summer, depending on species and elevation, and are pollinated by a variety of native bees (Rust and Clement, 1977; Armbruster, 1980). Reported chromosome numbers for tribe Collinsieae are $n = 21$, for *C. torreyi*, and $n = 7$ for all other species (Garber, 1956, 1958b, 1975). More recently, British Columbian plants, apparently closely related to *C. parviflora* and *C. grandiflora*, have been found to have $n = 14$ (Ganders and Krause, 1986). *Tonella* comprises two species restricted to western North America and is vegetatively similar to a diminutive *Collinsia*.

Flowers in both genera are zygomorphic and consist of a five-lobed calyx, five-lobed corolla, four epipetalous stamens, and one pistil, containing 2 to ~16 ovules (Fig. 1). The corollas of *Collinsia* are unique among the Scrophulariaceae s.l. in resembling pea flowers: an upper lip of two lobes forms the ''banner,'' and a lower lip of three corolla lobes includes a pair of wings and a folded (conduplicate) keel, enveloping the style and stamens. At the base of the banner, wings, and keel, the corolla is constricted into a narrow aperture, thus forming a constricted ''mouth'' at the top of a saccate tube (Fig. 1). *Tonella* flowers are similar but are more open and lack the banner, folded keel, and constricted mouth. The stigmas are receptive to pollen-tube growth either early in anthesis, in *Tonella* and most *Collinsia* species, or late in anthesis in a minority of *Collinsia* species (see below). In *Collinsia* flowers, nectar is secreted by a small nectary (probably derived from the staminode) at the base of the asymmetrically saccate corolla tube (Fig. 1) and is accessible to long-tongued bees that can reach the nectar by inserting their tongues through the opening in the constricted mouth at the base of the banner (Rust and Clement, 1972, 1977; Armbruster, 1980).

The four stamens in both genera are borne in two pairs: one upper and one lower. Members of the same staminal pair are of similar length for much of the life of the flower, and they dehisce at similar, but not identical, times. The anthers dehisce usually one at a time over the course of usually 3–4 d (occasionally 2–5 d), starting with one of the lower stamens, followed by the second lower stamen, one of the upper, and finally the second upper stamen (Fig. 1; see Kalisz et al. [1999] for a detailed description of late stages of floral development in *C. verna*). In most species, the style elongates during this period until the stigma reaches the dehisced anthers (pollen zone), when self-pollination can occur.

Pollinating bees at elevations below ~1000 m include species of *Bombus* (Apidae), *Anthophora*, *Emphoropsis*, and *Synhalonia* (Anthophoridae), and *Osmia* (Megachilidae). At higher elevations species of *Osmia* are the main pollinators (Armbruster, 1980). These bees land on the flowers and stand on the platform-like pair of wings with their heads at the base of the banner, obtaining nectar by inserting their proboscides through the constricted corolla mouth. Unlike in most other Scrophulariaceae s.l., the bees do not brush across the anthers and stigma as they move into the flower. Instead, as on a legume flower, the bees land directly in the foraging position and are excluded from entering the floral tube by the appressed lips of the corolla mouth. One exception is the oligolectic specialist *Osmia glauca* (Rust and Clement, 1972), which is small enough to be able to enter the saccate corolla tube to reach the nectar of some large-flowered species (Armbruster, 1980). However, this activity does not cause the depression of the keel or contact with anthers and stigmas; the latter only occurs when the bee is ''properly'' positioned on the platform (Armbruster, 1980). Other pollinating bees are large enough that their mass immediately depresses the keel and wings, but not the stamens and style. Hence pollen deposition and/or pickup occurs when the stamens and style move upwards, relative to the bee, and anthers and/or stigma contact the bee in a consistent position on the underside of its head, thorax, or abdomen (depending on species of bee and *Collinsia*; Armbruster, 1980). The clump of pollen placed on the bee is generally 1–3 mm in length. In addition to obtaining nectar, many bees also collect pollen by manipulating the anthers with their hind legs while the keel is depressed (Rust and Clement, 1972, 1977; Armbruster, 1980).

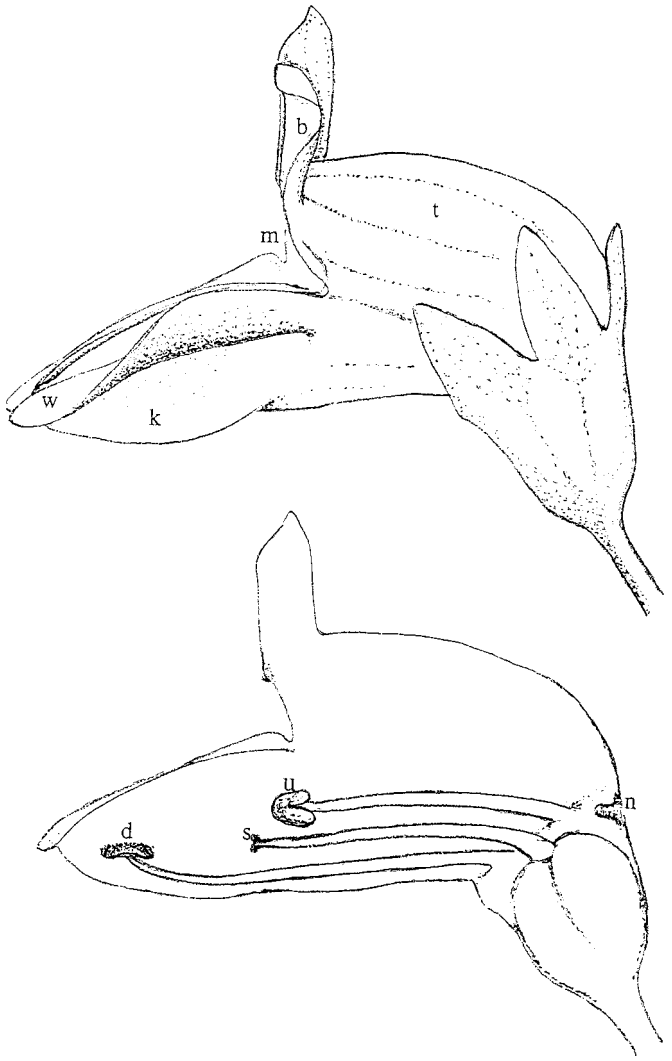

Fig. 1. Diagrammatic depiction of the flower of *Collinsia torreyi*. (A) Lateral external appearance of flower. Symbols: b = banner; k = keel; t = saccate corolla tube; m = mouth-like aperture of corolla tube; w = wing. (B) Longitudinal-section view; note only one of each of the two pairs of stamens is depicted. Symbols: d = dehisced anther; n = nectary; s = stigma; u = undehisced anther. The distance between d and s represents the degree of herkogamy and decreases to zero near the end of anthesis (exact timing depends on the species). The area denoted by d is also the pollen zone in this depiction.

Phylogenetic methods—Leaf material was collected from ~10 individuals from each of 19 populations representing 18 *Collinsia* species and one pop-

ulation each of the two *Tonella* species (Appendix 1: URL: http://ajbsupp.botany.org/v89/armbruster/) and kept on ice until returned to the laboratory, where samples were kept at −80°C. Total DNA of each sample was extracted from leaves using a modification of Doyle and Doyle's (1987) CTAB (hexadecyltrimethylammonium bromide) procedure (plus a phenol extraction, RNase digestion, and two ethanol precipitations). The 18S–26S nuclear ribosomal DNA internal transcribed spacer (ITS) region (i.e., *ITS-1*, *5.8S*, *ITS-2*) was PCR-amplified and directly cycle-sequenced using the methods of Baldwin and Wessa (2000). Sequences of both strands of the ITS-region were resolved on 4.8% polyacrylamide gels using an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, California, USA). Sequences of the ITS-region of *Collinsia* and *Tonella* were unambiguously aligned manually with outgroup sequences of *Chelone* L., *Keckiella* Straw, and *Penstemon* Schmid. generously provided by Andrea Wolfe. Representatives of tribe Chelonieae were chosen as the outgroup based on molecular phylogenetic evidence that tribe Chelonieae is sister to tribe Collinsieae, comprising *Collinsia* and *Tonella* (Wolfe et al., 1997). Parsimony analyses were conducted using PAUP* 4.0 (Swofford, 1998) using the heuristic option and 100 random addition sequences of the taxa. Analyses were conducted on the entire aligned sequence matrix plus indel characters (recoded using ''simple indel coding''; Simmons and Ochoterena, 2000), with all characters and character-state transformations given equal weight. We estimated reliability of clades by bootstrap and decay analyses, using heuristic searches (20 random addition sequences of the taxa) for each of the 100 bootstrap replicates and for the decay analysis. Decay values (Bremer support) for each clade were assessed using the reverse constraints approach implemented in AutoDecay 4.0 (Eriksson, 1998).

Floral development—Flowers from individuals of 22 populations in 16 species of *Collinsia* and one population from each of the two *Tonella* species were collected between 1976 and 1999 (Appendix 1: URL: http://ajbsupp.botany.org/v89/armbruster/), fixed in FAA (1 part formalin, 1 part acetic acid, 18 parts ethanol), and stored in 70% ethanol. Some morphological and ecological information on two additional *Collinsia* species (*C. concolor*, *C. parryi*) was obtained from the literature (Newsom, 1929; Munz, 1959). Flower samples were examined under a dissecting microscope and measured with digital calipers or an ocular micrometer. Flowers were classified into five stages based on the number of anthers dehisced (zero to four anthers). For each population we measured six to ten flowers (each usually from a separate plant) at each of the five developmental stages, recording keel length, corolla-tube length (mouth to nectary), length of the style beyond the mouth of the corolla tube, and length of each stamen beyond the mouth of the corolla tube (to the tip of each anther). Anther length was measured on a sample of 12 or more anthers per population. Although curvature of organs was minimal except when very young, all measurements were taken on straightened organs to improve repeatability.

Similar measurements were conducted on flowers of plants grown in the greenhouse for comparison with the measurements of preserved flowers and to obtain information about the actual duration of anthesis and each stage. Species examined in the greenhouse were: *C. childii*, *C. heterophylla*, *C. linearis*, *C. parviflora*, *C. rattanii*, *C. sparsiflora*, *C. torreyi*, and *C. verna*.

On preserved flowers we also measured the distance from the base of the anther of the shortest stamen with a dehisced anther to the apex of the anther of the longest stamen with a dehisced anther to estimate the length of the pollen ''zone'' at each floral stage. Thus, the length of the pollen zone is a function of both anther size and the positions of the dehisced anthers. The size of the pollen zone affects the degree of herkogamy because when the stigma is within this region it is subject to spontaneous self-pollination. As the style elongates, it drives the stigma through the pollen zone, effecting self-pollination. The size and position of the pollen zone also influence the size and position of the clump of pollen deposited on pollinating bees. We compared the length of the pollen zone at floral stage 3 across populations because this is the last stage at which herkogamy is maintained in any species. As flower size increases, the absolute size of the pollen zone could be expected to increase through isometric/allometric scaling effects. Hence, selection for increased herkogamy, and hence closer placement of anthers (greater ''precision'') in large-flowered species, should reduce the slope of the allometric relationship of pollen-zone size to flower size; this hypothesis was tested using regression of log-transformed data (Sokal and Rohlf, 1981).

The relative developmental precision index (DPI) was calculated as DPI = $1 - [(S1_3 - S3_3)/(S1_1 - S3_1)]$, where $(S1_3 - S3_3)$ is the distance between the tip of the first dehisced anther (S1) and the tip of the third dehisced anther (S3) at stage 3 and $(S1_1 - S3_1)$ is the corresponding distance at stage 1. The DPI measures the degree of precision in the position of the dehiscing anthers relative to the positions of the undehisced anthers, and it varies from <0 (developmental divergence) to 0 (parallel stamen growth) to 1 (developmental convergence/precision). This index is mensurally independent of both anther size and flower size. We calculated DPI for floral stage 3 because we estimated pollen-zone lengths at this stage.

We used two approaches to estimate the stage at which the stigma contacts the dehiscing anthers in each population. The first measure of the timing of anther-stigma contact was based on the visual classification of each flower as having the stigma in contact with, or not in contact with, dehiscing anthers and used logistic regression (SAS PROBIT procedure; SAS Institute, 1996) to estimate the stage of development at which 50% of the flowers in a sample had stigmas and dehiscing anthers in contact (ASC-50). The second approach to assessing timing of anther-stigma contact was based on graphs of style and stamen elongation. We determined the average stage at which the style entered the pollen zone (see RESULTS). These graphs were based on means of flower parts at each stage of development (five to ten flowers at each stage per population), and the metric is therefore sensitive to sample-size problems and differences in mean flower size of different floral cohorts. The two indices were highly correlated ($r = 0.90$, $P < 0.001$), so only ASC-50, which has known statistical properties (Sokal and Rohlf, 1981), was used in statistical analyses.

Stigma receptivity—We determined stigmatic receptivity of fresh flowers at each of the five stages of anther dehiscence in two ways. First, in the field we tested for stigmatic peroxidase activity (SPA) using the method of Kearns and Inouye (1993). Intact styles were placed on a glass slide in a drop of 3% hydrogen peroxide and covered with a cover slip. Bubble production from the stigma within 2–3 min indicates the activity of stigmatic peroxidases and hence receptivity of the stigma. The stage at which 50% of the stigmas in the sample tested positive for peroxidase activity (SPA-50) was calculated using logistic regression (SAS PROBIT procedure, SAS Institute 1996) and was our metric for population comparisons. Second, to verify the relationship of a positive SPA and actual receptivity to pollen-tube growth, generally 5–10 styles at each floral stage for one population each, from a subset of species and varieties, were preserved in ethyl alcohol, and, in the laboratory, were cleared, stained, and examined for pollen tubes using epifluorescence microscopy (see Kalisz et al. [1999] for details on the method).

Comparative statistical analyses—Statistical analysis of among-population relationships among traits was conducted using correlation analyses, factor analysis (principal components analysis [PCA] and Varimax rotation), and path analysis (Li, 1975) implemented with regression models in Statistica (StatSoft, 1994). All length measurements were log transformed to assess proportional variation and to correct for heteroscedasticity (Sokal and Rohlf, 1981). Correlation, factor, and path analyses were conducted on phylogenetically corrected (PC) independent contrasts (see below) and, for comparison, on the original ''phylogenetically naïve'' data (see Harvey and Pagel, 1991; Armbruster, 1992). This comparison gives insights not only into the value of using phylogenetic information in the analysis, but also into the nature of the phylogenetic ''signal.''

Independent contrasts were calculated using the program CAIC (Purvis and Rambaut, 1995) for calculating all the paired contrasts (differences) of trait values on the molecular phylogenetic tree. In a few cases we used conspecific or con-subspecific populations as phylogenetic pairs even though one (or both) of the populations was not represented in the molecular tree. This procedure was justified by the obvious close relationship between conspecific populations (all conspecific populations treated this way shared numerous morphological synapomorphies). Only two populations of minimal taxonomic rank

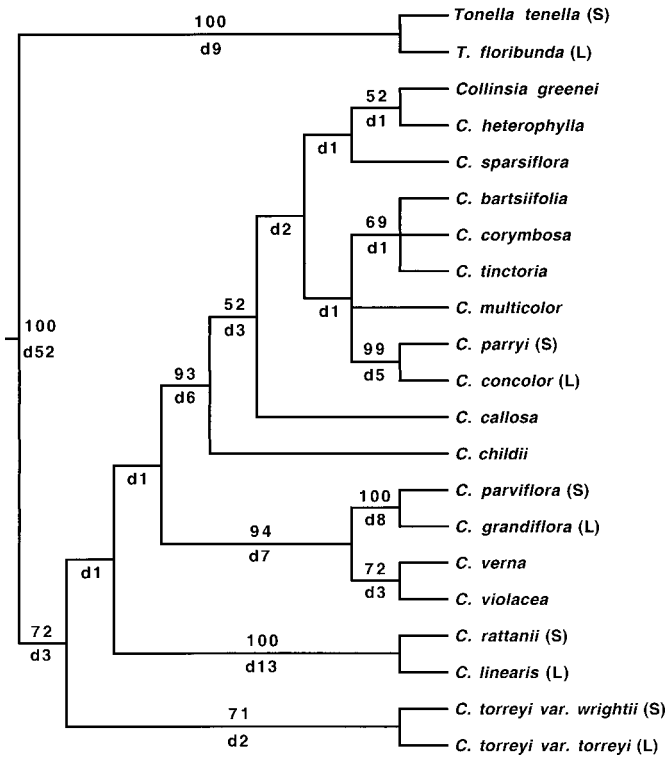

Fig. 2. Strict consensus of the two minimum-length trees of tribe Collinsieae based on ITS-region sequence variation and recoded insertion/deletion mutations, with bootstrap values >50% (above) and decay (Bremer) support (below branches). Rooted with ITS data for Cheloneae (*Chelone*, *Keckiella*, *Penstemon*). Consistency index = 0.69. Retention index = 0.78. The (S) and (L) next to the species epithet indicate the relative flower size (small and large, respectively) of clear small- and large-flowered species pairs.

were used (the two with largest sample sizes) in order to have unambiguous sister relationships.

Contrasts of continuous traits were analyzed using path analysis (Li, 1975) and multiple regression through the origin (Purvis and Rambaut, 1995) with Statistica (StatSoft, 1994). Simple paired comparisons of extant sister taxa were calculated using a modification of Ridley's (1983) method, employing the paired-sample *t* test (Sokal and Rohlf, 1981).

An index of relative expected outcrossing was calculated as the mean of the relative flower size and the relative ASC-50. The variables were relativized by dividing each observed value by the maximum observed value for that variable. Thus the index varies from near zero to one.

RESULTS

Phylogenetic relationships—Results of phylogenetic analyses based on nuclear rDNA ITS sequence data support monophyly of *Collinsia* and a sister group relationship between *Collinsia* and *Tonella* (Fig. 2). These results also demonstrate that the large- and small-flowered species pairs first recognized on morphological grounds (Newsom, 1929; Munz, 1959; Armbruster, 1980; Neese, 1993) constitute separate clades and hence separate changes in flower size and potentially in mating system (e.g., *C. linearis* [large] and *C. rattanii* [small], *C. grandiflora* [large] and *C. parviflora* [small], *C. concolor* [large] and *C. parryi* [small], *T. floribunda* [larger] and *T. tenella* [smaller]; Fig. 2). Support for each of the large- and small-flowered species pairs was very high, with bootstrap values generally approaching 100% (Fig. 2). In addition, these findings suggest that the two eastern species (*C. verna*, *C. violacea*) constitute a lineage that is sister to *C. grandiflora* and the widespread *C. parviflora*.

Developmental patterns of stamen elongation—Stamen elongation in most species of *Collinsia* occurs intermittently over the 2–5 d of anthesis. All species of *Collinsia* and *Tonella* (not shown) showed similar patterns: the stamens elongate gradually until they approach the tip of the keel, and then the anthers dehisce (Fig. 3). In all species, the lower stamens (stamens 1 and 2) are initially longer than the upper stamens and elongate a relatively short distance to reach the keel tip (Fig. 1). The upper stamens (stamens 3 and 4) are initially shorter and, in some species (e.g., *C. linearis*, *C. grandiflora*, *C. heterophylla* [population 2]), elongate proportionately more than the lower stamens prior to dehiscence, whereas in other species (e.g., *C. parviflora*, *C. sparsiflora collina*) the proportional elongation of upper and lower stamens is similar (Fig. 3). This difference in patterns of stamen elongation reflects converging developmental trajectories in the former group of species and nearly parallel developmental trajectories in the latter (Fig. 3).

Style elongation and timing of anther-stigma contact—Most species of *Collinsia* showed similar patterns of style elongation (Fig. 4), as did the sister genus *Tonella* (not shown). In general the style elongates with increasing flower age or remains relatively constant in length. The four anthers dehisce one at a time during anthesis (stages 1–4), and the proximal margin of the pollen zone advances basally as additional anthers dehisce (Fig. 4).

In all populations sampled but one, the style either elongated into the pollen zone or was in the pollen zone throughout anthesis. The exception was a population of *C. sparsiflora* var. *arvensis*, in which the stigma was positioned distal to the anthers from the earliest stages. In this taxon self-pollination appears to be achieved by elongation of staminal filaments, which brings the anthers into contact with the receptive stigma (Fig. 4). This mechanism was evident from observations both of the series of preserved flowers and of the development of individual flowers in the greenhouse. The median time (stage) of anther-stigma contact (ASC-50) ranged from ~0.5 in the earliest self-pollinators, such as *C. callosa*, to 3.05 in the large-flowered *C. tinctoria* (Table 1).

Stigma receptivity and flaring—Because pollen tubes can be present only after receptivity occurs, a strict correspondence between the stage at which peroxidase activity is first detected and the stage at which pollen tubes are first detected in the

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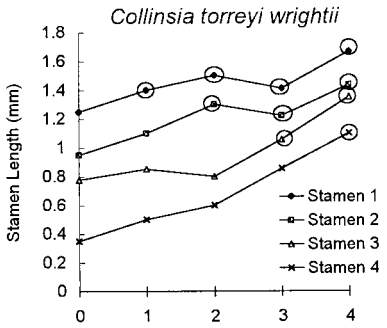
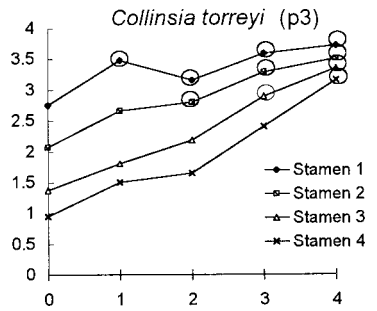
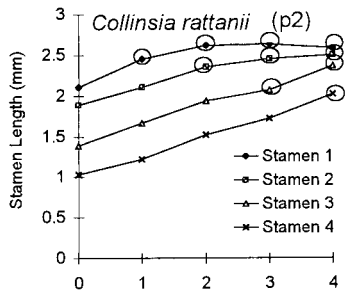
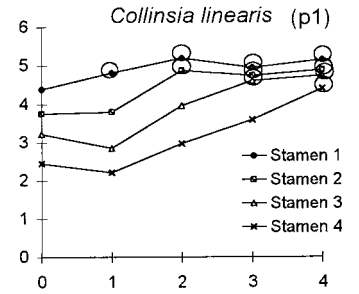
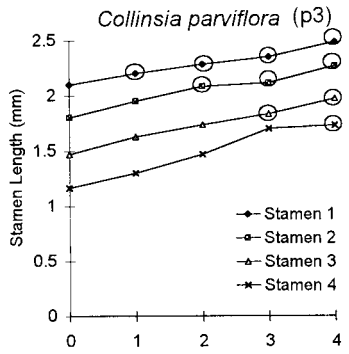
Fig. 3. Patterns of stamen development in five phylogenetic pairs of sister lineages of *Collinsia*. The left column contains relatively small-flowered lineages and the right column contains the larger-flowered sister lineages. Occasional reduction in length of floral parts with flower age is an artifact of sampling multiple flower cohorts at a single time. No error bars are shown to make developmental trends more obvious. Sample sizes are presented in Table 2. (Statistical analyses are based on data in Table 2.) A circle indicates that the anther has dehisced. In each panel stamens 1 and 2 belong to the lower pair in the flower, and stamens 3 and 4 belong to the upper pair in the flower.

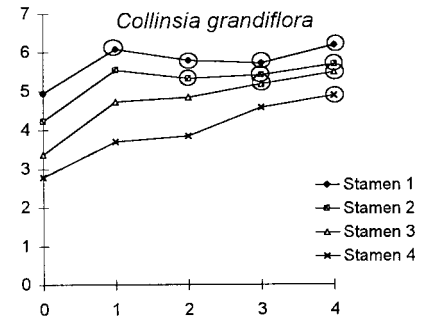

Collinsia torreyi wrightii
Stamen Length (mm)
Stamen 1
Stamen 2
Stamen 3
Stamen 4

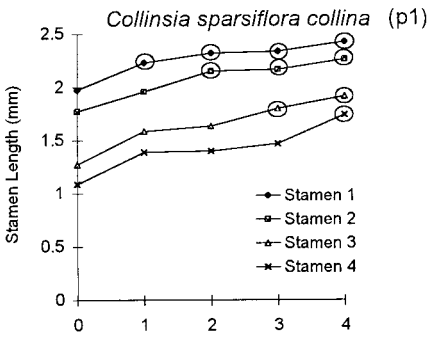

Collinsia torreyi (p3)
Stamen 1
Stamen 2
Stamen 3
Stamen 4

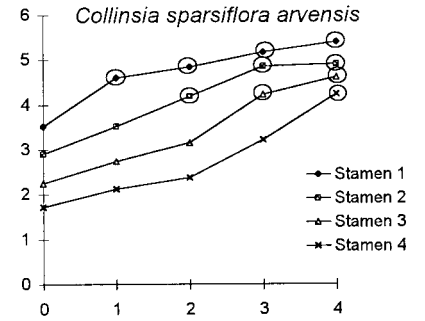

Collinsia rattanii (p2)
Stamen Length (mm)
Stamen 1
Stamen 2
Stamen 3
Stamen 4

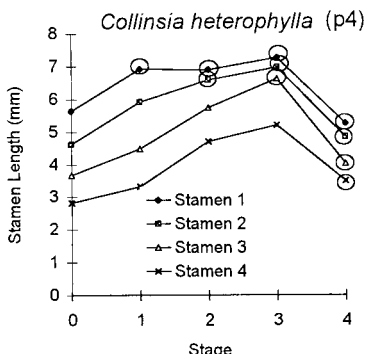

Collinsia linearis (p1)
Stamen 1
Stamen 2
Stamen 3
Stamen 4

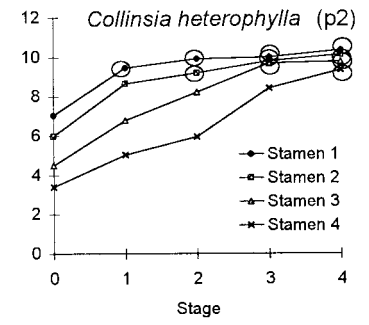

Collinsia parviflora (p3)
Stamen Length (mm)
Stamen 1
Stamen 2
Stamen 3
Stamen 4


Collinsia grandiflora
Stamen 1
Stamen 2
Stamen 3
Stamen 4


Collinsia sparsiflora collina (p1)
Stamen Length (mm)
Stamen 1
Stamen 2
Stamen 3
Stamen 4


Collinsia sparsiflora arvensis
Stamen 1
Stamen 2
Stamen 3
Stamen 4


Collinsia heterophylla (p4)
Stamen Length (mm)
Stamen 1
Stamen 2
Stamen 3
Stamen 4
Stage


Collinsia heterophylla (p2)
Stamen 1
Stamen 2
Stamen 3
Stamen 4
Stage

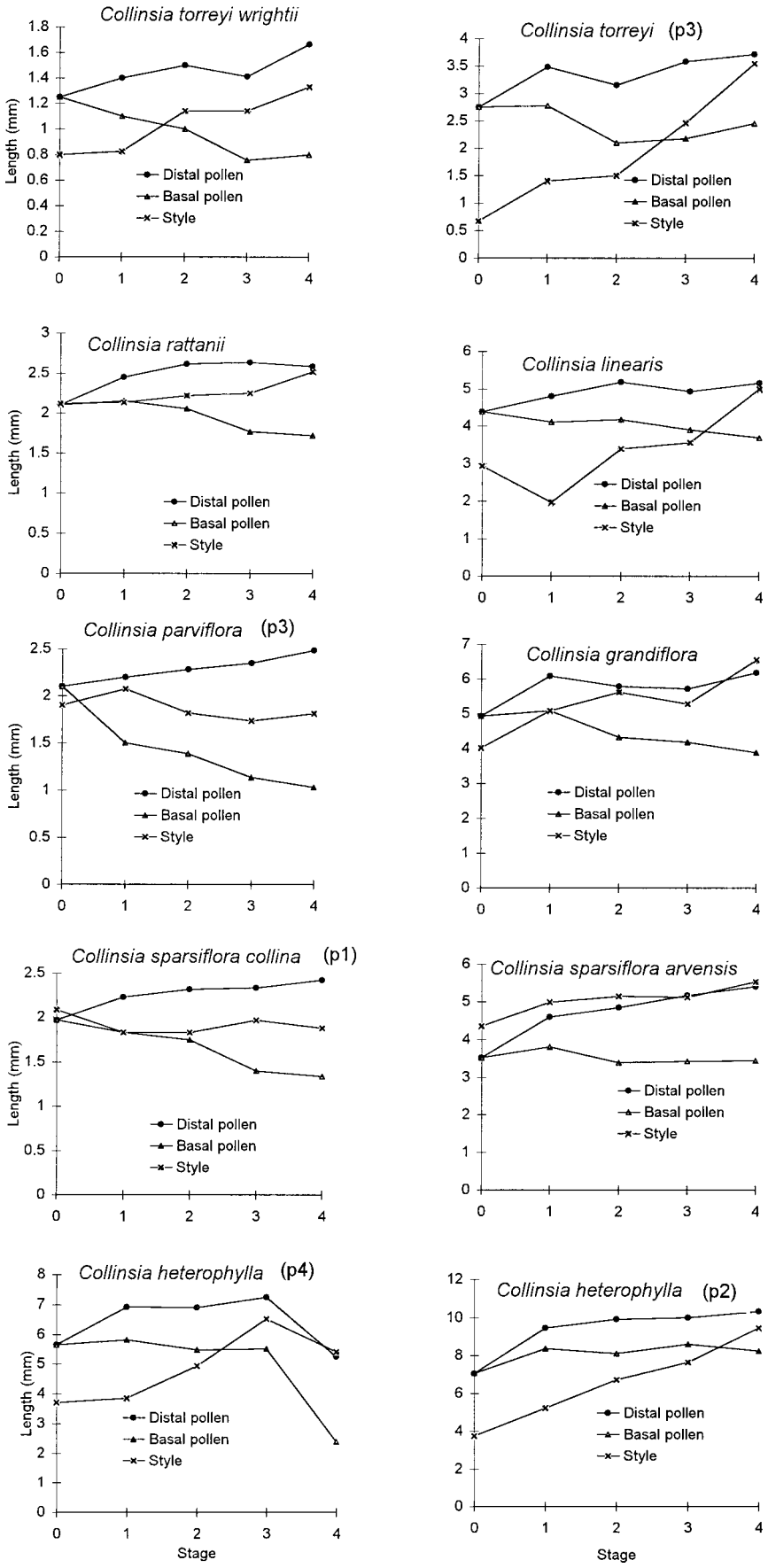

Collinsia torreyi wrightii
Collinsia torreyi (p3)
Collinsia rattanii
Collinsia linearis
Collinsia parviflora (p3)
Collinsia grandiflora
Collinsia sparsiflora collina (p1)
Collinsia sparsiflora arvensis
Collinsia heterophylla (p4)
Collinsia heterophylla (p2)
Length (mm)
Stage
Distal pollen
Basal pollen
Style

TABLE 1. Means and confidence intervals (when calculable) of the ASC-50 selfing index, SPA-50 stigma receptivity index, stage of first stigma flaring, flower length, number of ovules per ovary, length of anthers, relative developmental precision in stamen elongation, and length of the pollen-bearing zone in the keel for each *Collinsia* and *Tonella* population (pop.) included in this study. The *N* in column 1 refers to number of flowers used for morphological measurements and for the ASC-50. The 95% confidence interval (CI) in column 2 could not be calculated in some cases due to small sample size. The standard error of anther length was between 0.01 and 0.02. Missing data are indicated with —.

Species (*N*)	ASC-50 (95% CI)	SPA-50 (*N*)	First stage with flared stigmas	Flower length (mm) (1 SE)	Number of ovules	Anther length (mm)	Relative precision (1 SE)	Length of pollen zone (mm) (1 SE)
Collinsia bartsiifolia (51)	2.72 (2.30–3.14)	2.69 (66)	—	16.05 (0.25)	16	1.35	0.63	2.07 (0.13)
C. callosa (51)	0.50	0 (24)	—	7.16 (0.09)	7	0.8	−0.41	1.47 (0.07)
C. childii pop. 1 (30)	1.01 (0.54–1.34)	—	0	6.64 (0.10)	2	0.6	0.12	1.16 (0.14)
C. concolor (—)	—	—	—	12.0	16	—	—	—
C. corymbosa (59)	2.26	2.94 (64)	0	17.89 (0.31)	16	1.5	0.82	1.88 (0.17)
C. grandiflora (29)	1.04	—	0	9.75 (0.20)	4	1.0	0.66	1.54 (0.14)
C. greenei (3)	—	—	—	12.1 (0.50)	3	0.63	—	1.10[a]
C. heterophylla pop. 2 (Sierra form) (30)	2.69 (1.79–3.86)	2.17 (est. 25)[b]	—	15.13 (0.47)	16	1.1	0.88	1.42 (0.15)
C. heterophylla pop. 4 (41)	1.84 (1.08–2.61)	1.4 (23)[b]	0/1	8.53 (0.34)	16	1.1	0.74	1.73 (0.10)
C. linearis pop. 1 (32)	2.89 (2.49–3.24)	—	—	9.31 (0.40)	4	0.7	0.83	1.03 (0.11)
C. multicolor (53)	1.98	2.0 (34)	—	15.64 (0.32)	10	1.4	0.82	1.88 (0.15)
C. parryi (—)	—	—	—	8.5	10	—	—	—
C. parviflora pop. 1 (31)	1.70 (0.45–2.63)	—	0	6.8 (0.48)	4	0.4	0.18	1.00 (0.45)
C. parviflora pop. 2 (32)	0.5	0 (11)[b]	—	4.96 (0.41)	4	0.7	0.10	1.22 (0.30)
C. rattanii pop. 1 (30)	1.0	0 (26)[b]	0	5.50 (0.45)	4	0.3	0.28	0.87 (0.46)
C. sparsiflora var. *arvensis* pop. 1 (31)	1.72	0 (44)[b]	—	10.36 (0.43)	8	0.8	0.49	1.75 (0.48)
C. sparsiflora var. *collina* pop. 1 (30)	0.5	0 (27)[b]	—	4.88	8	0.4	0.17	0.93
C. sparsiflora var. *collina* pop. 2 (51)	1.41	0 (10)	—	4.88 (0.17)	8	0.39	0.09	0.99 (0.08)
C. tinctoria pop. 1 (30)	3.05	3.38 (28)	0	14.72 (0.40)	4	1.1	0.84	1.55 (0.14)
C. torreyi var. *wrightii* (43)	1.06	0 (17)	—	3.76 (0.09)	2	0.3	0.36	0.66 (0.05)
C. t. var. *torreyi* pop. 2 (30)	1.97	—	—	7.12 (0.39)	2	0.8	0.62	1.33 (0.11)
C. t. var. *torreyi* pop. 3 (30)	2.0	—	—	7.77 (0.44)	2	0.7	0.58	1.40 (0.29)
C. verna (45)	1.73 (1.19–2.26)	3.5[c]	2	12.87 (0.13)	4	1.54	0.66	2.52 (0.19)
C. violacea (15)	1.88	0 (14)	—	11.44 (0.39)	9	1.26	0.16	1.91
Tonella floribunda (34)	0.5	0 (16)	—	3.70	2	0.88	0.18	1.58
T. tenella (14)	0.5	0 (18)	—	2.35 (0.11)	2	0.40	−0.12	0.63 (0.21)

[a] Estimated from stage 4.
[b] Estimated from another similar or nearby conspecific population.
[c] Estimated from data published in Kalisz et al. (1999).

←

Fig. 4. Patterns of style elongation and stigma placement relative to the pollen zone in five phylogenetic pairs of sister lineages of *Collinsia*. The left column contains relatively small-flowered lineages and the right column the larger-flowered sister lineages. Occasional reduction in length of floral parts with flower age is an artifact of sampling multiple flower cohorts at a single time. No error bars are shown to make developmental trends more obvious. Sample sizes are presented in Table 2. (Statistical analyses are based on data in Table 2.)

TABLE 2. Relationship between SPA-50, peroxidase activity, and presence of pollen tubes in the styles of a sample of species of tribe Collinsieae. Values in parentheses for SPA-50 were estimated from nearby or similar populations (pop.). Ranges for the first stage with pollen tubes indicate uncertainty in estimation. The high value is the earliest stage at which tubes were observed, and the low value is the earliest possible stage. The low stage in the range and the intervening stages were missing from the sample, so we could not ascertain whether tubes were absent. A value in parentheses for the first stage with pollen tubes indicates only one tube seen at this stage. *N* is the number of styles examined for pollen tubes. Missing data are indicated by —.

Species	SPA-50	Stage with first observed peroxidase activity	First stage with pollen tubes	*N*
Collinsia bartsiifolia	2.7	2	4	64
C. callosa	0	0	1	21
C. corymbosa	2.9	2	3	63
C. multicolor	2.0	3	(0) 3	46
C. parviflora (pop. 3)	(0)	—	1	10
C. rattanii (pop. 2)	(0)	0	1–3	6
C. sparsiflora var. *arvensis* (pop. 2)	(0)	0	3	44
C. s. var. *collina* (pop. 3)	(0)	0	3	25
C. s. var. *sparsiflora* (pop. 2)	1	1.2	3	57
C. tinctoria (pop. 3)	(3.38)	3	4	22
C. torreyi var. *wrightii*	0	0	0–4	9
Tonella tenella	0	0	1–3	12

style was not expected nor observed (Table 2, Kalisz et al., 1999). Nevertheless, the two variables were significantly correlated ($r = 0.703$, $P = 0.016$), in the subset of species tested. We conclude therefore that the peroxidase test is a good measure of stigmatic receptivity for tribe Collinsieae.

The timing of stigma receptivity, as assessed by peroxidase activity, varied markedly among species. The stages with receptive stigmas ranged from stage 0 (stigma receptive when the flower first opens, prior to dehiscence of the first anther) in a number of species with small- to medium-sized flowers, to stage 3.28 (stigma receptive after the dehiscence of the third anther) in *C. tinctoria*, a large-flowered species. Early stigma receptivity was observed in all species with known early anther-stigma contact. Early stigma receptivity was also observed in some species with intermediate times of anther-stigma contact (e.g., *C. sparsiflora arvensis*: SPA-50 = 0, ASC-50 = 1.72; *C. violacea*: SPA-50 = 0, ASC-50 = 1.80; Table 1). Because the position of the style in the keel allows the stigma to contact bees that depress the keel at early as well as late stages, these observations suggest that the receptive period prior to anther-stigma contact in some species may provide a significant opportunity for cross-pollination prior to self-pollination.

Promotion of cross-pollination is less clear in species with late-receptive stigmas. In a few species (e.g., *C. heterophylla*) the stigmas are receptive shortly before anther-stigma contact, providing a short period during which cross-pollination can occur before self-pollination. In other species (e.g., *C. multicolor*, *C. tinctoria*), receptivity is nearly simultaneous with, or in two cases (*C. corymbosa*, *C. verna*) shortly after, anther-stigma contact. This last developmental condition, in particular, appears to preclude cross-pollination prior to self-pollination, yet *C. verna* is moderately outcrossing (Kalisz et al., 1999), as is *C. heterophylla* (Mayer, Charlesworth, and Meyers, 1996). Thus the length of the period between stigma receptivity and anther-stigma contact only partly accounts for the dynamics of pollen arrival and likelihood of cross-fertilization. Interestingly, stigma flaring generally occurred early in floral development, even in species with late receptivity (Table 1, see also Kalisz et al., 1999).

Trait covariance—Inspection of the patterns of floral development during anthesis (Fig. 3, Table 1) suggests that populations and species with large flowers (Fig. 3, right column) have more closely placed (precise) anthers at dehiscence than do populations and species with smaller flowers (Fig. 3, left column). Upper (initially shorter) stamens tend to grow more than lower (initially longer) stamens prior to anther dehiscence in large-flowered species, and hence the anther positions tend to converge with filament elongation. The resulting pollen zone is smaller than would result from isometric development. The trajectories of stamen growth tend to be closer to parallel in smaller-flowered populations and species. In each of the five *Collinsia* sister-species/population pairs (*C. torreyi* var. *wrightii* vs. *C. t.* var. *torreyi*; *C. rattanii* vs. *C. linearis*; *C. parviflora* vs. *C. grandiflora*; *C. sparsiflora* var. *collina* vs. *C. s.* var. *arvensis*; and *C. heterophylla*, population 4 vs. *C. heterophylla*, population 2) the larger-flowered species or population had more closely positioned anthers (greater developmental-precision index) than the small flowered species or population (see Table 1; phylogenetically corrected paired comparison test; $t = 7.35$, $P < 0.002$).

However, species with larger flowers generally had larger pollen zones in absolute size than species with small flowers, as might be expected from allometric/isometric scaling (see Table 1), although this pattern was not significant (paired comparison test; $t = 1.10$, $P > 0.1$). This tendency towards increased absolute size of the pollen zone indicates that increased relative precision does not usually fully compensate for the positive allometric effect of increasing flower and anther size. Nevertheless, the slope of the logarithmic scaling of pollen zone with flower size was 0.50, which was significantly shallower than the isometric scaling of pollen zone with flower size (i.e., $\beta = 1.0$; $P < 0.001$; Fig. 5). Anther length also increased allometrically with flower size, but its slope (0.74) was not significantly shallower than isometric or significantly steeper than that of the pollen zone (Fig. 5). These relationships suggest that smaller-than-expected (isometrically) pollen zones in large flowers are the result of more closely placed anthers rather than smaller anthers.

Inspection of the left and right panels in Fig. 4 and values

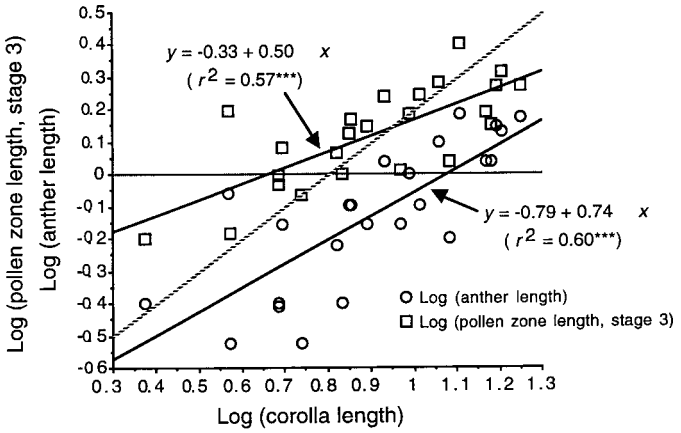


Fig. 5. Log mean pollen-zone length in stage 3 (squares) and log mean anther lengths (circles) regressed against the log mean corolla length for all 24 populations/species. The dotted line shows isometric increase. Note both relationships are negatively allometric (shallower than the isometric line), but only the relationship between pollen zone and flower length differs significantly in slope from the isometric line.

in Table 1 suggest correlated evolution between flower size and the timing of entry of the stigma into the pollen zone. In all five comparisons of species or population pairs, the small-flowered taxon showed the style elongating into the pollen zone earlier than the large-flowered taxon (paired comparison test; $t = 4.84$, $P < 0.01$). This pattern indicates correlated evolution of flower size and the timing of self-pollination: late self-pollinating species generally have larger flowers than early self-pollinating species.

Correlation analysis showed a complex pattern of intercorrelation among the various morphological and developmental variables. The naïve and phylogenetically corrected (PC) correlation analyses gave some strikingly different results (compare above vs. below diagonal, Table 3). Phylogenetically naïve analyses indicated that the time of stigma receptivity (SPA-50) was significantly positively correlated with flower size, anther length, size of pollen zone, and ASC-50 and was significantly negatively correlated with relative precision (Table 3). Incomplete sampling precluded PC analysis of the covariance patterns of SPA-50, so we cannot be certain that these trends would remain after correcting for phylogeny.

TABLE 4. Results of factor analyses on naïve and phylogenetically corrected data. Numbers for flower length, ASC-50, relative precision, ovule number, anther length, and pollen zone are variable loadings on each factor. Major loadings are in boldface type.

Variable	Factor 1 (naïve)	Factor 2 (naïve)	Factor 1 (PC)	Factor 2 (PC)
Eigenvalue	4.05	0.93	2.81	2.28
Percent variance	67.4	15.4	46.9	38.0
Flower length	**0.692**	**−0.655**	0.252	**−0.908**
ASC-50	0.249	**−0.919**	−0.401	**−0.827**
Relative precision	−0.251	**0.902**	−0.485	**0.798**
Ovule number	**0.696**	−0.157	**0.725**	0.500
Anther length	**0.877**	−0.351	**0.967**	−0.141
Pollen zone	**0.905**	−0.247	**0.839**	−0.234

Interpretation of the covariance pattern is simplified by factor analysis. Although the naïve and phylogenetically corrected data gave highly divergent results in the unrotated principal components analysis (not shown), the results were very similar after factor rotation, and only the latter are reported (Table 4). The six variables loaded three each on the two primary axes. Flower length, ASC-50, and relative precision loaded together in both naïve and PC analyses, indicating that they covary, perhaps in response to correlational selection related to mating system. Ovule number, anther size, and pollen zone loaded together on the same axis, perhaps covarying in response to selection on pollen-ovule ratio and the mechanical influence of anther size on pollen-zone size. In the phylogenetically naïve analysis, flower size loaded on both axes. Otherwise the results were nearly identical.

Because some floral traits are influenced causally by others and additional causal relationships can be reasonably inferred, path analysis was used to conduct a more integrated and quantitative analysis of the partial responses of relative precision and size of the pollen zone to time of anther-stigma contact, flower size, anther size, and number of ovules. We conducted conventional path analysis on the phylogenetically naïve data and compared the results with those of path analysis conducted on independent contrasts.

Path analysis of the phylogenetically naïve data showed a strong positive correlation between the time of anther-stigma contact and flower size (Fig. 6A). Relative precision of stamen position responded positively to ASC-50 but not to flower size. Ovule number and anther size both increased with flower size,

TABLE 3. Pairwise correlations among morphological and developmental variables. Naïve correlations are below the diagonal and phylogenetically corrected correlations are above the diagonal. N is in parentheses for naïve data, $N = 21$ for phylogenetically corrected data. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$.

Variable	Flower length	ASC-50	Relative precision	Ovule number	Anther length	Pollen zone
Flower length		0.621**	−0.850***	−0.171	0.348	0.315
ASC-50	0.780*** (23)		−0.370	−0.629**	−0.235	−0.112
Relative precision	−0.711*** (23)	−0.798*** (23)		0.046	−0.551*	−0.506*
Ovule number	0.522** (26)	0.362 (23)	−0.330 (23)		0.615**	0.320
Anther length	0.778*** (24)	0.522* (23)	−0.554** (23)	0.504* (24)		0.848***
Pollen zone	0.753*** (24)	0.433* (23)	−0.455* (23)	0.467* (24)	0.927*** (24)	
SPA-50	0.808*** (16)	0.826*** (15)	−0.755*** (16)	0.399 (16)	0.750*** (16)	0.661** (16)

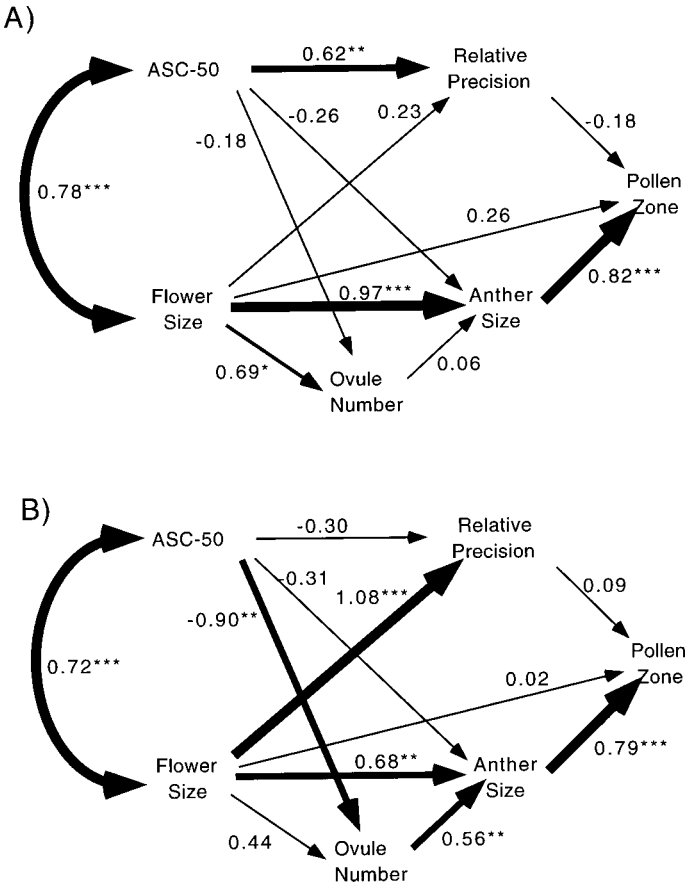


Fig. 6. Path diagrams of the partial responses of relative precision in placement of dehiscing anthers and size of the pollen zone to time of anther-stigma contact, flower size, anther size, and number of ovules. (A) Based on phylogenetically naïve data. (B) Based on phylogenetic independent contrasts.

but there was no discernible effect of ovule number on anther size. The size of the pollen zone was strongly affected by anther size.

The path analysis of independent contrasts derived from the ITS phylogeny (Fig. 6B) gave somewhat different results to those of the naïve analysis. Flower size and ASC-50 were again positively correlated. However, the relative precision responded positively to flower size rather than to ASC-50. Ovule number appeared to be influenced by ASC-50 rather than flower size. In this analysis anther size was significantly related to ovule number ($P < 0.01$), as well as to flower size. As in the naïve analysis, the size of the pollen zone was strongly affected by anther size.

DISCUSSION AND CONCLUSIONS

Floral development during anthesis—The overall pattern of post-anthesis floral development was largely similar, for the traits examined, across most members of tribe Collinsieae, despite substantial diversity in pollination ecology, flower size, and floral morphology (flowers ranging from ~2 mm in length and slightly zygomorphic in *Tonella* to nearly 20 mm in length and extreme zygomorphy in *Collinsia*). All species showed similar patterns of elongation of stamens and dehiscence of anthers, with one (or rarely two) anthers dehiscing at a time during the period of anthesis. Most species also showed a gradual elongation of the style during anthesis. Species varied in the floral stages during which stigma receptivity first occurred and during which stigmas came in contact with anthers, and in degree of convergence of anther position during stamen elongation and maturation. Much of this variation in developmental patterns is assignable to two syndromes: small-flowered species with widely spaced anthers at dehiscence (low developmental precision) and early self-pollination and large-flowered species with closely spaced anthers at dehiscence (high developmental precision) and late self-pollination. This variation is to a surprising extent independent of phylogeny: sister species often belong to opposite syndromes.

Variation in timing of stigma receptivity was incompletely assessed here, but enough data are available to reveal a pattern and raise a question. The data presented here support our interpretation that stigmatic peroxidase activity corresponds closely with stigma receptivity to pollen germination (see also Kalisz et al., 1999), although additional data are needed. All populations and species studied with early anther-stigma contact showed early peroxidase activity (i.e., stigma receptivity) and generally early pollen-tube growth. These small-flowered populations are almost certainly predominantly self-pollinating; self-pollen arrives and germinates on the receptive stigma shortly after the flower opens.

Several studied populations with medium to late anther-stigma contact have stigmas that become receptive well before anther-stigma contact. This developmental pattern presumably creates an herkogamous period (dehiscing anthers and receptive stigma spatially separated) during which cross-pollination can occur prior to self-pollination (Rust and Clement, 1977; Armbruster, 1980). Self-pollination occurs with anther-stigma contact, but long after stigmas become receptive to pollen-tube growth. Hence, the likelihood of experiencing cross-fertilization should be much greater for these large-flowered populations than for populations with early anther-stigma contact.

Finally, a few large-flowered taxa studied have stigmas that become receptive at about the same time as, or shortly after, anther-stigma contact. This pattern, seen most strongly in *C. verna* (see also Kalisz et al., 1999), *C. corymbosa*, and *C. tinctoria*, suggests that self-pollination (1) occurs as early as or even before cross-pollination and (2) occurs later than indicated by ASC-50. Absence of an herkogamous period (during which cross-pollination could precede self-pollination) in these large-flowered species is puzzling, especially because *C. verna*, at least, has moderately high rates of outcrossing (about as high as *C. heterophylla*, which appears to have an herkogamous period). More study is needed before we can understand the dynamics of cross- vs. self-pollen arrival in these species.

One possible mechanism that may promote outcrossing in populations that have stigmas becoming receptive at the same time as, or after, anther-stigma contact involves the flaring of the stigmatic lobes and expansion of papillae prior to physiological receptivity. Early flaring appears to occur in most *Collinsia* species (Table 1; Kalisz et al., 1999). Cross pollen may arrive and lodge on these stigmas after flaring but before receptivity (and thus before anther-stigma contact), although the pollen apparently does not germinate. Upon receptivity, cross-pollen tubes may grow down the style prior to deposition of self-pollen on the stigma. Alternatively, cross and self-pollen may lodge on the receptive stigma simultaneously, but cross-pollen tubes may grow faster than the self-pollen tubes and reach the ovules first. Germination of pollen after accumula-

tion of a pollen load has been observed in another member of the Scrophulariaceae s.l. (Stewart, Stewart, and Canne-Hilliker, 1996) and a lily (Kingston, 1998).

A possible advantage of receiving pollen on the stigmas over a protracted period but delaying receptivity until near the end of anthesis is that it may allow a large pollen load to accumulate on the stigma prior to pollen germination, leading to more intense pollen competition and higher offspring fitness. This possible advantage may hold both for pollen arriving from genetically diverse fathers (e.g., Snow and Spira, 1996) and for self-pollen, with pollen competition possibly reducing the effects of inbreeding depression (W. S. Armbruster and D. G. Rogers, unpublished data).

Trait covariance—Correlation, factor, and path analyses all helped to describe the covariation between morphological and developmental traits. Generally, the results of naïve and phylogenetically corrected analyses were similar, which suggests that most of the morphological and developmental traits are evolutionary labile and not constrained by factors associated with phylogeny. This interpretation is consistent with sister taxa often having contrasting flower sizes and probably mating systems. ''Phylogenetic lag'' is suggested when phylogenetically corrected analyses detect relationships not detected by naïve analysis. This reflects the influence of ancestral character states on current phenotype in addition to selection. In contrast, phylogenetic inertia (stasis) and pseudoreplicatory sampling is indicated when naïve analyses yield (mistakenly) significant results not shown by the corresponding phylogenetically corrected analyses. This reflects the influence of ancestral character states alone.

While the factor analysis yielded similar results for both naïve and phylogenetically corrected data, the correlation and path analyses showed several differences between the two types of data. For example, PC path analysis indicated significant positive covariance between anther size and ovule number and a significant decrease in ovule number associated with delayed anther-stigma contact, relationships not detected by the naïve analysis. (Both are predicted from the theory of optimal pollen/ovule ratios; Cruden, 1977.) These differences suggest that ancestral traits have influenced, but not prevented, the response to selection for optimal pollen/ovule ratios (phylogenetic lag).

The PC path analysis also indicated that relative precision in placement of dehiscing anthers varied in response to flower size rather than to ASC-50, whereas the naïve analysis indicated the reverse. However, this difference is more simply interpreted as a result of analytical instability generated by colinearity between ASC-50 and flower size ($r = 0.78/0.72$), rather than as the effect of phylogenetic inertia and/or pseudoreplication.

Tests of predictions—Our first prediction, that large-flowered species have delayed self-pollination was clearly supported, with highly significant PC and naïve correlations between the two traits (PC $r = 0.72$). This result leads us to conclude that small-flowered taxa are largely self-pollinating inbreeders and large-flowered taxa are more often outcrossing. Preliminary genetic data on mating systems support this interpretation (Weil and Allard, 1964; Charlesworth and Mayer, 1995; Mayer, Charlesworth, and Meyers, 1996; Kalisz et al., 1999; A. Kahler, University of California, Davis, personal communication, 1980)

Our second prediction, that large-flowered taxa have greater relative precision in anther position than small-flower taxa at the end of stamen elongation, was supported by both PC and naïve analyses. Thus, it appears that more consistent anther-stigma separation (herkogamy) is associated with delayed selfing, higher levels of outcrossing, and greater reliance on pollinators. We also found that the absolute size of the pollen zone increased through allometric scaling with flower and anther size, but significantly less steeply than the isometric relationship. This allometric relationship suggests also that selection for greater herkogamy, and hence a proportionally smaller pollen zone, has operated on larger flowered, outcrossing populations.

The third prediction, that self-pollinating populations will have smaller stamens and/or more ovules (i.e., lower pollen-ovule ratios) than cross-pollinating populations, was supported by the positive relationship between anther size and flower size in both phylogenetically corrected and naïve analyses. However, these results could also be interpreted as a result of scaling. In neither analysis were larger anthers associated with later anther-stigma contact after the effect of flower size was removed. Hence, the biological significance of these covariance relationships is difficult to assess.

Ovule number generally increased with flower size (strongly so in naïve analysis but only weakly so in the PC analysis). To the extent that flower size is a measure of mating system, this positive association between ovule number and flower size is the opposite of our prediction but consistent with flower-size scaling. However, in the phylogenetically corrected analysis ovule number was negatively related to the timing of anther-stigma contact, after the effect of flower size was removed. In other words, late-selfing species tended to have fewer ovules than earlier-selfing species of similar flower size. This pattern is consistent with the prediction of higher pollen/ovule ratios with greater outcrossing. Anther size was positively related to ovule number after removing the effect of flower size ($P < 0.01$; PC analysis), also as expected.

The fourth prediction was that flower size, anther size, precision of anther placement, and time of anther-stigma contact vary continuously and are intercorrelated. Indeed we found continuous variation in each trait and positive covariance between them. Although clusters of populations occurred at either end of the hypothesized mating-system continuum, over half the populations occurred in the middle of the distribution (Fig. 7). We suggest two possible explanations for this continuous variation: (1) that selection is driving populations towards the two extreme syndromes (two adaptive peaks), but genetic constraint or phylogenetic lag (manifested as a partial inertial pattern on the phylogeny) causes most populations to fall in between the extremes or (2) that continuous variation occurs in the fitness function (e.g., an adaptive ridge or cordillera) because correlational selection (Endler, 1976, 1995; Armbruster and Schwaegerle, 1996) acts on the various floral traits affecting mating system, and both extreme and intermediate mating systems represent stable optima. The limited phylogenetic inertia seen in this study system tends to support the first hypothesis, but the obvious evolutionary lability of traits that seem to influence mating system even more strongly supports the second hypothesis.

Both pollen zone and anther size could be expected to covary with flower size if species and population divergence simply follow a ''constrained'' path of allometric scaling. How can we decide whether the small flower, small anther, small

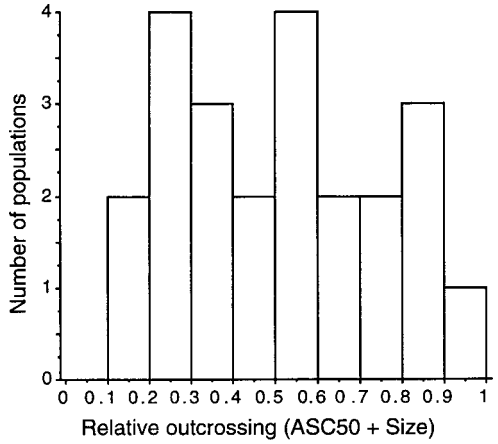


Fig. 7. Joint distribution of the relative flower length and time of self-pollination (taken together as a rough proxy for mating system). The index was calculated as the average of the relative value of flower size and the relative value of ASC-50. Values near zero are inferred to be self-pollinating and near one to be outcrossing.

pollen zone, and early-selfing combination is an adaptive complex associated with self-pollination and hypothesized inbreeding or is simply the result of constrained evolution toward smaller, faster-developing, and/or physiologically cheaper flowers (see Galen 1999, 2000; Galen, Sherry, and Carroll, 1999; Jonas and Geber, 1999; Runions and Geber, 2000)? And what if the evolutionary sequence is the reverse, from small-flowered to large-flowered, as some preliminary phylogenetic data suggest? Does the directionality of evolutionary change alter the likelihood of the adaptive vs. constraint scenario?

Various lines of evidence suggest that the above and related floral traits covary adaptively rather than as a result of genetic or developmental constraints. Most simply, time of anther-stigma contact and relative precision are size-independent traits, and yet they strongly covary positively and negatively, respectively, with flower size in the phylogenetically corrected analyses, as expected from our adaptive-covariance hypothesis. Second, by holding flower size statistically constant in path analysis we can assess the relationship between time of self-pollination (ASC-50) and ovule number more or less independently of confounding effects of flower size. Doing so, we see a significant negative relationship between time of self-pollination (ASC-50) and ovule number, as expected. Similarly, after correcting for phylogeny, the relationship between anther size and ovule number is positive even when flower size is held constant, as expected from the adaptive covariance hypothesis.

The adaptive scenarios presented above relating to covariation of mating system, flower size, anther size, ovule number, relative precision in anther position during anthesis, and size of the pollen zone are reasonable both in the context of evolution toward more cross-pollination and in the context of evolution toward more self-pollination. However, concluding that the above trait covariance reflects correlated response to selection for more faster, economical flowers makes sense (the above counterevidence notwithstanding) only if predominant selfers have evolved from cross-pollinating species rather than vice versa. Thus, resolving the historical events that have led to the present distribution of mating systems across the phylogeny of tribe Collinsieae may allow discrimination between hypotheses explaining floral trait covariation. More detailed phylogenetic information may also allow us to ascertain the order of change of functionally related traits (see Donoghue, 1989; Armbruster, 1992; Pagel, 1994, 1997).

In summary, floral traits in Collinsieae tend to be integrated into covarying suites, probably associated with high, intermediate, and very low outcrossing rates in different populations. Populations with small flowers have early anther-stigma contact, early stigma receptivity, and parallel stamen elongation leading to widely spaced dehiscing anthers. These flowers are almost certainly mostly autogamous. In contrast, populations with large flowers have delayed anther-stigma contact, often delayed stigma receptivity, and convergent stamen elongation leading to closer positioning of the dehiscing anthers. These larger-flowered populations appear to experience more cross-pollination and probably have outcrossing or mixed mating systems. Thus, we found a complex web of covariation that could best be interpreted as the result of multitrait adaptation for early selfing and high levels of autogamy and/or for delayed selfing and higher levels of outcrossing. However, the variation in these traits is continuous, indicating either continuous variation in selective optima or the combined effects of divergent selection (bimodal fitness optima) and phylogenetic lag. Evaluating the importance of these two processes remains a challenge for future comparative studies of tribe Collinsieae.

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