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Journal

Annals of Botany, 118(5)

ISSN

0305-7364

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Publication Date

2016-10-01

DOI

10.1093/aob/mcw134

Peer reviewed

Outcrossing and photosynthetic rates vary independently within two *Clarkia* species: implications for the joint evolution of drought escape physiology and mating system

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Received: 13 May 2016 Returned for revision: 13 May 2016 Accepted: 19 May 2016

• **Background and Aims** Mating systems of plants are diverse and evolutionarily labile. Abiotic environmental factors, such as seasonal drought, may impose selection on physiological traits that could lead to transitions in mating system if physiological traits are genetically correlated with traits that influence mating system. Within *Clarkia*, self-fertilizing taxa have higher photosynthetic rates, earlier flowering phenology, faster individual floral development and more compressed flowering periods than their outcrossing sister taxa, potentially reducing the selfing taxa's exposure to drought. In theory, this contrast in trait combinations between sister taxa could have arisen via correlated evolution due to pleiotropy or genetic linkage. Alternatively, each trait may evolve independently as part of a life history that is adaptive in seasonally dry environments.

• **Methods** To evaluate these hypotheses, we examined relationships between photosynthetic rates (adjusted for plant height and leaf node position) and outcrossing rates (estimated by allozyme variation in progeny arrays) during two consecutive years in multiple wild populations of two mixed-mating *Clarkia* taxa, each of which is sister to a derived selfing taxon. If the negative association between photosynthetic rate and outcrossing previously observed between sister taxa reflects correlated evolution due to a strong negative genetic correlation between these traits, then a similarly negative relationship would be observed within populations of each taxon. By contrast, if the combination of elevated photosynthetic rates and reduced outcrossing evolved independently within taxa, we predicted no consistent relationship between photosynthetic rate and outcrossing rate.

• **Key Results** We found no significant difference in outcrossing rates within populations between groups of plants with high versus low photosynthetic rates.

• **Conclusions** Overall, these results provide support for the hypothesis that the joint divergence in photosynthetic rate and mating system observed between *Clarkia* sister taxa is the result of independent evolutionary transitions.

Key words: *Clarkia unguiculata*, *Clarkia xantiana* ssp. *xantiana*, drought escape, life history, mating system, outcrossing, photosynthetic rate, physiology, self-fertilization.

INTRODUCTION

The evolution of self-fertilization from predominantly cross-fertilizing ancestors is likely the most common convergent transition in angiosperms (Stebbins, 1970). It is also consequential, as plant mating systems explain much of the observed variation among populations and taxa in genetic diversity and floral morphology (Wyatt, 1988; Hamrick and Godt, 1989; Sicard and Lenhard, 2011). There are two leading hypotheses to explain why self-fertilization may be favoured by natural selection. The first acknowledges that a self-fertile mutant arising in an outcrossing population has, on average, three means by which to transmit nuclear genes to subsequent generations, whereas strictly outcrossing individuals have only two (Fisher, 1941). The second hypothesis is that a strong advantage of selfing is gained from predictable mating opportunities (i.e. reproductive assurance), particularly where pollen vectors are scarce or unreliable, which is of keen import for sessile organisms such as

plants (Schoen *et al.*, 1996; Elle and Carney, 2003; Goodwillie *et al.*, 2005; Busch and Delph, 2012).

A third hypothesis that has received recent attention suggests self-fertilization may evolve indirectly due to genetic correlations with other traits that are themselves adaptive (Guerrant, 1989; Mazer *et al.*, 2004, 2010; Elle *et al.*, 2010; Ivey and Carr, 2012). Under this scenario, self-fertilization evolves along with other life-history traits that are adaptive in particular environments because of genetic correlations between traits conferring self-fertilization and other traits that are favoured in these environments. Self-fertilization can evolve through this mechanism even where it confers no direct fitness benefits (i.e. where it is neutral or even maladaptive), if the genetic correlation and selection are both sufficiently strong. Genetic correlations within ancestral populations may impose constraints on phenotypic divergence between descendant lineages (*sensu* Armbruster, 1991; Baker and Wilkinson, 2003). As an example, in one study, genetic variance–covariance matrices for five morphological traits did not

significantly differ across seven species of field cricket, and the sign of correlations in the phylogenetically reconstructed ancestral matrix explained observed patterns of phenotypic divergence among taxa (Bégin *et al.*, 2004). This result suggests that the ancestral G-matrix constrained the macroevolutionary history of this lineage. Similar patterns have been reported in various other taxa, including *Dalechampia* plants (Bolstad *et al.*, 2014) and *Anolis* lizards (Revell *et al.*, 2007). The hypothesis that mating system traits are genetically correlated with physiological traits could explain why traits that reduce exposure to water stress are often observed in self-fertile taxa (Kiang and Hamrick, 1978; Mazer *et al.*, 2004; Hall and Willis, 2006; Elle *et al.*, 2010; Ivey and Carr, 2012). In climates with pronounced seasonal drought, individual annual plants that complete their life cycle prior to becoming exposed to substantial water stress can have a strong advantage over those that delay maturation (Hall and Willis, 2006; Levin, 2006; Dudley *et al.*, 2012; see below). If traits promoting selfing are genetically correlated with those that contribute to drought escape, then self-fertilization may evolve as a correlated response to direct selection on drought escape traits in such environments. Genetic correlations among life history and physiological traits could arise via linkage if, for example, major quantitative trait loci segregate together. Genetic correlations can also reflect the pleiotropic effects of genes, and this is often reported for life-history and other quantitative traits (Conner, 2002; Latta and Gardner, 2009; Sletvold *et al.*, 2010; Friedman *et al.*, 2015; Schneider and Mazer, 2016; Smith, 2016). Within the genus *Clarkia*, predominantly self-fertilizing taxa flower earlier than their outcrossing progenitors, and have individual flowers that mature more rapidly, which leads to autonomous self-fertilization via synchronous and spatially proximal stigma receptivity and anther dehiscence (Mazer *et al.*, 2004; Dudley *et al.*, 2007). Rapid development in these taxa is accompanied by higher rates of carbon assimilation and respiration (Mazer *et al.*, 2010), which is adaptive in the ephemeral wet environments in which they occur (Dudley *et al.*, 2012). Thus, rapid development and autonomous self-fertilization in ancestral populations of the selfing taxa may have evolved as a response to selection on rapid photosynthetic rates if these traits are genetically correlated through pleiotropy or linkage (Mazer *et al.*, 2010; Dudley *et al.*, 2012; Schneider and Mazer, 2016).

An association between drought escape and mating system could also evolve adaptively if natural selection independently affects the evolution of both drought response and mating system traits. When an accelerated life cycle facilitates drought escape, self-fertilization may be favoured as an additional component of a rapid life cycle (e.g. Aarssen, 2000; Mazer *et al.*, 2004; Dudley *et al.*, 2015). Evaporative water loss from petals, for example, is reduced by relatively compressed flowering periods, shorter individual floral lifespans and smaller flowers (Galen, 1999; Lambrecht, 2013), but these are also transitions that may facilitate the evolution of self-fertilization (Fenster *et al.*, 1995; Armbruster *et al.*, 2002; Mazer *et al.*, 2004; Dudley *et al.*, 2007; Ivey and Carr, 2012; Jorgensen and Arathi, 2013). Life history changes associated with adaptation to drought conditions may favour self-fertilization through reproductive assurance as well. For example, the selfing *Clarkia xantiana* ssp. *parviflora* occurs in drier habitats than its outcrossing progenitor *C. x.* ssp. *xantiana* (Eckhart *et al.*, 2010; Dudley *et al.*, 2015) – a difference in distribution that appears

to be maintained in part by divergence in drought adaptation (Eckhart *et al.*, 2004; Geber and Eckhart, 2005). Notably, self-fertilization appears to be maintained in *C. x.* ssp. *parviflora* due to a reproductive assurance advantage conferred by the marked difference in pollinator communities across the ranges of the two subspecies (Moeller and Geber, 2005; Moeller, 2006; Runquist and Moeller, 2013).

Plant adaptation to drought is accomplished through coordinated suites of physiological as well as phenological traits (Ludlow, 1989; Kooyers, 2015). The constellation of traits associated with drought response in annual herbaceous plants in seasonally dry climates includes high photosynthetic rates, in addition to rapid growth and early flowering (i.e. ‘drought escape’ *sensu* Ludlow, 1989; Kooyers, 2015; but see Sherrard and Maherali, 2006; Maherali *et al.*, 2008). Higher photosynthetic rates in such plants may evolve by selection for high-activity variants of the Rubisco enzyme involved in carbon fixation. Plants with higher peak activity of Rubisco are able to maintain the photosynthetic capacity necessary for growth and flowering even as leaf internal carbon dioxide concentrations drop during stomatal closure (Wright *et al.*, 2001). In field and greenhouse populations of *Mimulus guttatus*, for example, leaf nitrogen content, which is often interpreted as a proxy for photosynthetic capacity (Reich *et al.*, 1997), was negatively phenotypically correlated with flowering date (Ivey and Carr, 2012), which likely mediates drought stress in the seasonally wet environments in which this species occurs. Similarly, in an 8-year study of a community of perennial understory herbs, the earliest-flowering taxa had the highest photosynthetic rates (Kudo *et al.*, 2008). Consistent with the idea that elevated Rubisco activity may evolve in response to water stress, Rubisco maximum carboxylation capacity (V_{cmax}) was found to be negatively associated with mean annual precipitation across a broad range of plant species (Vogan and Maherali, 2014). If physiological processes such as photosynthesis drive the evolution of drought escape, and if mating system and drought escape evolve together, then divergence in physiological traits would be expected between taxa with divergent mating systems. Comparisons of sister taxa with respect to mating system and physiological traits that may promote drought escape are consistent with this prediction (Mazer *et al.*, 2010; Wu *et al.*, 2010; Ivey and Carr, 2012). A study in the genus *Clarkia*, for example, found that facultatively autogamous taxa had higher photosynthetic rates, conductance rates and transpiration rates than their predominantly outcrossing sister taxa (Mazer *et al.*, 2010). Thus, the pattern observed within *Clarkia* is consistent with the hypothesis that mating system and drought escape evolve jointly.

To explore the nature of relationships between mating system and drought escape traits further, we examined phenotypic covariation between photosynthetic rates and outcrossing rates during two consecutive years within multiple field populations of two pollinator-dependent *Clarkia* taxa. *Clarkia* sister taxa differ with respect to both photosynthetic rate and mating system: *C. unguiculata* and *C. xantiana* ssp. *xantiana* have lower photosynthetic rates and greater dependence on outcrossing than their sister taxa, *C. exilis* and *C. xantiana* ssp. *parviflora* respectively (Mazer *et al.*, 2010). We considered two hypotheses regarding the joint evolution of these traits. First, if a genetically based correlation between outcrossing and photosynthetic

rate in ancestral populations of these taxa contributed to phenotypic divergence between taxa (the ‘correlated response to selection’ hypothesis, *sensu* Mazer *et al.*, 2004; Dudley *et al.*, 2012), then we would expect to observe negative relationships between outcrossing rate and photosynthetic rate within populations as well. In other words, strong phenotypic correlations within wild populations that mirror evolutionary divergence between taxa would provide corroborative evidence that ancestral genetic correlations influenced their joint evolution (*sensu* Begin *et al.*, 2004), assuming that the phenotypic correlations are a reliable proxy for genetically based correlations. Specifically, we predicted that within populations of *C. unguiculata* and *C. xantiana* ssp. *xantiana*, plants with higher average photosynthetic rates would also have lower average outcrossing rates. This pattern, if observed, would not determine whether phenotypic divergence were a consequence of genetic correlations. Nonetheless, such a pattern, if observed across multiple taxa and multiple populations, would provide supportive evidence for the correlated response to selection hypothesis. Moreover, given that phenotypic change results from natural selection, whereas genotypic change responds only indirectly, a strong phenotypic correlation between photosynthetic rates and outcrossing rate would necessarily result in a change in mating system if selection favours individuals with rapid photosynthetic rates, at least in the short term. The alternative hypothesis we considered is that photosynthetic rate and outcrossing rate evolved independently. A similar between-taxon pattern could result if, for example, high photosynthetic rate and high selfing rate were each adaptive for drought-sensitive genotypes where the growing season is sharply truncated by seasonal drought (i.e. the ‘drought escape’ hypothesis; Mazer *et al.*, 2004; Dudley *et al.*, 2015). Under this scenario, we would not expect to observe a consistent pattern of association between photosynthetic rate and outcrossing rate within populations.

MATERIALS AND METHODS

Study system

Clarkia unguiculata and *C. xantiana* ssp. *xantiana* are winter-annual wildflowers native to the California Floristic Province. Both are self-fertile and in pollinator-free glasshouse conditions both are capable of low levels of autonomous self-fertilization (Mazer *et al.*, 2010). Compared with each of their derived sister taxa, however, both *C. unguiculata* and *C. x.* ssp. *xantiana* have pronounced herkogamy and protandry and are more reliant on outcrossing (Moore and Lewis, 1965; Vasek, 1965; Dudley *et al.*, 2007). Previous estimates of outcrossing rate in *C. unguiculata* range from 0.79 to 1.0 (Vasek, 1965; A. Hove, unpubl. res.) and those of *C. x.* ssp. *xantiana* range from 0.59 to 0.85 (Moeller *et al.*, 2012; A. Hove, unpubl. res.). Geitonogamy may contribute to self-fertilization, although a recent emasculating study reported that geitonogamous pollen deposition did not contribute significantly to pollen loads on stigmas (A. Hove, unpubl. res.). Like most plants in the genus *Clarkia*, the two taxa studied here have a relatively late flowering phenology, most notably when compared with their highly autogamous sister taxa, *C. exilis*, and *C. x.* ssp. *parviflora*, respectively (Dudley *et al.*, 2007). The populations studied grew in the Lake Isabella region of Kern County, CA, USA, in 2009 and 2010

and were within 75 km of one another (Supplementary Data Table S1). We were able to study two populations of *C. unguiculata* during both 2009 and 2010, but herbivory during 2010 reduced seed production in the *C. x.* ssp. *xantiana* populations studied during 2009 below what was needed for estimating outcrossing rates (see below), so we used different populations of this taxon for the 2010 season. Monthly precipitation totals during the 2009 and 2010 growing seasons at these sites peaked during December to February at 1–1.5 cm, when plants were in early stages of development, and declined to zero by June or July, when plants were mature (Dudley *et al.*, 2015). Mean monthly maximum temperatures, on the other hand, were lowest from December to February at 10–15 °C and increased to 35 °C by July (Dudley *et al.*, 2015). As a consequence, leaf predawn water potentials, which become increasingly negative as plants experience stronger drought stress, declined steadily as plants matured (Dudley *et al.*, 2015). Significant variation among sites in monthly mean temperature and precipitation totals was reported for 2009 and 2010 (Dudley *et al.*, 2015), although during 2008 populations did not differ significantly in an index of drought stress calculated from temperature and precipitation (Mazer *et al.*, 2010).

Physiological measurements

At two times in the growing season (prior to flowering [Pre] and during peak flowering [Flower]), carbon assimilation (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) was measured in the field on 90 plants (without replacement) within each of two populations per taxon using a Li-Cor 6400 (Li-Cor, Lincoln, NE) for each of two years (2009 and 2010). Plants were randomly chosen along three 30-m transects within each site from among those plants bearing three or more leaves that were >2 cm long and appeared healthy and undamaged. Most measurements were recorded between 1000 and 1200 h, beginning about 30 min after direct sunlight exposure. Any plants that were not measured before photosynthetic rates dropped toward zero due to midday stomatal closure were measured the following morning. A single leaf was sampled for measuring A on each plant while the leaf remained attached to its stem. Leaves were selected based on size (large enough to enter the Li-Cor leaf chamber and touch the thermocouple) and appearance (no apparent herbivore or fungal damage). Internal chamber settings were as follows: photosynthetically active radiation (PAR) = 1500 $\mu\text{mol s}^{-1}$; stomatal ratio = 1.0; flow = 500 $\mu\text{mol s}^{-1}$ and reference chamber CO_2 concentration = 400 ppm. Some leaves were too narrow to completely fill the area of the 2-cm² leaf chamber. In these cases, the leaf area inside the chamber was calculated manually by tracing the edges of the leaf inside the chamber with ink, removing the leaf from the plant, taping the leaf to a piece of paper with transparent tape, and then scanning it using a digital scanner. The program ImageJ (Schneider *et al.*, 2012) was used to estimate the measured area of each leaf, and gas exchange estimates were adjusted based on these values using the Li-Cor recompute utility. Each leaf measured occurred along the primary stem axis and its node position (relative to the first true leaf) was also recorded. For additional details on field sampling methods and gas exchange measurements, see Dudley *et al.* (2015).

Previous studies have found that A covaries with node position and plant size (Mazer *et al.*, 2010; Dudley *et al.*, 2012). Thus, to control for some of the microenvironmental or developmental influences associated with plant size and leaf node position on photosynthetic rates, residual values from a multiple linear regression of A on $\ln(\text{plant height})$ and leaf node number were used in lieu of raw values of A . Additional unmeasured environmental factors or intrinsic (e.g. leaf N content) variables may have contributed to variation in A , but controlling for plant size and leaf node reduced environmentally induced variation in A and therefore increased the degree to which estimates of the phenotypic correlation between A and outcrossing rate within populations are likely to reflect the genetic correlation between these traits. We assumed that variance in these corrected measurements among plants was greater than variance within plants. Within each population and measurement time, plants with the 20 highest and 20 lowest residual values from this multiple regression were placed into Fast and Slow photosynthetic rate categories, respectively. These 640 plants (20 plants \times 2 measurement times \times 2 photosynthetic rate categories \times 4 populations \times 2 years) were the focus of subsequent analyses. At the end of the 2009 and 2010 growing seasons, seeds were collected from mature capsules on these plants, placed in paper coin envelopes, stored in zip-lock plastic bags with silica gel, and kept at -20°C until analysed.

Isozyme assays and mating system estimation

Approximately 40–50 seeds per maternal plant were placed on 8 % agar in 9-cm Petri plates and kept in the dark at 4°C for 7–10 d, after which they were placed under six-lamp fixtures with 32-watt, 2700-lumen, 6500-K tubes at room temperature and a 16-h photoperiod. Seeds of *C. x. ssp. xantiana* were gently nicked with a razor blade sanitized in 70 % EtOH prior to placing under light to improve germination. After 2–3 d, up to 20 germinated seedlings per family were transplanted to 79-cm² pots filled with moist Promix HP (Premier Tech Horticulture, Rivière-du-Loup, Quebec, Canada) soilless medium, maintained under the same light regime, and bottom-watered as needed. After about 2 weeks, leaves from up to ten seedlings per family were individually ground to a slurry in a chilled Tris–EDTA buffer (Gottlieb, 1984) and allozymes of each individual were resolved using horizontal starch gel electrophoresis (Wendel and Weeden, 1989). We screened a total of 20 enzymes on six buffer systems, and on the basis of this initial screen we chose seven enzymes reflecting nine putative loci that had consistent expression and polymorphism to use in the study.

Our primary aim in examining isozyme variation in progeny arrays was to estimate outcrossing rates within populations and maternal families of plants. Covariation between these estimates and photosynthetic rates were used to evaluate hypotheses concerning their joint evolution (see Introduction). We also used the isozyme phenotypes, however, to examine variation in additional mating system components for which we had no *a priori* predictions regarding covariation with photosynthetic rate and that to our knowledge had not previously been estimated in these taxa.

Mating system components were estimated using the Newton–Raphson procedure in MLTR (Ritland, 2002). In addition to estimates of the maximum likelihood multilocus outcrossing rate (t_m), we estimated population-level fixation indices (F), which provides an estimate of deviation from expected heterozygosity; biparental inbreeding ($t_m - t_s$), calculated as the difference between t_m and the mean of single-locus outcrossing estimates (t_s); the correlation of paternity among siblings within families (r_p), which can be interpreted as the probability that two siblings share the same outcrossed pollen parent; and the correlation of outcrossing among families (r_t), which can be interpreted as the normalized variance in outcrossing rate among families (Ritland, 2002). Within each population, mating system parameters were estimated for each combination of measurement time and photosynthetic rate as the mean of 1000 bootstrap replicates, in which the progeny array was the unit of resampling. Standard errors were estimated as the standard deviation of the bootstrap estimates. Maternal genotypes were inferred for each locus according to their likelihood, using a Monte Carlo modification of Brown and Allard (1970). For each population, estimates of F , r_p or r_t were considered to be stable if the absolute value of the mean divided by its standard error exceeded 1.96 (Zar, 1999). Parameter estimates that were unstable for all groups (Fast, Slow, Pre and Flower) within a population based on initial model runs were constrained to 0 for subsequent runs to increase power for estimating remaining parameters (Ritland, 2002). Population-level pollen and ovule allele frequencies were estimated separately, and estimates of allele frequencies from constrained models within each population were used to refine estimates of family-level t_m . Family estimates of t_m were generated for each family with at least five progeny using a multilocus method of moments procedure (Ritland, 2002). We used five progeny as a threshold for family-level estimation to improve the precision of estimates (see Ritland, 2002 and references therein for discussion of precision and accuracy in family-level mating system estimates). Imprecision in estimates of family-level outcrossing is widely reported (Morgan and Barrett, 1990; Ivey and Wyatt, 1999; Ritland, 2002); these estimates are best used for questions that transcend the value of the estimate as, for example, when investigating relationships between outcrossing rates and other traits (Ritland, 2002).

Analyses

Within each population and year, mating system parameters for each combination of measurement time (Pre or Flower) and photosynthetic rate category (Fast or Slow) were compared using pairwise two-sided Z -tests, based on the distribution of differences between estimates in bootstrap replicates. To correct for conducting six pairwise comparisons within populations, we used a Bonferroni correction ($\alpha = 0.008$) as a criterion for inference. In addition, we used mixed model analysis of covariance (ANCOVA) to evaluate the overall relationship between family-level t_m and residual photosynthetic rate within each species. Estimates of t_m and residual photosynthetic rate within populations did not deviate significantly from a normal distribution (Shapiro and Wilk, 1965), so analyses were performed on untransformed data. Fixed effects included residual

photosynthetic rate and measurement time (Pre or Flower). Random effects for the analysis of *C. x. ssp. xantiana* included population nested within year. Because the same *C. unguiculata* populations were sampled during both years, we included a population \times year interaction term as a random effect. Significance tests of fixed effects used Satterthwaite's approximation for degrees of freedom, and the significance of random effects was tested using log-likelihood ratio tests (Littell *et al.*, 1996). Analysis was conducted using PROC MIXED in SAS (SAS Institute, 2003).

RESULTS

Photosynthetic rate was variable within populations (Supplementary Data Table S2; Fig. 1). Residual photosynthetic rates were, on average, two orders of magnitude higher in plants categorized as Fast than in those categorized as Slow, indicating that the classification successfully distinguished groups of plants with extreme values of photosynthetic rate adjusted for plant height and leaf node position (Table S2; Fig. 1). Residual photosynthetic rates did not overlap between Fast and Slow groups for any population (Fig. 1). Measurements of carbon fixation (A) that were not adjusted for plant height and leaf node position showed a similar pattern (Table S2). With few exceptions, the maximum value of A observed within plants categorized as Slow was lower than the minimum value observed within Fast plants.

A mean (s.d.) of 459 (170) seedlings per population were analysed for allozyme variation (Supplementary Data Table S3). Polymorphism was sufficient for outcrossing rate estimation (Ritland, 2002); the mean (s.d.) expected frequency of the most common multilocus genotype within populations was 0.07 (0.05) (Table S3). Four of the eight population samples had fixation index estimates that deviated significantly from zero, two of which were negative (both *C. x. ssp. xantiana*) and two of which were positive (both *C. unguiculata*).

Most population-level outcrossing rates were >0.5 , with the exception of the 2010 Stark Creek population of *C. unguiculata* (Supplementary Data Tables S4, S5). Outcrossing rates did not significantly differ between Fast and Slow groups within any population (Tables S4, S5). Estimates of r_p were significantly positive in most cases within populations of *C. unguiculata* during both years (Tables S4, S5). These estimates indicate that the number of sires contributing to arrays (estimated as $1/r_p$; Ritland, 1989) ranged from 1 to 12 (Tables S4, S5). For pre-flowering plants in one population (2010 Live Oak, Table S5), r_p was nearly twice as high in the Slow photosynthetic rate group as in the Fast group; otherwise, these estimates did not significantly differ between any pair of groups within any population. Estimates of r_t were significantly positive for several groups within the *C. unguiculata* populations in both years, which indicates significant variation among families in outcrossing rate (Ritland, 1989). Only one population of *C. x. ssp. xantiana* had significantly positive estimates of r_p or r_t (Table S5). Biparental inbreeding ($t_m - t_s$) estimates were similarly more often significantly positive within *C. unguiculata* than *C. x. ssp. xantiana* populations (Tables S4, S5).

Of the 439 maternal plant families involved in the study, 77 had fewer than five progeny that could be assayed for allozyme

variation because of poor germination or low numbers of viable seed. Of the remaining 362 families, mean (s.d.) progeny array size was 9.1 (1.1). Family-level estimates of t_m among the families analysed ranged from 0 to 1.48 (Fig. 1) and standard errors of these estimates ranged from 0.001 to 1.058; the mean (s.d.) within-population standard error of family t_m was 0.19 (0.07), ranging from 0.10 to 0.46. Estimates of family t_m and residual photosynthetic rate varied broadly within each population in both years (Fig. 1). For *C. x. ssp. xantiana*, we found no significant relationship between t_m and residual photosynthetic rate (Table 1). Within *C. unguiculata*, there was an overall weak but significant negative relationship between outcrossing rate and residual photosynthetic rate [$t_m = 0.53 - 0.004$ (residual photosynthetic rate); Table 1].

DISCUSSION

Although several adaptive hypotheses for the evolution of self-fertilization are well characterized theoretically and empirically (e.g. Goodwillie *et al.*, 2005), alternative evolutionary pathways have received less attention. Here, we examined associations between the frequency of self-fertilization and photosynthetic performance within populations of *Clarkia unguiculata* and *C. x. ssp. xantiana*. We found little support for the hypothesis that relationships between photosynthetic rate and outcrossing rate in these taxa are influenced by genetic correlations between these traits. Under this hypothesis ('correlated response to selection'; Mazer *et al.*, 2004; Dudley *et al.*, 2015), the higher photosynthetic rate observed in autogamous *Clarkia* taxa relative to their outcrossing sister taxa would be mirrored by within-population covariation between A and t_m in progenitor outcrossing taxa (Mazer *et al.*, 2004, 2010; Ivey and Carr, 2012). The only significant relationship observed – a weak negative relationship between family-level outcrossing rates and residual photosynthetic rates within *C. unguiculata* (Table 1, Fig. 1) – was consistent with this prediction. There was no difference in mean outcrossing rate, however, between Fast and Slow photosynthetic groups within any population.

Overall, the results reported here are more consistent with the hypothesis that the taxonomic associations between photosynthesis and outcrossing rate within *Clarkia* occur because each trait evolved independently. Under this hypothesis, traits that contribute to rapid completion of the life cycle in the ephemeral mesic, early spring season are each independently favoured by natural selection. We found no difference in outcrossing rate estimates within multiple populations between groups of plants differing in photosynthetic rate. This suggests that there is no consistent pattern of association between these traits within populations. Thus, given available data, it appears that the taxonomic associations observed between outcrossing rate and photosynthetic rate within *Clarkia* (Mazer *et al.*, 2010) are a result of independent evolutionary transitions, likely having evolved through natural selection. In a study involving nine populations, Dudley *et al.* (2012) found that the autogamous *C. exilis* experienced stronger and more consistent selection favouring high photosynthetic rate when compared with *C. unguiculata*, its mixed-mating sister taxon and likely progenitor. This pattern of selection parallels phenotypic differences between taxa: *C. exilis* has higher photosynthetic rates than *C.*

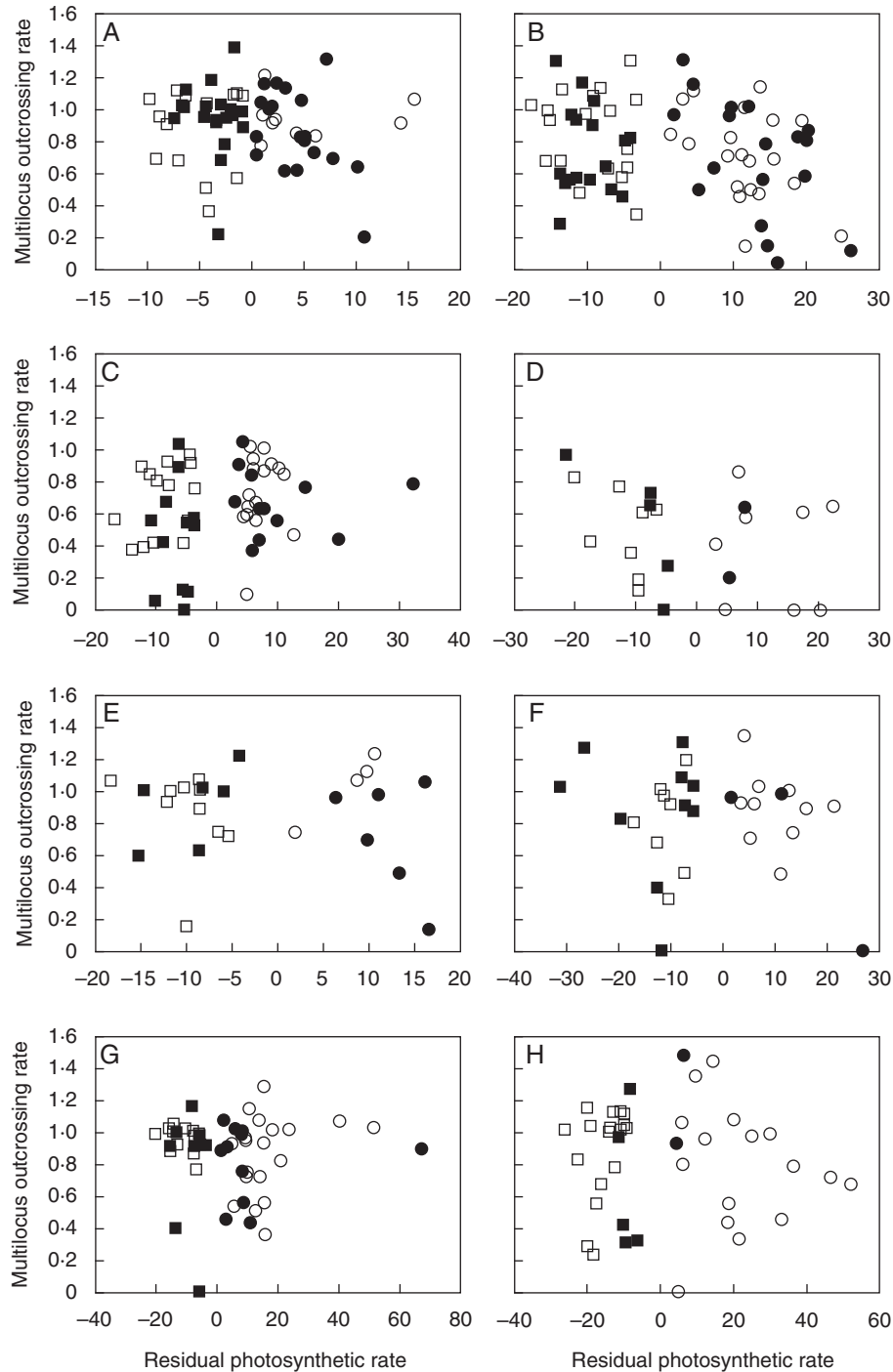


FIG. 1. Open-pollinated family-level multilocus outcrossing rate estimates of *Clarkia unguiculata* (A–D) and *C. xantiana* ssp. *xantiana* (E–H) plotted against residuals from a multiple linear regression of photosynthetic rate on leaf node number and plant height measured in the field prior to flowering (open symbols) or during peak flowering (closed symbols). Families classified as Fast versus Slow are plotted as circles (values of residual photosynthetic rate >0) and squares (<0), respectively. Data presented include only families with at least five progeny. Estimates were from the 2009 (A, B, E, F) and 2010 (C, D, G, H) growing seasons. Plant populations (Live Oak, A, C; Stark Creek, B, D; Lucas Creek, E; Sawmill, F; Borel Road, G; Camp Three, H) were located in Kern County, CA, USA.

unguiculata (Mazer et al., 2010; Dudley et al., 2012). Thus, historical selection in ancestral populations similar to what has been observed in contemporary populations may have contributed to existing phenotypic differences between taxa. In our study, it is noteworthy that we found phenotypic variation in

photosynthetic rate as well as outcrossing rate in all populations (Fig. 1); if this variation reflects standing genetic variance, the potential for adaptive phenotypic evolution still exists.

We did not estimate genetic correlations between outcrossing and photosynthetic rate, and thus cannot conclusively reject the

TABLE 1. Mixed-model analysis of covariance testing effects of residual photosynthetic rate (see Materials and methods), measurement time (flowering or pre-flowering) and interactions on family-level multilocus outcrossing rate estimates within two species of *Clarkia*

Species	Effect type	Source	$F_{\text{ndf, ddf}}$ or χ^2	P
<i>C. x. ssp. xantiana</i>	Fixed	Year	0.94 _{1,143}	0.9
		Residual photosynthetic rate	1.11 _{1,143}	0.3
		Measurement time	1.43 _{1,143}	0.2
		Photosynthetic rate × measurement time	0.27 _{1,143}	0.6
		Population (year)	<0.00	0.99
<i>C. unguiculata</i>	Fixed	Year	5.23 _{1,197}	0.15
		Residual photosynthetic rate	4.07_{1,209}	0.044
		Measurement time	1.28 _{1,210}	0.3
		Photosynthetic rate × measurement time	0.06 _{1,208}	0.8
		Population × year	11.6	<0.0003

Estimates of photosynthetic rate were recorded during the 2009 and 2010 growing season.

Statistically significant model terms ($P < 0.05$) are in bold. All populations were located in Kern County, CA, USA.

possibility that a genetic correlation exists, at least in some populations studied. There have been few direct tests for genetic correlations between mating system and drought escape traits, and, to our knowledge, none that have been conducted in multiple populations of wild plants growing in the field. In a greenhouse study, Dudley *et al.* (2007) reported positive broad-sense genetic correlations between dichogamy and age at first flower for both *C. unguiculata* and *C. xantiana ssp. xantiana*. In another greenhouse study, maternal families of *C. unguiculata* that flowered late had higher herkogamy than those that flowered early (Schneider and Mazer, 2016). These studies suggest that rapidly developing genotypes, which likely experience reduced exposure to drought, also have more synchronous and compressed within-flower development, which may increase the chance of autogamy. In another greenhouse study, positive additive genetic correlations were reported between flower size and age at first flower in *Mimulus guttatus*, which similarly may indicate a higher probability of autogamy in rapidly developing genotypes, because flower size is strongly and positively correlated with herkogamy in this species (Ivey and Carr, 2012).

In an observational field study such as ours, multiple environmental factors may contribute to phenotypic variation. We were able to statistically control for variation in photosynthetic rate due to node position of the sampled leaf and plant height, which is a proxy for plant vigour, and carbon assimilation was measured under constant conditions of light, CO₂ concentration and temperature (see Materials and methods). Thus, several potential environmental influences on phenotypic variation in photosynthetic rate were reduced. Other unmeasured environmental variables, however, may have also contributed to phenotypic variation (e.g. Zangerl and Bazzaz, 1983). Experimental fertilization treatments, for example, altered carbon assimilation rates in *Populus deltoides* saplings (Funk *et al.*, 2007). Similarly, variation in outcrossing rates are likely influenced by both environmental and genetic factors (Barrett and Harder, 1996; Karron *et al.*, 2012). If plant reproduction is strongly pollen-limited and pollinator visitation is rare, low outcrossing rates may primarily indicate autonomous selfing (Schoen and Lloyd, 1992; Goodwillie *et al.*, 2005), which may be a constitutive expression of plant genotype (e.g. *S*-allele expression; Levin, 1995). On the other hand, self-pollen deposition for some taxa requires pollinator visitation, and thus restricted

visits may reduce both self- and cross-fertilized seed production (Knight *et al.*, 2005). A 3-year study reported no pollen limitation in *C. unguiculata* and only slight pollen limitation in *C. xantiana ssp. xantiana* (A. Hove, unpubl. res.). In that study, both species depended on pollinators for a majority of pollen deposited onto stigmas, which suggested that variation observed in outcrossing rates in these taxa was mostly likely governed by the behaviour or abundance of floral visitors. Under such circumstances, the influence of plant genotype on the expression of outcrossing rates is likely to be weak.

CONCLUSIONS

Plant mating systems are remarkably diverse and labile (Barrett, 2002; Charlesworth, 2006), and the observed variation in mating systems likely is the result of a variety of mechanisms influencing their expression and their evolution. Here, hypotheses were evaluated concerning the evolution of associations between mating system and physiological traits across taxa within the genus *Clarkia* (Mazer *et al.*, 2010; Dudley *et al.*, 2012, 2015). Specifically, variation in outcrossing rates and photosynthetic rates were examined within populations of two mixed-mating *Clarkia* taxa. A pattern within populations in which plants with higher photosynthetic rates also had lower outcrossing rates would suggest that the similar genus-level taxonomic association previously reported (Mazer *et al.*, 2010) might have evolved via correlated evolution. However, little evidence supporting this pattern within populations was observed. Thus, the observed association between photosynthetic rate and outcrossing rates among taxa within *Clarkia* may reflect independent evolutionary transitions. Physiological adaptations to compressed growing seasons are associated with self-fertilization in other taxa (Arroyo, 1973; Elle *et al.*, 2010; Wu *et al.*, 2010; Ivey and Carr, 2012). Identifying the selective pressures behind these transitions will illuminate our understanding of the evolution of these key plant life-history characteristics.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: coordinates and elevations of studied populations of *Clarkia* from Kern

County, CA, USA. Table S2: least-squares mean (s.e.) A ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), minimum and maximum A and least-squares mean (s.e.) residual photosynthetic rate of *Clarkia* measured in the field after controlling for variation in plant height and leaf node position with a multiple regression. Table S3: summary of allozyme data in *Clarkia* populations from Kern County, CA. Table S4: mean (s.e.) mating system estimates for *Clarkia* populations based on allozyme loci, using arrays of open-pollinated seeds collected in Kern County, CA, 2009. Table S5: mean (s.e.) mating system estimates for *Clarkia* populations based on allozyme loci, using arrays of open-pollinated seeds collected in Kern County, CA, 2010.

ACKNOWLEDGEMENTS

We thank D. Ha, M. Ha, N. Habecker and K. Ronan for laboratory assistance, the Spring 2015 Faculty Learning Community writing group at Chico State for encouragement, and reviewers for helpful comments. This work was supported by a US National Science Foundation Research Opportunity Award supplement to S.J.M. (grant number OIS-0718227) and a sabbatical leave to C.T.I. from California State University, Chico.

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