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Incomplete loss of a conserved trait: function, latitudinal cline, and genetic constraints

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Retention of nonfunctional traits over evolutionary time is puzzling, because the cost of trait production should drive loss. Indeed, several studies have found nonfunctional traits are rapidly eliminated by selection. However, theory suggests that complex genetic interactions and a lack of genetic variance can constrain evolution, including trait loss. In the mustard family Brassicaceae the conserved floral condition includes four long and two short stamens, but we show that short stamens in the highly self-pollinating mustard *Arabidopsis thaliana* do not significantly increase selfed seed set, suggesting that the trait has lost most or all of its function after the transition to selfing. We find that short stamen loss is common in native populations. Loss is incomplete and decreases with increasing latitude, a cline unexplained by correlations with flowering time or ovule count (which also vary with latitude). Using recombinant inbred lines derived from a cross between plants at the latitudinal extremes of the native range, we found three QTLs affecting short stamen number, with epistasis among them constraining stamen loss. Constraints on stamen loss from both epistasis and low genetic variance may be augmented by high selfing rates, suggesting that these kinds of constraints may be common in inbred species.

KEY WORDS: constraint, epistasis, nonfunctional trait, *Arabidopsis thaliana*.

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While evolutionary biologists often focus on adaptive traits, it has been recognized since Darwin (1859) that reduction and loss of traits under relaxed selection is also a dominant theme in evolution. This may occur after a shift in ecology renders formerly adaptive traits nonfunctional (Fong et al. 1995), such as during colonization of a new environment. Well-known examples of this type of trait loss include elimination of eyes in cave dwelling fish (Yoshizawa et al. 2012) and reduced armor in freshwater sticklebacks (Le Rouzic et al. 2011). However, retention of nonfunctional traits is also common (Lahti et al. 2009), including feeding structures in larvae with no digestive tract (Pernet 2003), retinal circadian rhythms in blind cave fish (Espinasa and Jeffery 2006), oil glands in orchids that are no longer pollinated by the bees the oil rewarded (Steiner 1998), and antirattlesnake behaviors in ground squirrel populations isolated from the predator for >70,000 years (Coss 1999).

The evolutionary fate of nonfunctional traits depends on several factors. For traits requiring few resources, loss may be nearly neutral and occur only slowly through mutation accumulation (Lahti et al. 2009). Loss of a costly nonfunctional trait may be accelerated by selection; this has been shown in both of the best-understood examples of nonfunctional trait evolution, cavefish

eyes and freshwater stickleback armor (Le Rouzic et al. 2011; Yoshizawa et al. 2012). Trait loss may also be constrained by a lack of additive genetic variance; this can include pleiotropy, which can cause lack of variance in multivariate space (Dickerson 1955; Kirkpatrick 2009; Walsh and Blows 2009; but see Agrawal and Stinchcombe 2009, Conner 2012). In addition, epistasis can either facilitate or constrain adaptive loss (Hansen 2013). Synergistic epistatic interactions can lead to the release of standing genetic variation and an accelerated response to selection, as Carlborg et al. (2006) showed in chickens under artificial selection for body size. In the opposite scenario, adaptive evolution can be slowed by epistasis—in the most extreme case, the adaptive phenotype is only expressed in the presence of certain combinations of alleles across multiple loci (e.g., Blount et al. 2008; Futuyma 2010). This constraint would be augmented by a reduction in recombination among the epistatic loci, as would happen in asexual or highly self-fertilizing populations. Understanding the mechanisms constraining nonfunctional trait loss requires studies of similar depth as the cavefish and stickleback examples, but in taxa where the evolutionary loss is incomplete.

We present an example of incomplete loss of a nonfunctional trait in the model organism *Arabidopsis thaliana. Arabidopsis thaliana* is a member of the Brassicaceae, a family in which almost all of the >3700 species (Al-Shehbaz et al. 2006) have flowers with four long stamens and two short stamens (Endress 1992) (Fig. 1A). This condition, called tetradynamy, is diagnostic for this important plant family (Zomlefer 1994). There is evidence that tetradynamy is adaptive in outcrossing species (Conner et al. 2003; Kudo 2003); available data suggest that short stamens function to reduce the rate of pollen depletion under high per-flower visitation rates (Conner et al. 1995, 2003; cf., Harder and Thomson 1989). On the other hand, *A. thaliana* is highly (97–99%) self-pollinating (e.g., Abbott and Gomes 1989; Platt et al. 2010), reproducing by pollinating autonomously within flowers without the aid of insect pollinators. Because short stamens are thought to be adaptive for outcrossing, and the short stamen anthers are often placed far from the stigma, a shift to self-pollination should relax selection for their production.

The evolution of self-pollination from outcrossing is one of the most common transitions in flowering plants (Stebbins 1957; Barrett 2002), and is associated with a suite of trait changes known the "selfing syndrome" (Darwin 1876; Ornduff 1969); there is evidence that these trait changes are adaptive (Sicard and Lenhard 2011). A switch to self-pollination generally involves shifting resources away from attracting pollinators and increasing investment in female fitness compared to male fitness (Charlesworth and Charlesworth 1981). It includes less nectar, less scent, smaller flowers, reduced herkogamy (distance between anthers and stigma) and a diminished pollen-to-ovule (P/O) ratio relative to outcrossing plants (e.g. Lloyd 1965; Ritland and Ritland 1989; Goodwillie and Ness 2005), the last largely due to a reduction in pollen production (Darwin 1876; Ornduff 1969).

One way to reduce pollen investment in selfers is to eliminate some of the stamens. When stamens of two lengths exist in a flower, not producing the stamens that are further from the stigma—and therefore less likely to facilitate self-pollination—could increase fitness. Stamens of different lengths have been shown to have changed functions with selfing in the Ericaceae; Escaravage et al. (2001) showed experimentally that long stamens in *Rhododendron ferrugineum* did not function in selfing—in this species, only short stamens directly contact the stigma. Although no stamen loss has been recorded in *R. ferrugineum*, a pattern of loss of stamens whose anthers are farther from the stigma has been noted in other selfing taxa (Svensson 1990; Matsuhashi et al. 2012). Thus, stamen loss may not be simply a method of reducing the P/O ratio, but also a way to preferentially eliminate the stamens that are least likely to function in selfing. In the Brassicaceae, long stamens are usually closer to the stigma, making short stamens the candidates for elimination with the evolution of self-pollination.

Although tetradynamy is generally diagnostic in the Brassicaceae, some stamen loss exists in the family. The genus *Lepidium* features the most variation in stamen production, with reduction in stamen number due to loss of both short and long stamens in nearly every possible combination (Bowman et al. 1999). Selfing in this genus is associated with the two-staminate condition (Al-Shehbaz 1986), and two-staminate species always have long stamens only (Bowman et al. 1999). However, loss of short stamens in particular seems to be a side-effect of allopolyploidization in *Lepidium* (Lee et al. 2002) rather than a response to natural selection. Partial loss of short stamens has been noted in some populations of the selfing mustard *Cardamine hirsuta*, (Matsuhashi et al. 2012), and a similar pattern of occasional production of flowers lacking short stamens has been noted previously in *A. thaliana* (Müller 1961). In *A. thaliana*, the anthers of short stamens have been shown to remain below the level of the stigma throughout floral development (Müller 1961; Smyth et al. 1990) (Fig. 1A).

A trait that loses its primary function may still be adaptive if it increases the organism's fitness in some other way (Lahti et al. 2009), but that seems unlikely in this case. "Staminodes," organs evolved from stamens but currently performing alternative functions, occur in nearly a third of angiosperm families; however, they universally have roles in facilitating outcrossing via insect pollinators (Walker-Larsen and Harder 2000). Similarly, feeding anthers, sometimes with sterile pollen, likely function for outcrossing rather than selfing (Vallejo-Marin et al. 2009). Therefore, we might expect selection to reduce investment in short stamens in *A. thaliana* if they are indeed ineffective in self-pollination. In that case, reallocating resources such as lipids and proteins abundant in pollen (Evans et al. 1991) to other functions

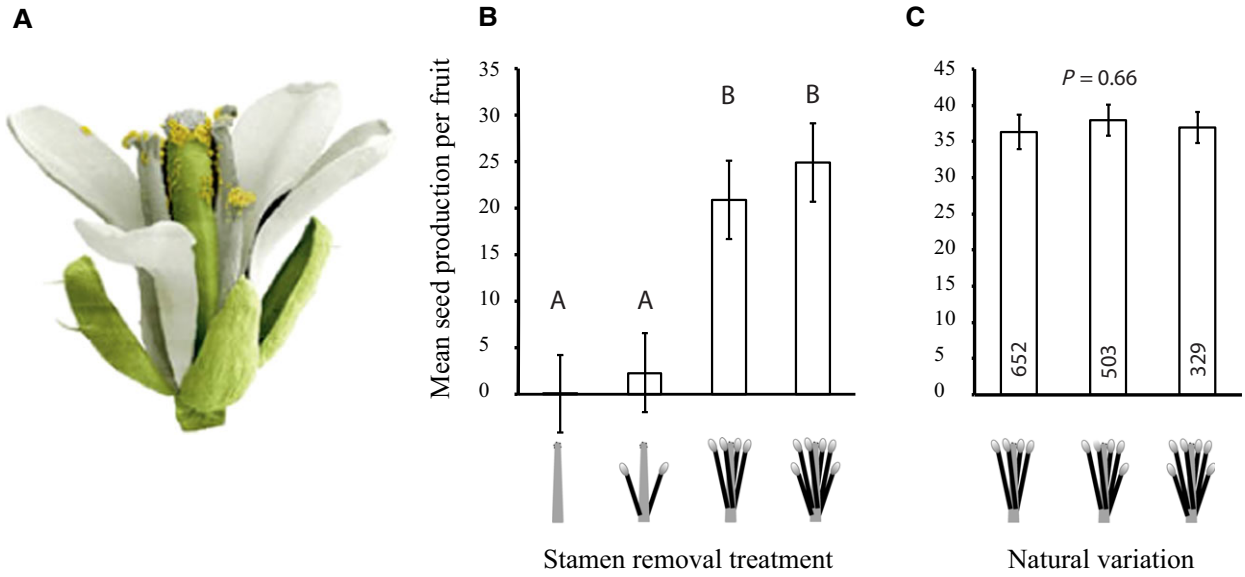

Figure 1. **Effects of short stamen number on per-flower seed set. (A) *Arabidopsis thaliana* flower showing the pollen from the short stamen deposited on the side of the pistil rather than the stigma. (Source: Jürgen Berger/Max Planck Institute for Developmental Biology, Tübingen, Germany.) (B) Least square means ±2 SEM from a model including stamen removal treatment, population, and plant nested in population as fixed effects. Shared letters above the bars indicate lack of significant difference according to a Tukey HSD test. There were 32–24 flowers per treatment (see Fig. S1). (C) Least square means ±2 SEM from a model including natural stamen number, with population, population × stamen number, and plant nested in population as fixed effects. Number of flowers with each stamen number shown within each bar, and the *P* value is from the stamen number main effect.**

by eliminating, or reducing investment in, short stamens would increase fitness.

The combination of a trait with loss that is incomplete, is easy to quantify (including effects on fitness) and manipulate, and is in a model organism with abundant genetic resources has allowed an unusually detailed glimpse of trait loss in progress. We used three approaches to understand the evolution of short stamens in *A. thaliana.* (1) We investigated the function of short stamens using experimental stamen removal and observations of flowers with natural variation in short stamen number to compare the contributions of short and long stamens to seed set. (2) Using a common garden approach with matrilines sampled from 45 populations across the native range, we measured the frequency of short stamen loss, its geographic distribution, and correlations with flowering time and ovule number, both of which affect fitness and show latitudinal clines (Stinchcombe et al. 2004; see Results). (3) Finally, we described the genetic basis of short stamen loss and identified potential genetic sources of constraint using quantitative trait locus (QTL) analysis.

Methods

PLANT MATERIAL

Except where specified, plants were grown in chambers at 22˚C at Michigan State University's main campus in East Lansing, Michigan (MSU), or at MSU's Kellogg Biological Station in Hickory Corners, Michigan (KBS) (see Supporting Information for details). Most seeds were obtained from the Arabidopsis Biological Resource Center (abrc.osu.edu). Collections in Sweden and Italy were made by D. Schemske, and Ukrainian collections were from J. Beck (Wichita State University) (Table S1).

MODELING STAMEN NUMBER AS A CONTINUOUS TRAIT

The number of short stamens within one flower is discrete. However, this discrete trait can be treated as the observed outcome of a binomial process, which depends on a continuous variable, *P*, the probability of short stamen loss. Estimates of mean short stamen number provide unbiased estimates of *P* at the plant level (see Appendix A). This continuous trait (Fig. S1) is the subject of all of our analyses occurring at the level of individual plants (or above). As for any trait, the value for an individual is what is relevant to selection; in the case of a serially repeated trait, the mean is appropriate and commonly used (e.g. Byars et al. 2007; Reynolds et al. 2010; Kulbaba and Worley 2013).

FUNCTION OF SHORT STAMENS IN SELF-POLLINATION

To test whether short stamens in *A. thaliana* are functional, we compared seed set across four stamen treatments: all six stamens removed, only long or only short stamens removed, or all stamens

intact but buds probed with forceps to control for stamen manipulations (Fig. 1B). Each treatment was performed on at least one bud per plant, with all four treatments performed on adjacent buds; one complete set of treatments was performed per plant. These treatments were carried out before anther dehiscence in unopened buds using fine forceps under a dissecting microscope. Pedicels were painted with unique colors indicating the treatment applied.

Because we only used flowers that produced two short stamens for the treatments, populations with high short stamen production were used (Table S1). In 2007, the stamen removal treatments were performed on 18 plants grown at MSU from two Swedish populations (seed originally collected by Jon Ågren and D. Schemske near the towns of Rödåsen and Skuleberget). In 2011, we repeated the experiment on two to four individuals from Rödåsen and four other populations grown at KBS (total 16 plants; grand total of 34 plants and 80 flowers). The 2011 plants were placed in the field after the treatments were applied, and returned to growth chambers after treated flowers senesced. Resulting seeds from each flower were counted, and seeds per fruit for the manipulated flowers were analyzed in JMP version 10.0.0 (SAS Institute 2012) with a model including treatment, population, and plant ID nested in population as fixed effects. Differences between treatment means were tested using a Tukey HSD test, also carried out in JMP (Table S2).

The challenges of floral manipulation on this small scale produced some outliers in the data. We tried to control for bud damage during stamen removal with the control in which buds were probed but not manipulated further. However, the control treatment was likely not aggressive enough to mimic damage levels from actual short stamen removal. The position of short stamens deeper in the buds makes it likely that short stamen removal will either disrupt long anther position (reducing contact with the stigma) or that the pistil itself will be damaged. Evidence for this comes from the greater number of flowers with zero seed set in the short stamen removal treatment compared to the manipulated control (Fig. S2). Four flowers had unusually high seed set (≥ 24) in the long and all stamens removed treatments (Fig. S2). These were likely the result of accidental pollinations; three of the four (including the highest in both treatments) occurred in growth chambers, where no potential insect pollinators were present. To investigate the effect of these outliers, we also performed the analysis of seed set in manipulated flowers with outliers excluded (flowers with seed set of zero when no stamens or short stamens were removed, and flowers with seed set over 23 when all stamens or long stamens were removed).

For a complementary test of short stamen function using natural variation, we grew two plants from each of eight matrilines that exhibited stamen loss from across the native range. Short stamen number was scored nondestructively on most or all flowers produced by these 16 plants, and then the pedicel of each flower was marked with paint indicating the number of short stamens present. The 1484 fruits produced by these flowers were collected and seeds counted. To test for a relationship between short stamen number and seeds set by individual flowers, we ran a fixed-effects model in JMP with number of seeds in each fruit as the response variable, and number of short stamens, matriline, the short stamen $\times$ matriline interaction, and plant nested within matriline as predictors.

GEOGRAPHIC VARIATION IN SHORT STAMEN PRODUCTION AND CORRELATIONS WITH OTHER TRAITS

To determine if short stamen loss is common in *A. thaliana* and to test for geographic trends for this and correlated traits, we recorded stamen production, ovule production, and date of flower collection in plants from 45 populations sampled as evenly as possible from latitudinal and longitudinal bands across the native range (Fig. 2; Table S1). We grew seven to 10 plants from each population, with up to five matrilines per population (mean = 2.67), for a total of 428 plants from 119 matrilines (Table S1). Plants were grown at KBS with matrilines randomly interspersed in trays. Tray positions in growth chambers were rotated weekly to minimize position effects. Replicate plants for each line were divided evenly in two blocks grown under identical chamber settings two months apart. For stamen counts, one flower was sampled from each plant every two to three days for an average of six flowers per plant (2570 total flowers), with 13–117 flowers sampled per population (mean 56.6, SD 22.0). Flowers were preserved in 70% ethanol for later stamen counts using a dissecting microscope. Short stamen number was used in the analysis because a reduction in total stamen count is nearly always due to loss of short stamens (only 31 of 2570 flowers examined, or 1.2%, produced fewer than four long stamens, compared to 1496, or 58.2%, producing fewer than two short stamens).

Ovule counts were performed on a subset of 304 preserved flowers from 37 of the native range populations (Table S3). We stripped sepals, petals, and stamens from the pistil, placed the pistil on a glass slide, and pressed it gently under a coverslip with a drop of blue food coloring to highlight the ovules. Ovules were then counted with a hand tally counter. Counts were repeated at least once to verify, using the average of three counts if no consensus was reached.

To estimate flowering time (which was not recorded directly) in the common garden, we used the subset of 257 individual plants with data on floral rank and collection date, set the earliest collection date for each block as 1, regressed collection date on floral rank for each plant, and used the *Y*-intercept as the estimate of flowering time. To eliminate poor estimates of flowering time, we excluded individuals with less than three flowers collected and/or

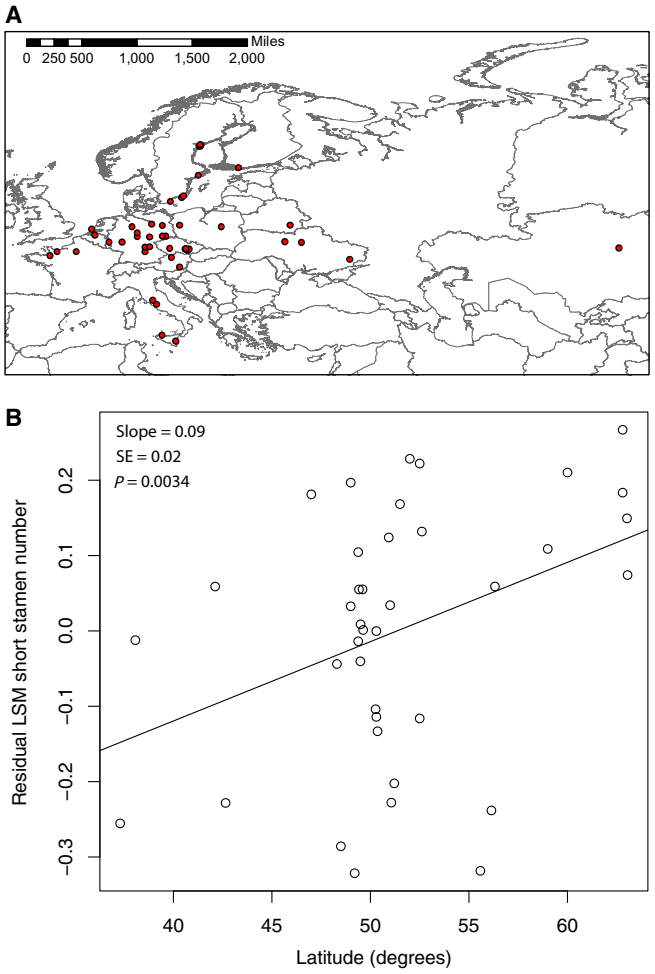

Figure 2. Geographic variation in short stamen number. (A) Populations were sampled in latitudinal and longitudinal bands of the native range. (B) Short stamen production increases with latitude. The plot shows residual population mean short stamen number after removing the effects of longitude and flowering time (cf. Table 2B).

flowers collected on only one day (representing 32.7% of plants), and those with regression r^2 values below 0.9 (12.1% of plants), leaving a total of 142 individuals in 40 populations (this reduction in sample size did not qualitatively change the results for the analysis including flowering time). We tested this method on a set of 10 plants from a line not used in this study; the correlation between the regression estimate and known flowering time was 0.993. We estimated population mean flowering time from the individual plant estimates as least square means (LSM) from a model including population and matriline nested in population as random effects. Population mean stamen number and ovule number were estimated as LSMs from models including population as a fixed effect, matriline nested within population as a random effect, and plant nested within matriline as a random effect.

We calculated pairwise correlations between stamen number, ovule number, flowering time, latitude, and longitude in the native range populations. To test for geographic patterns in stamen loss, and determine if there is a relationship with flowering time after accounting for geography, we then performed a multiple regression of short stamen number on the standardized variables latitude, longitude, and flowering time. To test if resources saved by stamen loss result in increased ovule number after correcting for latitude, we used a multiple regression of ovule number on standardized stamen number and latitude. Longitude and flowering time were not included to reduce collinearity, and because they were not significant in the stamen number regression (see Results). Analyses were performed in JMP 10.0.0 (SAS Institute 2012).

QTL MAPPING

To gain insight into the genetics of short stamen loss, we mapped quantitative trait loci (QTL) involved in loss using a set of 519 recombinant inbred lines (RILs) produced using parents from the latitudinal extremes of *A. thaliana*'s native range, Sweden and Italy (Ågren et al. 2013). Successful QTL mapping requires that the parents of the mapping lines differ in the trait of interest; in our study, the Italian parent produced 0.92 short stamens on average and the Swedish 1.96. Each RIL was genotyped at 348 SNP markers spaced every ~1 cM across the genome (Ågren et al. 2013). Seeds were sown at MSU; plants used were a subset of those in Dittmar et al. (2014). After stratification, seedlings were transferred to growth chambers programmed to mimic the temperature fluctuations and day lengths encountered during the *A. thaliana* growing season in Italy.

On the day of flower collection, we selected the newest flowers on plants with at least 10 open flowers. If there were fewer than three healthy open flowers, we took whatever was available; if the plant was nearly done flowering but still healthy, we took the entire flowering end of the inflorescence, which occasionally resulted in more than three flowers sampled per plant. More plants flowered toward the end of the experiment, with some senescing before they could be sampled. This resulted in reduced sampling of later-flowering RILs and the later-flowering RIL parent (Swedish). Short stamen number was scored on one to seven flowers (mean = 3.01) from one to seven plants (mean = 4.76) from each of 519 RILs, for a total of 2468 plants and 7435 flowers (mean = 14.33 flowers/RIL). Additionally, 502 flowers from the Italian and 224 from the Swedish parental lines were phenotyped (Table S4). Flowers were stored in 70% ethanol. Stamens were counted under a dissecting microscope. RIL mean stamen number (an estimate of the continuous probability of short stamen loss) was used for the QTL analysis. RIL means are well-estimated, with an average of nearly 15 flowers sampled across five plants (Table S4). This standard approach of analyzing line means

Table 1. Geographic variation in population mean short stamen production, ovule production, and flowering time across the native range of *Arabidopsis thaliana*.

	SS#	*FT*	*Lat*	*Ovule #*	*Long*
A. Correlations					
Short stamen #	–	0.50**	0.60***	−0.45**	−0.14
Flowering time	0.0013	–	0.60***	−0.54**	0.18
Latitude	<0.0001	<0.0001	–	−0.51**	0.09
Ovule #	0.0051	0.0008	0.0010	–	−0.01
Longitude	0.3465	0.2751	0.5508	0.9474	–
	Slope	SE	*F*	*P*	Partial r^2
B. Short stamen production model					
Latitude	0.09	0.02	9.89	0.0034**	0.23
Longitude	−0.04	0.03	2.14	0.1523	0.08
Flowering time	0.05	0.03	2.15	0.1512	0.05
Full model $r^2 = 0.44$ $P = 0.0001$					
C. Ovule production model					
Latitude	−0.62	0.23	4.88	0.0337*	0.12
Short Stamen #	−0.34	0.27	1.61	0.2131	0.04
Full model $r^2 = 0.30$ $P = 0.0021$					

(A) Correlations are in the upper right with stars indicating significance (* = <0.05, ** = < 0.01, * = < 0.001); full *P*-values for the correlations are in the lower left. (B) Results of multiple regression of short stamen number on standardized latitude, longitude, and flowering time; *N* = 39 populations. (C) Multiple regression of ovule number regressed on standardized latitude and short stamen number; *N* = 38 populations. Partial r^2 estimated by squaring partial correlation coefficients.**

produces similar results as QTL analyses using a full dataset with replication within lines, with little loss of power (Zou et al. 2006).

The main-effect QTL analyses were carried out using the R (R Core Team 2015) package R/*qtl* (Broman et al. 2003). We performed Haley–Knott regression with 10,000 permutations to set a LOD threshold, followed by automated stepwise analyses (Broman et al. 2003) without epistasis, with alpha set at 0.05. We tested for epistasis between the main-effect QTL with an ANOVA in JMP (SAS Institute 2012) based on RIL genotypes at the marker closest to the QTL peaks. The model included all QTL and all possible interactions between them.

Although the distribution of RIL phenotypes spanned the parental means, it was highly skewed (stamen loss is relatively rare in the RILs) (Fig. S1) and could not be normalized through transformation. Because the automated stepwise procedure in stepwiseqtl is sensitive to nonnormal residuals, we analyzed the untransformed data and then made two new, complementary versions of the dataset: one binary (lines coded as loss vs. no loss), and one quantile-normalized, including only lines with stamen loss. These two new datasets were analyzed in the same way as the complete raw data to look for consistencies across all three analyses (Broman et al. 2003).

Previous work shows that segregation distortion in this set of RILs is limited and most pronounced only on chromosome 4 (Ågren et al. 2013), which does not include a QTL for short stamen loss in our analyses. Regardless, simulations show this level of segregation distortion does not affect the QTL mapping with such a large number of RILs (Ågren et al. 2013).

We examined how variation in short stamen production was structured in both the RILs and the RIL parents. Observations of binomial traits, such as short stamen number, are expected to be variable (unless *P* is exactly 0 or 1). However, empirical data may be over- or underdispersed relative to this expectation, often due to the presence of additional structure in the data (Appendix B). We used generalized linear mixed effects models to account for overdispersion in both the RIL and the RIL parent datasets. These models provided estimates of the value of the binomial parameter *P* for each dataset, which allowed us to calculate the expected level of variance attributable to the binomial distribution ($P \times (1 - P)$). This also allowed us to quantify overdispersion due to the hierarchical structure of our data, by including nested random effects accounting for variation among genetic lines, plants (within line), and flowers (within plants). Any remaining overdispersion unexplained by our model was estimated by a residual error term. Collectively, these terms allowed us to perform a variance partitioning on short stamen number data, comparing the amount of variation attributed to each nested level of organization and other sources of variation.

To fit these models, we used a hierarchical Bayesian approach and the MCMCglmm() function of the R library MCMCglmm (Hadfield 2010). Other commonly used generalized linear mixed

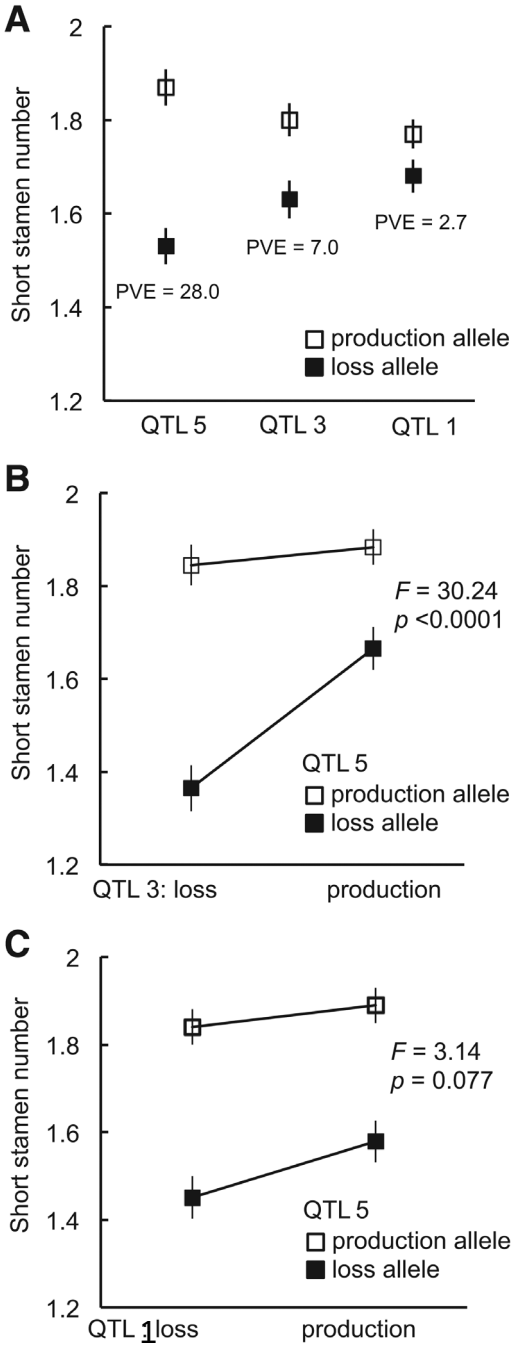

Figure 3. **Main effect QTL and pairwise epistasis. The dark squares indicate the phenotype of individuals homozygous for the short stamen loss allele at each locus, while the white squares represent individuals homozygous for short stamen production alleles (n = 519 RILs). Bars indicate 2 SEM. (A) Main effects from stepwise analysis with full, untransformed data in R/qtl. QTLs are arranged in order of effect size/percent variance explained, and named by which chromosome they are on. All three are significant at $P < 0.0001$. (B) Epistatic interactions between QTLs 5 and 3, and (C) between QTLs 5 and 1, from ANOVA including main effects and all possible interactions. The other interactions were not statistically significant (3 × 1, $F = 0.10$, $P = 0.75$; 5 × 3 × 1, $F = 1.41$, $P = 0.24$.)**

modeling (GLMM) methods do not report the error variance we needed to complete the variance partitioning. For the RIL dataset, we estimated a single intercept, corresponding to the mean short stamen loss probability across all RILs. For the RIL parent dataset, we allowed plants descended from the Italian and Swedish parents to have different intercepts. As expected, these means were significantly different ($P < 1 \times 10^{-}4$; Table S5). Methods for calculating variance partitions were adapted from Nakagawa and Schielzeth (2010) (see Appendix B for a detailed description; associated R code is available at https://github.com/ctkremer/VPC_binom).

Results

FUNCTION OF SHORT STAMENS IN SELF-POLLINATION

If long stamens alone are responsible for autogamous seed set in *A. thaliana*, we would expect seed set in intact flowers and those with short stamens removed to be equally high, and seed set in completely emasculated flowers and those with long stamens removed to be equally low. The results supported these predictions (Fig. 1B; Table S2A); short stamens do not contribute significantly to seed set, and seed set in the absence of long stamens is not significantly greater than zero. When outliers are removed from the analysis, the nonsignificant increase in seed set with short stamens present nearly disappears (with long stamens absent) or is reversed in the normal case of long stamens present (Table S2B). There was also no significant effect of natural variation in short stamen number on seed set (Fig. 1C; Table S2C). Under these circumstances, we might expect evolutionary loss of short stamens in natural populations of *A. thaliana*.

GEOGRAPHIC VARIATION IN SHORT STAMEN PRODUCTION AND CORRELATIONS WITH OTHER TRAITS

Population mean short stamen production varied from 1.00 to 1.77 (Table S1). Thus, there is some loss in every population, but loss of short stamens is never complete. Across the native range, we found significant positive correlations between short stamen production, flowering time, and latitude; plants in the north show less stamen loss (Fig. 2B) and flower later than in the south. There are significant negative correlations between these three variables and ovule number, and no correlation between these variables and longitude (Table 1A). The significant correlations were all similar in absolute magnitude, between 0.45 and 0.6. Multiple regression suggests that the latitudinal gradient in short stamen number is not caused by a pleiotropic correlation with flowering time, but rather something else correlated with latitude (Table 1B). The multiple regression of ovule number on latitude and short stamen number suggests that the latitudinal gradient in ovule number, with more

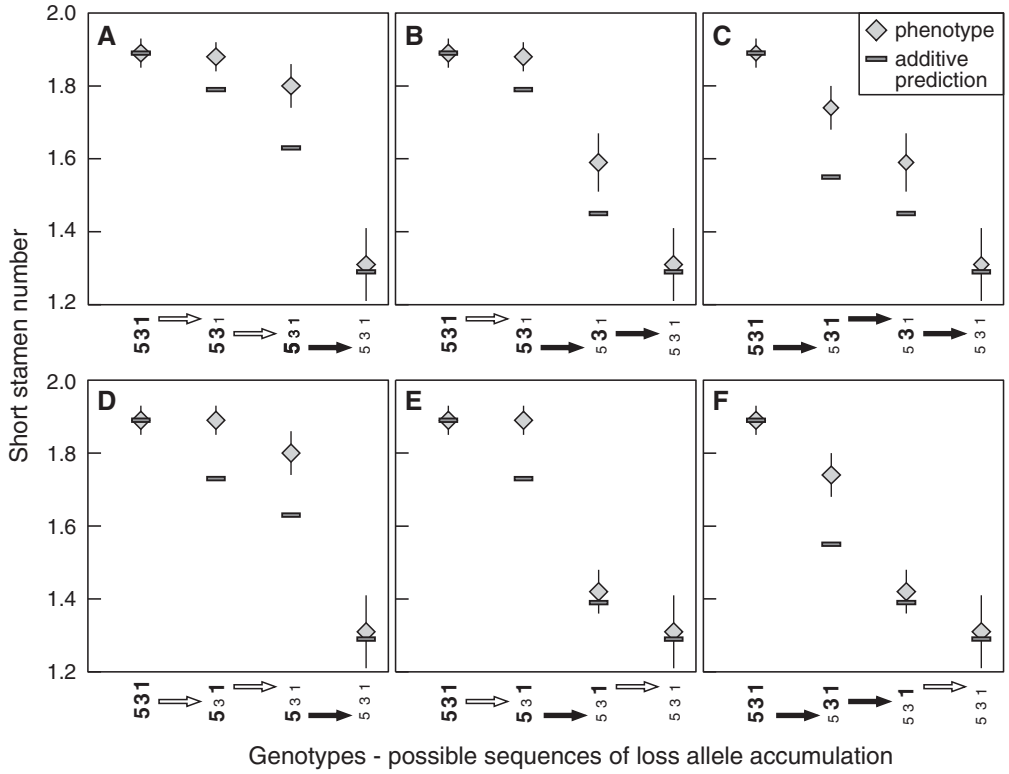

Figure 4. **Epistasis imposes evolutionary constraint on short stamen loss. All possible sequences of accumulating loss alleles are shown, illustrating that epistasis slows loss of short stamens relative to the additive prediction (n = 519 RILs). QTLs are arranged in order of effect size/percent variance explained, and named by the chromosome on which they are located. Small numbers indicate the Italian genotype; large, bold numbers the Swedish genotype. Arrows show which QTL is transitioning from Swedish to Italian in each step. Steps that result in a significant difference according to Tukey's HSD test are indicated by a black arrow; white arrows indicate nonsignificant steps. In five of the six intermediate genotypes between the all-Swedish (531) and the all-Italian (531), the additive expectation for short stamen number is lower than the actual phenotype; the exception is 531. In four of the six scenarios (A, B, D, and E), the first step results in little or no short stamen loss; in only one scenario (C) does each step produce a significant drop in short stamen number. The additive prediction was made by subtracting the main effect sizes (difference between Swedish and Italian in Fig. 3A) from the mean short stamen production with the Swedish genotype at all three QTL. Bars indicate 2 SEM.**

ovules in southern plants, is unlikely to be caused by resources saved by losing stamens, but again by some other factor correlated with latititude (Table 1C).

QTL FOR STAMEN LOSS

We found three highly significant QTL for short stamen loss (one each on chromosomes 1, 3, and 5; Figs. 3 and S3). While the existence of minor loci affecting short stamen production below the threshold of detection is likely, our smallest detected QTL (PVE ~3%) shows that our study has the power to detect small-effect QTL. The QTL on chromosome 5 has the largest effect (Table S6; Fig. S3), and is the only QTL in the binary analysis. Quantile-normalization of the data did not change the results relative to the analysis of raw data (Table S6). There is a significant epistatic interaction between the QTLs on chromosomes 3 and 5, and a second nearly significant epistatic interaction between QTLs on chromosomes 1 and 5 (Fig. 3). In both interactions, epistasis decreased short stamen loss; the stamen-loss alleles on chromosomes 1 and 3 only have a significant phenotypic effect in the presence of the stamen-loss allele on chromosome 5 (Figs. 3 and S4). This pattern of epistasis would slow stamen loss by decreasing the loss produced relative to the additive prediction and limiting the number of possible evolutionary paths with significant stamen loss at each allele replacement (Figs. 4 and S4).

The results of our variance partitioning show that in both the RILs and the RIL parents, most of the variance in short stamen number is an inherent property of binomially distributed traits (distributional variance, see Appendix B) (Tables S7, S8, S9; Figs. S5 and S6). As expected, most of the remaining variance is between lines in the RILs and between individuals in the parents (the parental "lines" are sublines that have been separated for only nine generations, and variance due to differences between Italian and Swedish parents was removed by a fixed effect in the

model). Very little variance is left within individuals (Fig. S7; Table S9). For each model we show variance partitioning results for three categories, mean short stamen number for the RILs and both parents, to emphasize how the overall variance of this binomial trait decreases as mean stamen number increases towards 2 (Fig. S7).

Discussion

We find that short stamens do not significantly increase seed set in the highly selfing *A. thaliana*. Given the reduced or absent short stamen function we describe, evolutionary reduction in investment in this phylogenetically conserved trait could be adaptive, or at least neutral. We show that short stamen loss is in fact common in the native range, but it is never complete (either across independent maternal lines in a population, or within individual plants). We find that short stamen loss exhibits a latitudinal cline with greater loss in the southern part of the range, and that the pattern of epistasis in the major QTL responsible for short stamen loss may act as a constraint, slowing any potential adaptive loss. It is possible that the evolutionary loss of the trait is in process—i.e., given more time, loss will be complete.

Recent evolution of high selfing rates would increase the likelihood that we are seeing ongoing evolution of short stamens. The loss of self-incompatibility in *A. thaliana*, the first step required for the evolution of selfing, certainly occurred after the split with sister species *A. lyrata* (Vekemans et al. 2014). While speciation was complete at least 5 Mya (Koch et al. 2001; Beilstein et al. 2010), estimates of loss of self-incompatibility in *A. thaliana* range from <0.5 to 1 Myrs ago (Bechsgaard et al. 2006; Tang et al. 2007; Shimizu et al. 2011). The shift to reproducing primarily by self-pollination after loss of self-incompatibility may have been even more recent; thus it is possible we are observing ongoing evolution of stamen loss rather than static variation in the trait.

There are at least three hypotheses to explain the latitudinal cline: there is selection to maintain long stamens in the north but not in the south, stamen loss is pleiotropic with another trait that exhibits a latitudinal cline, or adaptive loss of short stamens is constrained more strongly at high latitudes. While the latitudinal cline in loss could be caused by direct selection favoring short stamen production in the north, this seems unlikely. Short stamens are predicted to increase fitness only when outcrossing rates and pollinator visitation are high. While small pockets of modestly increased outcrossing have been reported in the species (up to 14.5%) (Bomblies et al. 2010), direct observations of floral visitors to *A. thaliana* suggest that 7% or fewer of all flowers are visited (Hoffmann et al. 2003; Lundemo 2010). This falls far short of the multiple visits per flower thought to make short stamens adaptive (Harder and Thomson 1989; Conner et al. 1995, 2003). However, field tests of the fitness effects of short stamens across the native range are necessary to test the hypothesis that short stamens directly increase fitness at higher latitudes.

Selection on a correlated trait is another potential cause of the latitudinal cline. Adaptive latitudinal trait clines have been documented in many species, including adaptation to variation in temperature (e.g. Azevedo et al. 1998; Prasad et al. 2011; Yampolsky et al. 2014) and day length (reviewed in Hut et al. 2013), with several examples in *A. thaliana* (e.g. flowering time (Stinchcombe et al. 2004) and cold tolerance (Zhen and Ungerer 2008)). Although our data suggest that correlations with flowering time or ovule number are unlikely to constrain short stamen loss (Table 1), studies that measure genetic correlations between short stamen number and other unmeasured traits that co-vary with latitude are needed.

Postglacial population dynamics are another possible source of constraint; founder events with recolonization have resulted in lower genetic variance in the north for many species (Comes and Kadereit 1998). The same pattern is present in *A. thaliana*, both in 167 populations sampled from across the native range (Beck et al. (2008) and in 31 populations over nine degrees of latitude within Norway (Lewandowska-Sabat et al. (2010). While these results from neutral markers are suggestive, it is genetic variation for stamen number that is directly relevant to the hypothesis of constraints on adaptive loss in the north. In the 14 European populations in our study with four or five matrilines sampled, there was a negative (but nonsignificant) correlation between latitude and the percent of variance in stamen number within each population explained by matriline ($r = -0.35$; $P = 0.22$). This is consistent with less genetic variance for stamen number at higher latitudes. Clearly, more populations and more matrilines per population need to be sampled to test the hypothesis that stamen loss at high latitudes is constrained by a lack of genetic variance. Note that this is an argument for adaptive loss of stamens being constrained by a lack of genetic variance caused by founder effects, not the more common argument that drift can cause loss of a nonfunctional trait (Daehler and Strong 1997; Lahti et al. 2009). Our data do not support the latter, because drift would be predicted to cause more stamen loss in the north where founder effects have been prevalent, opposite to our latitudinal cline.

Regardless of the source of the latitudinal cline, epistasis between the three major stamen-loss QTL is a likely source of constraint on the evolution of short stamen loss. These interactions reduce the phenotypic effect of single loci alone (Figs. 3 and 4). Depending on the order in which the alleles for loss are accumulated at different loci, this can mean that alleles causing loss have no significant phenotypic effect, and thus might not be increased in frequency by natural selection. In fact, of the six possible evolutionary scenarios starting at the ancestral condition of two short stamens, only one results in a significant decrease

in short stamen production with the addition of each allele for loss (Figs. 4 and S4). Additionally, high homozygosity across the genome resulting from selfing reduces the effective recombination rate, slowing the rate at which stamen-loss alleles at different loci are brought together when rare outcrossing events occur. This would be a constraint even in a purely additive system, but by preserving combinations of alleles across loci, it also makes epistasis more enduring. It has long been thought that breakdown of linkage disequilibrium, by homogenizing genetic background, renders epistasis unimportant for long-term evolutionary trajectories (reviewed in Hansen 2013). However, exclusive selfing maintains linkage disequilibrium, stabilizing epistatic interactions. Thus, the shift to selfing that reduced or eliminated the function of short stamens may also be slowing their loss.

In summary, there is widespread but incomplete loss of short stamens that have lost most or all of their function in *A. thaliana.* Based on the pattern of neutral variance (Beck et al. 2008; Lewandowska-Sabat et al. 2010), the latitudinal cline in stamen loss is not likely due to loss through drift in the south. The cline is more likely due to stronger selection to maintain short stamens in the north, or constraints on adaptive loss in the north due to some combination of decreased genetic variance, genetic correlations with other traits exhibiting a cline, or epistasis; future work will be needed to determine which of these factors are responsible for the cline. The predominantly selfing mating system is likely to exacerbate constraint from both low genetic variance and epistasis. This suggests that the particular kind of epistatic constraint we see here may be more common in selfing species in general.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Distribution of RIL mean short stamen production.
Figure S2. Distribution of seed set in stamen removal treatments.
Figure S3. Main-effect QTL using stepwise analysis on the complete untransformed data with no epistasis in R/*qtl*.
Figure S4. Epistasis results in fewer effective paths to evolution of stamen loss by natural selection.
Figure S5. Diagnostic outputs showing the MCMC chains and posterior distributions obtained from hierarchical Bayesian model of RIL dataset.
Figure S6. Diagnostic outputs showing the MCMC chains and posterior distributions obtained from hierarchical Bayesian model of RIL Parent dataset.
Figure S7. Variance partitioning across nested levels of biological organization, for RIL Parents and RIL datasets.
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Appendix B. Background and detailed methods for binomial variance partitioning.