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The timing of molecular and morphological changes underlying reproductive transitions in wild tomatoes (*Solanum* sect. *Lycopersicon*)

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Abstract

Molecular mechanisms underlying the transition from genetic self-incompatibility to self-compatibility are well documented, but the evolution of other reproductive trait changes that accompany shifts in reproductive strategy (mating system) remains comparatively under-investigated. A notable exception is the transition from exserted styles to styles with recessed positions relative to the anthers in wild tomatoes (*Solanum* Section *Lycopersicon*). This phenotypic change has been previously attributed to a specific mutation in the promoter of a gene that influences style length (*style2.1*); however, whether this specific regulatory mutation arose concurrently with the transition from long to short styles, and whether it is causally responsible for this phenotypic transition, has been poorly investigated across this group. To address this gap, we assessed 74 accessions (populations) from 13 species for quantitative genetic variation in floral and reproductive traits as well as the presence/absence of deletions at two different locations (StyleD1 and StyleD2) within the regulatory region upstream of *style2.1*. We confirmed that the putatively causal deletion variant (a 450-bp deletion at StyleD1) arose within self-compatible lineages. However, the variation and history of both StyleD1 and StyleD2 was more complex than previously inferred. In particular, although StyleD1 was statistically associated with differences in style length and stigma exsertion across all species, we found no evidence for this association within two species polymorphic for the StyleD1 mutation. We conclude that the previous association detected between phenotypic and molecular differences is most likely due to a phylogenetic association rather than a causal mechanistic relationship. Phenotypic variation in style length must therefore be due to other unexamined linked variants in the *style2.1* regulatory region.

Keywords: inbreeding, mating system, outbreeding, pollination, self-incompatibility, speciation, stigma exsertion, *Style 2.1*, style length, tomato

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Introduction

Mating (breeding) systems are a critical component of reproductive strategies because they determine the number and identity of reproductive partners, as well as the genetic identity and variability of offspring from these partners. Consequently, mating systems influence the amount and distribution of genetic variation (Wright 1921; Stebbins 1957; Hamrick & Godt 1996; Wright *et al.* 2013), the potential strength of forces such as sexual selection and genetic conflict (Trivers 1974; Arnqvist & Nilsson 2000; Parker 2006; Bedhomme *et al.* 2009), and the evolutionary outcomes of these processes, including population extinction, trait divergence

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and speciation (Thornhill 1993; Parker & Partridge 1998; Gavrilets *et al.* 2001; Brandvain & Haig 2005; Lankinen & Larsson 2009). Accordingly, the traits that contribute to mating system variation can be critical in determining the evolutionary trajectory and persistence of lineages.

In some cases, critical aspects of mating system transitions are mechanistically well understood. For example, the transition from genetically determined self-incompatibility (SI) to self-compatibility (SC) in plant species is due to the mutational breakdown/loss of either male (pollen)- or female (stigma/style)-acting components of the self-incompatibility locus ('S-locus') and/or of modifier genes with which these S-locus components interact (e.g. Charlesworth & Charlesworth 1979; Stone 2002; Takayama & Isogai 2005; Mable 2008; Covey *et al.* 2010; Tao & Iezzoni 2010). SI to SC transitions have repeatedly occurred in many different plant groups (Stebbins 1950; Igic *et al.* 2008; Nasrallah 2011), and such transitions often initiate a shift in the mating system from outcrossing to more frequent selfing (Busch & Schoen 2008). However, transitions from complete outcrossing enforced by self-incompatibility to complete or predominant self-fertilization often require floral morphological changes in addition to the loss of SI. This is because the effective mating system (i.e. the frequency of outcrossing vs. inbreeding events) can be affected by a wide range of additional behavioural, developmental and structural traits. In animal-pollinated plants, for example, these include traits that affect pollinator attraction (such as the size of floral displays and the amount and quality of floral reward), the likelihood that self-mating can take place (including the number of flowers open at a given time (which affects pollen transfer among flowers within the same plant; 'geitonogamy'), and the separation of male and female reproductive structures in time ('dichogamy') and/or space ('herkogamy') (Webb & Lloyd 1986; Lloyd & Barrett 1996)). Relative to outcrossing species, highly self-pollinating taxa tend to have smaller flowers and shorter distances between the receptive stigmatic surface of the (female) pistil and (male) pollen-bearing anthers ('stigma exsertion') (Lloyd & Barrett 1996; Sicard & Lenhard 2011) – traits that reduce outcrossing and increase self-fertilization rates (e.g. Rick *et al.* 1978; Brunet & Eckert 1998; Motten & Stone 2000; Takebayashi *et al.* 2006). In comparison with reproductive shifts from SI to SC that are based on mutations at the S-locus or its modifiers, however, the genetic basis of these other trait changes is relatively poorly understood (Sicard & Lenhard 2011).

The wild tomato group *Solanum* Section *Lycopersicon* contains both self-incompatible and self-compatible taxa. In addition to several transitions from SI to SC that are well documented (Igic *et al.* 2008; Grandillo *et al.* 2011), outcrossing and inbreeding populations also exhibit many of the reproductive and phenotypic trait differences characteristic of mating system variation, including differences in flower size and stigma exsertion (e.g. Rick *et al.* 1977, 1979; Rick 1982; Fig. 1). Moreover, field estimates of natural outcrossing rates have confirmed that variation in floral morphology – including stigma exsertion – significantly affects outcrossing vs. selfing rates, even in genetically self-compatible lineages (Rick *et al.* 1978; and see Discussion). Based on experimental evidence, it has been hypothesized that the phenotypic transition from exserted to inserted stigmas in this group (Fig. 1C, D) is due to a specific mutation in the regulatory region of a gene – *style2.1* – that influences style length (Chen *et al.* 2007). *Style2.1* is one

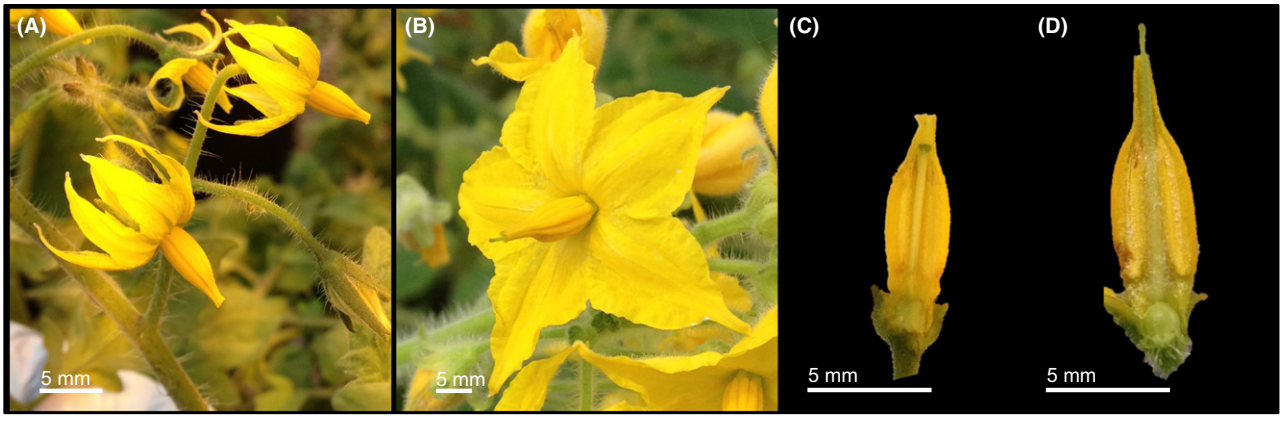

Fig. 1 Variation in reproductive morphology characteristic of mating system differences within *Solanum* section *Lycopersicon*. (A) Self-pollinating flower (*S. lycopersicum* LA4025) with recessed stigma within anther cone (stamens). (B) Outcrossing flower (*S. habrochaites* LA1777) with exserted stigma that extends beyond the anther cone. Cross-sections of each stigma phenotype, for (C) *S. lycopersicum* and (D) *S. habrochaites*.

of five genes that underlie *stigma exsertion2.1* (*se2.1*) – a quantitative trait locus (QTL) on chromosome two that accounts for the greatest change in stigma exsertion between outcrossing and inbreeding *Solanum* species (Bernacchi & Tanksley 1997; Fulton *et al.* 1997; Chen & Tanksley 2004). Of the five genes underlying this complex QTL, *style2.1* accounts for the greatest change in stigma exsertion, specifically via changes in cell elongation during style development (Chen *et al.* 2007). Using transformations, Chen *et al.* (2007) showed that domesticated, short-styled tomato individuals exhibit elongated styles when *style2.1* protein is expressed from the *style2.1* promoter region derived from wild, long-styled *Solanum pennellii*. A 12-kb regulatory region from the long-styled genotype is sufficient to produce elongated styles in the short-styled genotype, indicating that the relevant functional change is regulatory rather than coding (Chen *et al.* 2007). *Style2.1* protein does not differ between long- and short-styled genotypes.

A comparison of sequence differences between long-styled and short-styled regulatory regions revealed numerous molecular differences in this regulatory region, including two major deletions (Chen *et al.* 2007). Specifically, the short-styled allele harbours a 450-bp deletion located ~4 kb upstream of the *style2.1* start site (hereafter StyleD1) and a 750-bp deletion located ~8 kb upstream of *style2.1* (hereafter StyleD2). Based on a survey of natural variation at these two deletions, Chen *et al.* (2007) hypothesized that the functional trait change in the regulation of *style2.1* was due to the 450-bp deletion (StyleD1), as this mutation was more clearly associated with SC lineages (Fig S1, Supporting Information). Nonetheless, the precise evolutionary timing of origin of this candidate regulatory mutation was not investigated, and the strength of its association with phenotypic transitions in style length among wild populations with different mating systems is unknown. If StyleD1 is causally responsible for natural changes in style length, this would be one of the few cases in which the specific genetic lesion underlying morphological changes involved in a critical reproductive transition is known. However, data on the distribution and timing of this mutation with respect to other phenotypic and functional changes are required to confirm this mechanistic association and to understand the progression of mutational changes that contribute to such shifts.

In this study, we evaluated the phenotypic associations and historical timing of the *style2.1* regulatory lesion (at StyleD1) hypothesized to contribute to a major life history transition in wild tomatoes. To do so, we characterized quantitative genetic and molecular variation within and among species in the tomato clade, *Solanum* sect. *Lycopersicon*. Although this group is known to have experienced several SI to SC transitions (e.g. Igic *et al.* 2008; Miller & Kostyun 2011), our goal was to examine trait shifts that are independent of the S-locus, specifically morphological trait variation generally associated with mating system changes, and regulatory molecular variants at *style2.1*. Our specific aims were to:

- identify the distribution of variation within and between species of Solanum (wild tomatoes) in reproductive traits that accompany mating system transitions, including floral morphological traits and self-incompatibility status;
- identify the phylogenetic distribution and evolutionary timing of two large deletion mutations ('StyleD1' and 'StyleD2') in the regulatory region of *style2.1*, including the putatively causal locus (StyleD1); and
- evaluate the association between these molecular changes in *style2.1* and phenotypic variation in morphological and reproductive traits.

Materials and methods

Selection of Solanum populations

Our diversity panel included 74 accessions (populations), representing 13 (of 14 total) species in the tomato clade (Table S1, Supporting Information). Seeds of each accession were obtained from the C. M. Rick Tomato Genetics Resource Center at U. C. Davis (http://tgrc.ucdavis.edu). Because the relevant transition from long to short styles has been hypothesized to occur close to the origin of domesticated lineages (Chen *et al.* 2007; see also Results), our chosen accessions were enriched for populations from species that are most closely related to domesticated tomato (i.e. *S. lycopersicum* var *cerasiforme*, *S. pimpinellifolium*, *S. cheesmanieae* and *S. galapagense*). The taxonomic status of one of these species – *S. lycopersicum* var. *cerasiforme* – has recently been questioned (Peralta *et al.* 2007). Molecular marker analyses indicate that some accessions (populations) of *S. lycopersicum* var. *cerasiforme* are more closely related to domesticated tomato and therefore are likely to be 'landraces' or feral escapees from domesticated populations (Ranc *et al.* 2008). The same study revealed a second group of *S. lycopersicum* var. *cerasiforme* populations that clusters phylogenetically more closely with other wild species, and this group is thought to represent wild lineages (Ranc *et al.* 2008). Based on these previous delineations, our analysis included 16 wild accessions and 4 'feral' (domesticated escapee) accessions, of *S. lycopersicum* var. *cerasiforme* (Table S1, Supporting Information). We also included five South American cultivars of domesticated tomato (*S. lycopersicum*). In

comparison, Chen *et al.*'s (2007) analysis examined molecular deletion variation at *style2.1* in accessions primarily from domesticated tomato (*S. lycopersicum*) and more distant SI *Solanum* species. In general, at least three individuals per accession were assessed here for molecular and quantitative trait variation.

Assessment of reproductive trait variation

We assessed variation in four floral morphological traits: corolla diameter (CD), stamen length (AL), style length (SL) and stigma exsertion (SE) (Fig. S2, Supporting Information). Using digital callipers, floral traits were measured on two flowers from different inflorescences on each experimental individual; floral replicates were averaged to obtain a single trait value for each plant. To confirm self-compatibility status (SI vs. SC), at least one (generally two) and up to three flowers on each individual were hand-self-pollinated, tagged and checked 2, 7 and (if necessary) 14 days postpollination, to assess evidence for fruit development. Each pollination was scored as 1 (self-compatible) or 0 (self-incompatible), depending upon whether a fruit did or did not develop.

Assessment of variation at two deletions at Style 2.1

We assessed evidence for the presence/absence of StyleD1 and StyleD2 lesions within the *style2.1* regulatory region using primer pairs designed to flank each deletion site, as developed and reported in Chen *et al.* (2007). From each experimental individual, two hole punches of leaf tissue were collected and frozen for DNA extraction and genetic analysis ($N = 225$). DNA was extracted in 96-well plate format using a standard CTAB (phenol–chloroform) protocol, DNA content quantified, and each sample diluted to a PCR stock solution (10 ng/μL). PCRs were run using 20-μL mixtures containing 20 ng DNA, 30 mM tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl, 1.5 units of Taq DNA polymerase, 100 μM dNTPs and 0.1 μM of each of the primers. The PCR programmes for both StyleD1 and StyleD2 consisted of initial denaturation at 94 °C for 3 min followed by 34 cycles of 30 s at 94 °C, 45 s at 52 °C and 1 min at 72 °C with a final extension time of 10 min at 72 °C.

PCR products were separated by size on 1.5% agarose gels and scored by eye from digital images after staining with ethidium bromide. At each locus, alleles were classified according to the length of the amplified band, with longer (slower) bands generated from alleles with no deletion and shorter (faster) bands generated from alleles that contained a deletion. Deletion heterozygotes showed two clear bands (length variants). Unexpectedly, at both StyleD1 and StyleD2, we detected at least three classes of allele corresponding to long (L), intermediate (I) and short (S) bands (i.e. no deletion, small deletion and large deletion alleles, respectively). For a subset of individuals ($N = 36$) with heterozygous genotypes and/or alleles of unexpected length, a new DNA sample was extracted and analysed in order to verify the original genotyping result (see Table S2, Supporting Information).

To confirm the homology of comigrating bands revealed by gel electrophoresis (i.e. to verify that they shared the same deletion), for a subset of individuals ($N = 13$ or 8, for StyleD1 and StyleD2, respectively; Table S2, Supporting Information), we direct-sequenced the PCR amplification product of our genotyping step, using standard protocols. For each deletion locus, sequences were aligned in *Sequencher 4.5* (Gene Codes Corporation 2005), with manual adjustments for short insertion–deletion (indel) sites. The colocation of deletion breakpoints for each class of deletion allele (intermediate, short) within either StyleD1 or StyleD2 was confirmed from sequence alignments. In addition, for further verification, one to three representative alleles from each class ($N = 8$ or 5 sequences, for StyleD1 and StyleD2, respectively) were aligned against a contig containing the two BACs first used to identify both deletion sites (gbEU161283.1, gbEU161284.1); the contig also included mRNA sequences for two protein-coding loci (L02 (*style2.1*) [gbEU161281.1] and L04 (*MDR1*) [gbEU161279.1]) generated in the original sequence analysis (Chen *et al.* 2007), to verify the position of StyleD1 and StyleD2 upstream of L02.

Analysis and statistics

Reproductive traits. Quantitative reproductive traits were analysed using parametric correlations and a series of analyses of variance (ANOVAS). Correlations, and partial correlations, between the four reproductive traits were assessed with multivariate analyses, and all traits were significantly correlated (see Results), largely due to the variation in overall flower size. A principle components analysis, performed on trait correlations, revealed three principal components (PCs) that explained >98% of the variation (Table S3, Supporting Information). As expected from correlations, the first axis of variation captured variation in flower size. Interestingly, the second axis captured variation ranging from flowers with small corollas and short stamens but long styles and exserted stigmas, to flowers with larger corollas and long stamens but short styles and inserted stigmas (Table S3, Supporting Information).

Our goal was to examine the distribution, and associations, of trait variation specifically in style-related

Table 1 Pairwise correlations (above diagonal) and partial correlations (below diagonal) between reproductive traits. For all pairwise correlations, $N = 218$, $P < 0.00001$

	Corolla diameter	Stamen length	Style length	Stigma exsertion
Corolla diameter	—	0.745	0.735	0.570
Stamen length	0.343	—	0.857	0.544
Style length	0.085	0.761	—	0.808
Stigma exsertion	0.121	−0.495	0.770	—

traits, independently of flower size *per se*. Therefore, to account for correlated variation in flower size in our data set, for all reproductive traits (AL, SL and SE) except CD (Table 1), we included CD as a covariate in nested ANOVAS that included species and accession within species as effects. Tukey HSD contrasts were performed on trait least-squares means to assess pairwise differences between species and accessions.

Molecular genetic variation. We classified each allele at each deletion locus according to its band length class (long, intermediate, short). At each locus, DNA sequence comparisons determined that alleles within each length class were homologous for the same deletion location (see Results). In addition to these three major deletion classes, within each allelic class we also observed some minor length variation (up to ±50 bp) that sequencing revealed was due to the combined effect of multiple additional shorter indel variants that did not overlap with or occur close to our major deletions (data not shown). In addition, unlike StyleD1 and StyleD2 alleles (which were fixed in the majority of accessions examined – see Results), the occurrence of these minor indels was highly idiosyncratic (each variant was found only in one or two individuals within our data set). Because these secondary length variants were rare and not hypothesized to be involved in our morphological transitions, they were not further considered in our analysis. For each locus, StyleD1 and StyleD2, we calculated allele and genotype frequencies for each accession. To identify the phylogenetic distribution of each allele, these data were assessed on a cladogram of species relationships, previously constructed from 18 independent loci (Haak *et al.* 2014; see Results, and Supplementary Information).

Associations between molecular and morphological variation. To examine the association between alleles at each deletion locus and each of our three reproductive traits, we performed multivariate ANOVAS that included both StyleD1 and StyleD2 as main effects and CD as a covariate. Ideally, these analyses would also include species and/or accession within species, to simultaneously examine the effects of phylogenetic classifications. However, because deletion alleles were, in most cases, perfectly associated with species and/or accession within species, this was not possible in our data set (see Discussion).

In addition, for two of the taxa examined here (*S. lycopersicum* and *S. lycopersicum* var. *cerasiforme*), we detected substantial intraspecific polymorphism for deletion variants at StyleD1 (see Results). Therefore, within each of these species, we also evaluated the statistical association between deletion variants and phenotypic variation in our four reproductive traits using ANOVAS with StyleD1 and StyleD2 as main effects.

Results

Distribution of reproductive trait variation

All four morphological traits (corolla diameter, stamen length, style length and stigma exsertion) showed a significant variation among species and accessions, as revealed by preliminary ANOVAS on each trait, with species and accession nested within species as effects (Table S4, Supporting Information). In addition, multivariate correlations indicated that all four morphological traits were strongly correlated with each other (Table 1). In order to examine the variation specifically in SE-related traits independently of general differences in flower size, for most of the remaining analyses CD (flower size) was used as a covariate (see Methods). In addition, we confirmed most previous reports of SI vs. SC status for our accessions (Table S1, Supporting Information). For some species (*S. arcanum*, *S. corneliomulleri*, *S. habrochaites* and *S. pennellii*), we detected among-accession variation in SI/SC status, as previously reported for these species (except *S. corneliomulleri*; Grandillo *et al.* 2011). Although most populations were either SC or SI, three accessions were inferred to be segregating for SI/SC (one from *S. arcanum* and two from *S. corneliomulleri*; Table S1, Supporting Information).

We also found that SI vs. SC status had a large effect on the amount and distribution of reproductive trait variation, such that most variation occurred within and between SC species (Fig. 2), although note also our sample includes a larger number of SC ($N = 60$) than SI ($N = 11$) accessions. Interestingly, the SI accession with the smallest trait values (LA0385) is from a species that is segregating for SI/SC (*S. arcanum*), although we did not uncover evidence for SC individuals in this specific population. When ANOVAS were performed separately for SC and SI accessions, different patterns of reproductive trait variation were observed for each group (Table 2). For all traits, overall model fits were stronger

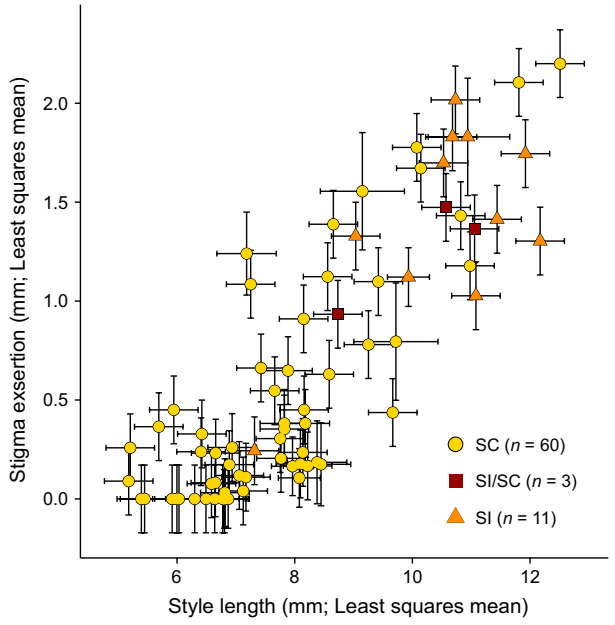

Fig. 2 Distribution in style length (*x*-axis) and stigma exsertion (*y*-axis) variation in self-compatible (yellow circles), self-incompatible (orange triangles) and mixed (segregating for SI/SC) (red squares) populations of wild *Solanum*. Bars represent standard errors (vertical = stigma exsertion; horizontal = style length). Note that these phenotypic traits are strongly correlated (see also Table 2). LS means for each accession are from nested ANOVAS with species and accession nested within species.

for SC accessions, and each trait had significant species, accession within species, and flower size (CD) effects. In contrast, in SI accessions, variation in the three traits was weaker and explained by different effects. Variation in style length (SL) was associated with species differences, variation in stigma exsertion (SE) was associated with differences among accessions within species, and variation in stamen length (AL) was associated with individual variation in flower size (Table 2).

Phylogenetic distribution and timing of regulatory deletions at style2.1

Our analysis of deletion alleles at StyleD1 and StyleD2 revealed two clear findings: i) the number of deletion variants in this region is greater than originally reported and ii) the putatively causal deletion allele (at StyleD1) did arise within SC lineages but both deletion loci have a more complex history than previously reported.

First, whereas the previous analysis revealed only a single deletion variant (i.e. only two alleles; Chen *et al.* 2007), we identified at least three allelic classes for each deletion locus: long (i.e. no deletion), intermediate and short (Table 3). Direct sequencing indicated that, within each locus, all I alleles at a locus shared the same deletion breakpoints/start and end sites. The same pattern held for the S alleles within loci.

The relationship between the two detected deletion alleles – I vs. S – differed by locus, however (Fig. 3). StyleD2-I and StyleD2-S alleles share the same deletion breakpoints, but the greater length of the StyleD2-I allele results from a secondary ~390-bp insertion adjacent to the original deletion site (Fig. 3). In contrast, StyleD1-I and StyleD1-S alleles do not share a common deletion breakpoint; instead, the region deleted in the StyleD1-I allele is nested within the region deleted in the StyleD1-S allele (Fig. 3). One possible interpretation is that the StyleD1-I deletion arose first, followed by a second deletion that generated the StyleD1-S variant. Although this scenario cannot be excluded based on our sequence data alone, it is not consistent with the phylogenetic distribution of allelic variants (Fig. 4). In particular, StyleD1-I is restricted to *S. cheesemanieae* and does not occur in lineages containing the StyleD1-S allele (*S. pimpinellifolium*, *S. cerasiforme*, *S. lycopersicum*) and *vice versa* (Fig. 4). Independent evolutionary origins of StyleD1-I and StyleD1-S alleles, from different StyleD1-L ancestors, is a more parsimonious explanation for this pattern. These inferences, based upon comparisons of directly sequenced PCR products (Table S2, Supporting Information), are consistent with our observed alignments of representative alleles at each locus against the BAC contig spanning the regulatory region of *style2.1* (Table S4, Supporting Information).

Second, as previously hypothesized (Chen *et al.* 2007), the phylogenetic distribution of variation indicates that the StyleD1-S allele arose within SC lineages (Fig. 4), as did the new StyleD1-I allele identified here. However, the phylogenetic distribution of variation at both StyleD1 and StyleD2 indicates a more complex evolutionary history of changes than originally hypothesized. In particular, several species previously reported as fixed for deletion alleles (based upon more limited sampling, Chen *et al.* 2007; Fig. S1, Supporting Information) are actually polymorphic at StyleD1 or StyleD2 (Fig. 4, Table 3). Most notably, the allele hypothesized to cause short styles at *Style2.1* (StyleD1-S) was found to be segregating with the nondeletion (StyleD1-L) allele in three species: *S. lycopersicum* (South American cultivars of domesticated tomato), *S. lycopersicum* var. *cerasiforme* (both 'feral' and wild lineages; Table S2, Supporting Information) and *S. pimpinellifolium*. In the case of *S. pimpinellifolium*, the StyleD1-S alleles were only found in 2 of 24 examined populations; in LA1245, we detected both homozygote classes and a heterozygote, whereas in LA1606 a single LS heterozygote was detected (Table S2, Supporting Information).

Unlike *S. pimpinellifolium*, the other two polymorphic species in our analysis showed substantial intraspecific

Table 2 ANOVA results from accessions blocked by accession self-incompatibility status (SI vs. SC). Analyses were performed separately on populations that were self-incompatible (SI) vs. self-compatible (SC). SI populations show little or no significant variation within and between species; in comparison, SC populations showed a strongly significant variation both within and between species. Significant results are highlighted in boldface. Flower size (CD) was included as a covariate in both analyses. Analyses treated accessions with both SI and SC individuals (i.e. our three 'mixed' accessions; Table S1, Supporting Information) as SC accessions; results do not differ if these accessions are treated as SI or excluded from analyses (data not shown)

SC/SI status	Trait	Whole model	DF	Sum of squares	Mean square	*F* Ratio	Prob > *F*	Effect	*N*	DF	Sum of squares	*F* Ratio	Prob > *F*
(A) SI accessions	Stamen length	Model	11	26.03	2.37	3.34	0.0094	Species	5	5	6.03	1.70	0.1809
		Error	20	14.19	0.709	—	—	Pop(Species)	5	5	5.96	1.68	0.1852
		C. Total	31	40.22	—	—	—	Corolla diameter	1	1	11.06	15.59	**0.0008**
	Style length	Model	11	59.61	5.42	5.09	0.0008	Species	5	5	26.40	4.96	**0.0041**
		Error	20	21.30	1.07	—	—	Pop(Species)	5	5	5.10	0.959	0.466
		C. Total	31	80.91	—	—	—	Corolla diameter	1	1	2.95	2.77	0.1115
	Stigma exsertion	Model	11	7.31	0.665	3.80	0.0047	Species	5	5	1.72	1.97	0.1277
		Error	20	3.49	0.175	—	—	Pop(Species)	5	5	4.64	5.31	**0.0029**
		C. Total	31	10.80	—	—	—	Corolla diameter	1	1	0.033	0.189	0.6686
(B) SC accessions	Stamen length	Model	63	231.42	3.67	20.64	<0.0001	Species	10	10	28.66	16.10	**<0.0001**
		Error	122	21.72	0.178	—	—	Pop(Species)	52	52	83.72	9.04	**<0.0001**
		C. Total	185	253.14	—	—	—	Corolla diameter	1	1	3.09	17.38	**<0.0001**
	Style length	Model	63	504.24	8.00	22.90	<0.0001	Species	10	10	118.58	33.90	**<0.0001**
		Error	122	42.68	0.350	—	—	Pop(Species)	52	52	159.42	8.76	**<0.0001**
		C. Total	185	546.91	—	—	—	Corolla diameter	1	1	6.65	19.01	**<0.0001**
	Stigma exsertion	Model	63	61.67	0.979	13.64	<0.0001	Species	10	10	17.75	24.74	**<0.0001**
		Error	122	8.75	0.072	—	—	Pop(Species)	52	52	29.45	7.89	**<0.0001**
		C. Total	185	70.42	—	—	—	Corolla diameter	1	1	0.383	5.34	**0.0225**

variation at StyleD1 (Fig. 4, Table 1, Table S2, Supporting Information). In most cases, populations were fixed for a specific allele (Table S2, Supporting Information). However, in five populations – two wild (LA1542 and LA2123), one feral (LA0292) *S. lycopersicum* var. *cerasiforme* accession and two (LA0358 and LA0134C) *S. lycopersicum* cultivar populations – we detected both alleles (i.e. these populations were polymorphic). Because our within-population sampling was limited to three individuals, deeper sampling might reveal more widespread intra-accession variation than we have detected here. Regardless, our finding that two species showed a substantial variation at StyleD1 allowed us to more directly assess the association between the putatively causal StyleD1-S allele and phenotypic trait variation in these taxa (see below).

Association between molecular and quantitative variation in reproductive traits

As previously inferred (Chen *et al.* 2007), we detected a significant association between StyleD1 deletion alleles and phenotypic variation in style length and stigma exsertion (Table 4). Interestingly, deletion alleles at the other deletion locus – StyleD2 – were also associated with variation in all four reproductive traits, independently of variation at StyleD1 (Table 4). Ideally, we would perform these analyses including both deletion locus effects and taxonomic categories (species, accession); however, we were unable to do so because in multiple cases species and accession are completely confounded with the presence/absence of these alleles. That is, the distribution of variation in both phenotypic traits and deletion loci is associated with phylogenetic relationships.

Nonetheless, because two species were polymorphic for deletion alleles at StyleD1, we could more directly evaluate the phenotypic effect of StyleD1-S on reproductive traits among individuals within a species, without the potentially confounding effect of deeper phylogenetic associations. Within these two polymorphic species, we detected no significant association between StyleD1-S and variation in style length (Fig. 5); style lengths in individuals with and without StyleD1

Table 3 Genotypic variation at StyleD1 and StyleD2 in 74 accessions from 13 *Solanum* species

					StyleD1				StyleD2			
Species	Species code	Status	Number accessions	Number individuals	Freq_LL	Freq_II	Freq_LS	Freq_SS	Freq_LL	Freq_II	Freq_LS	Freq_SS
S. arcanum	SARC	Wild	3	9	1	0	0	0	0	1	0	0
S. lycopersicum var. *cerasiforme*	SCER	Feral	4	12	0.417	0	0	0.583	0	0	0	1
		Wild	17	51	0.353	0	0.020	0.627	0	0	0	1
S. cheesmaniae	SCHE	Wild	2	7	0.429	0.571	0	0	0	0	0	1
S. chilense	SCHL	Wild	1	3	1	0	0	0	0	0	0	1
S. chmielewskii	SCHM	Wild	2	5	1	0	0	0	0	1	0	0
S. corneliomulleri	SCOR	Wild	3	9	1	0	0	0	0.111	0	0	0.889
S. galapagense	SGAL	Wild	3	11	1	0	0	0	0	0	0	1
S. habrochaites	SHAB	Wild	3	5	1	0	0	0	1	0	0	0
S. lycopersicum	SLYC	Cultivar	5	15	0.200	0	0	0.800	0	0	0	1
S. neorickii	SNEO	Wild	1	3	1	0	0	0	0	1	0	0
S. pennellii	SPEN	Wild	2	6	1	0	0	0	1	0	0	0
S. peruvianum	SPER	Wild	4	13	1	0	0	0	0	0	0.077	0.923
S. pimpinellifolium	SPIM	Wild	24	71	0.958	0	0.028	0.014	0	0	0	1

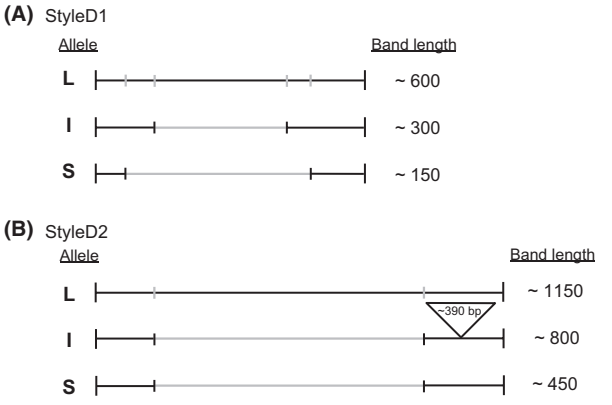

Fig. 3 Schematic of deletion alleles at (A) StyleD1 and (B) StyleD2 loci within the regulatory region of *style2.1*. Sequence alignments indicate homology among alternative deletion alleles detected at StyleD2, but not at StyleD1 (see also text).

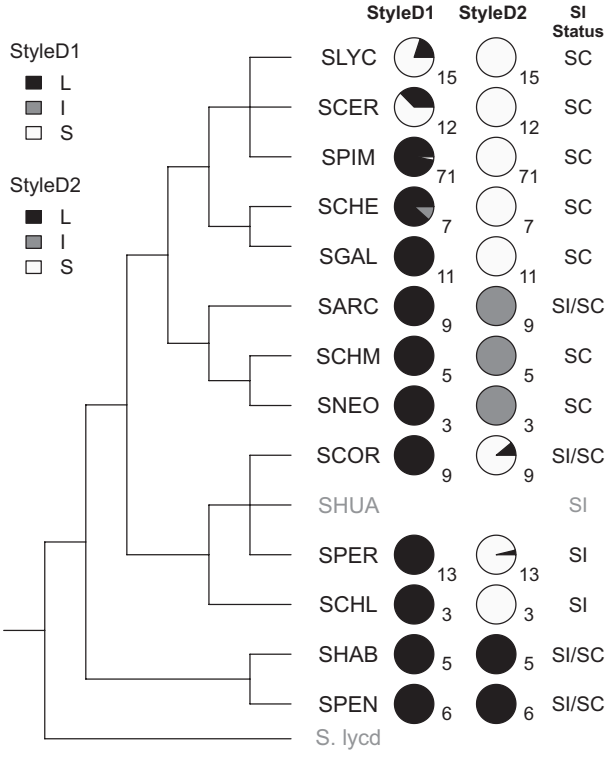

Fig. 4 Phylogenetic distribution of allelic variation at two regulatory loci of *style2.1*. Species names are given as four letter abbreviations (see Table S1, Supporting Information for complete names), except the outgroup *S. lyco = S. lycopersicoides*. Cladogram is based on species relationships inferred from 18 unlinked loci (Haak *et al.* (2014)). The number of individuals genotyped for each species is indicated to the lower right of each pie. Also indicated is the SI/SC status of each lineage, based on our assessment of selfing status in this study. *S. peruvianum* has also previously been reported as variable for SI/SC (Grandillo *et al.* 2011), although we did not detect the variation in SI status in the populations examined here.

Table 4 Reproductive trait variation within and between Solanum species associated with allelic variation at deletion loci StyleD1 and StyleD2 in the regulatory region of *style2.1*. For each trait, ANOVAS were performed with both StyleD1 and StyleD2 as effects. Tukey HSD contrasts with significant trait differences are indicated with different letters. Significant comparisons are highlighted in boldface

	Analysis of variance						Effect tests					Contrasts							
Trait	Source	DF	Sum of Squares	Mean Square	*F* Ratio	Prob > *F*	Effect	DF	Sum of squares	*F* Ratio	Prob > *F*	StyleD1	Least-sq Mean	Std Error	Tukey HSD	StyleD2	Least-sq Mean	Std Error	Tukey HSD
Corolla	Model	4	700.7	175.09	10.69	<0.0001	StyleD1	2	26.83	0.819	0.442	L	24.28	0.551	A	L	28.41	1.48	29.00 **A**
diameter	Error	206	3373.4	16.38	—	—	StyleD2	2	605.11	18.48	**<0.0001**	I	23.32	2.107	A	I	22.19	1.23	22.79 **B**
	C. Total	210	4073.7	—	—	—	—	—	—	—	—	S	23.46	0.816	A	S	20.47	0.710	20.81 **B**
Stamen	Model	4	17.2	4.30	2.68	0.033	StyleD1	2	1.10	0.342	0.711	L	7.87	0.172	A	L	8.45	0.463	8.61 **A**
length	Error	206	331.0	1.61	—	—	StyleD2	2	14.31	4.45	**0.0128**	I	7.51	0.660	A	I	7.45	0.385	7.61 **AB**
	C. Total	210	348.2	—	—	—	—	—	—	—	—	S	7.73	0.256	A	S	7.22	0.223	7.33 **B**
Style	Model	4	179.0	44.70	14.74	<0.0001	StyleD1	2	63.32	10.44	**<0.0001**	L	9.42	0.237	**A**	L	10.81	0.636	11.36 **A**
length	Error	206	624.8	3.03	—	—	StyleD2	2	86.93	14.33	**<0.0001**	I	9.08	0.907	**AB**	I	8.05	0.529	8.60 **B**
	C. Total	210	803.6	—	—	—	—	—	—	—	—	S	8.11	0.351	**B**	S	7.75	0.306	7.93 **B**
Stigma	Model	4	25.0	6.26	17.37	<0.0001	StyleD1	2	11.51	15.97	**<0.0001**	L	1.10	0.082	0.792	**A**	L	1.29	0.219	1.65 **A**
exsertion	Error	206	74.2	0.360	—	—	StyleD2	2	8.74	12.12	**<0.0001**	I	0.560	0.313	0.165	**AB**	I	0.584	0.182	0.946 **B**
	C. Total	210	99.3	—	—	—	—	—	—	—	—	S	0.553	0.121	0.158	**B**	S	0.343	0.105	0.542 **B**

deletions were indistinguishable (Fig. 5C, Table S6, Supporting Information). More specifically, the StyleD1-S allele was not associated with short styles; for example, in *S. l. var. cerasiforme*, StyleD1-S homozygotes had style lengths ranging from 4.31 to 9.57 mm, in comparison with a range of 5.04–9.22 mm for StyleD1-L homozygotes (Table S6, Supporting Information). StyleD1 variation was also not associated with corolla size differences (Fig. 5A; Table S6). Interestingly, however, StyleD1 alleles were significantly associated with variation in both stamen length (Fig. 5B) and stigma exsertion (Fig. 5D) in *S. lycopersicum* (SLYC). For *S. lycopersicum* var. *cerasiforme* (SCER), this association was marginally significant for AL, but not for SE (Fig. 5B, D).

Discussion

Mating systems are the product of suites of traits that collectively contribute to determining the frequency of mating and the identity of sexual partners. Even though single loci –for example, the S-locus in species with genetically determined self-compatibility systems – can strongly influence outcrossing rates, mating system transitions (such as from complete outcrossing enforced by self-incompatibility to complete or predominant self-fertilization) often require changes in other traits. For example, even when self-incompatible plant species evolve the ability to self-fertilize, outcrossing rates can remain high unless changes in floral morphology also occur (Takayama & Isogai 2005), a phenomenon observed in self-compatible tomato species (e.g. Rick & Dempsey 1969; Rick *et al.* 1978; Rick 1982).

Here, we examined evidence for a mechanistic association between molecular variation (deletion alleles) and variation in style length, a key quantitative trait known to influence effective mating system. Along with anther (stamen) length, the length of the style influences the degree to which male and female reproductive structures are separated in space (stigma exsertion; Fig. 1, Fig. S2, Supporting Information) and, therefore, the potential frequency with which self-pollination occurs within a single flower (Sicard & Lenhard 2011). In *Solanum*, the degree of stigma exsertion is known to influence natural selfing rates under field conditions (Rick & Dempsey 1969; Rick *et al.* 1978; Rick 1982). For example, comparisons between genotypes of *S. pimpinellifolium* (an SC species) with different floral traits showed that rates of cross-pollination in the field were strongly positively correlated with sigma exsertion and flower size (Rick *et al.* 1978). Similar strong associations between selfing rates and floral traits (including inflorescence size) have been inferred in intraspecific studies in several other wild tomato species (e.g. Rick *et al.* 1979 and see below).

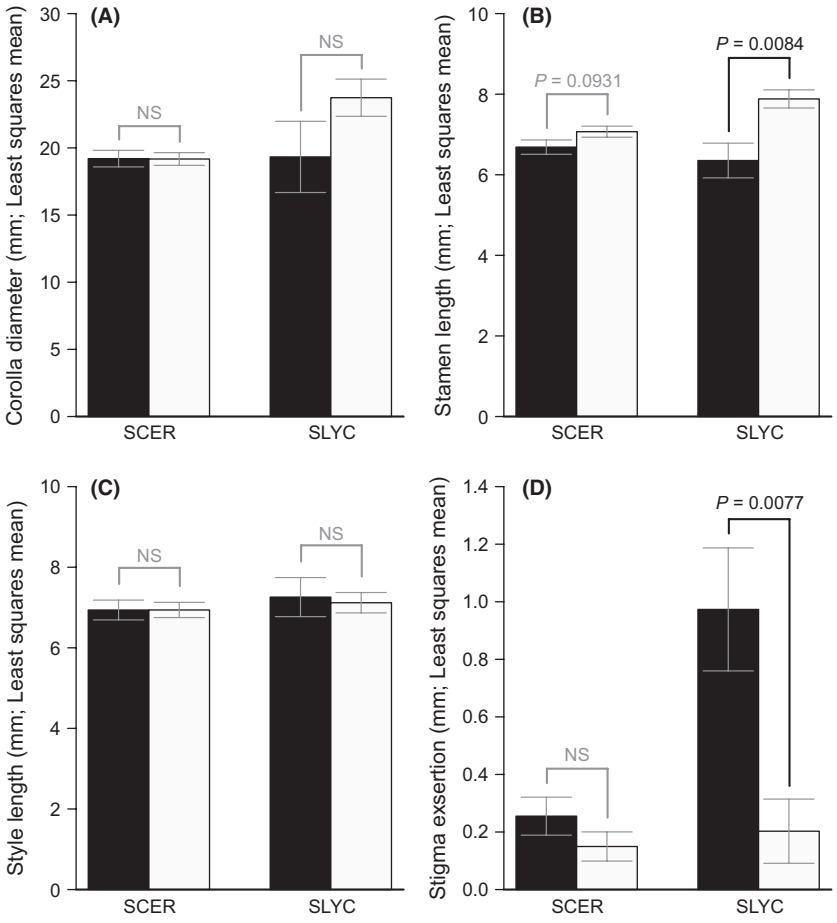

Fig. 5 Phenotypic differences in (A) corolla diameter, (B) stamen length, (C) style length and (D) stigma exsertion, associated with different alleles at StyleD1 (StyleD1-L on left and StyleD1-S on right) within *S. lycopersicum* var. *cerasiforme* (SCER) and *S. lycopersicum* (SLYC).

In previous work among short- and long-styled *Solanum* species, Chen *et al.* (2007) elegantly showed that allelic variation in style length was specifically due to differences within a 12-kb regulatory region of the locus, *style2.1*, located on chromosome 2. They proposed that a specific deletion variant in this *style2.1* regulatory region – here called StyleD1 – was responsible for this functional variation between alleles, such that individuals bearing the deletion (short or S) allele at this locus had significantly shorter styles. Because the genetic basis of such quantitative trait transitions is currently poorly understood, this would represent one of very few cases where the specific molecular variant contributing to this critical reproductive transition had been identified. Contrary to the previous hypothesis, however, our analysis indicates that the major (450-bp) deletion giving rise to the StyleD1-S allele is not functionally responsible for phenotypic variation in style length, suggesting that a different variant in this regulatory region is causal. Although we do not yet know the nature of this alternative variant, our data do support several substantive inferences about the phenotypic and molecular transitions involved in mating system shifts in this group.

Phenotypic changes in reproductive morphology occur after the transition to genetic self-compatibility

Our data confirm that there is substantial variation among wild tomato accessions in quantitative reproductive traits known to be associated with selfing rates. In particular, we found that floral traits are much more variable among genetically self-compatible individuals and accessions than among self-incompatible individuals and accessions. This difference is consistent with differences in the reproductive strategies of self-incompatible vs. self-compatible lineages. Self-incompatible lineages share a common reproductive strategy – maximizing pollinator-mediated outbreeding – that is often best achieved via a single configuration of reproductive traits, including larger, showier flowers and more exserted stigmas (Barrett & Harder 1996). In comparison, self-compatible lineages can have more variable reproductive strategies, ranging from largely outcrossing (with infrequent facultative selfing) to primarily selfing (autogamous). Which reproductive strategy is most effective for maximizing fitness for an SC lineage depends on many ecological factors, including the availability of mates (Fryxell 1957) and of pollinators to

effectively transport gametes (Baker 1959; Lloyd 1980; Lloyd & Barrett 1996; Barrett & Harder 1996; Eckert *et al.* 2006). Previously observed geographical patterns of floral trait variation within and between wild tomato species are consistent with spatial variation in such ecological factors, including the intensity of local pollen limitation due to pollinator scarcity (Kalisz & Vogler 2003) or low population densities (e.g. Baker 1955; Lloyd 1980). For example, 'selfing' morphologies are frequently observed at species range margins (e.g. Rick *et al.* 1977; Rick 1982; Caicedo & Schaal 2004), consistent with an increased premium on reproductive assurance at lower local densities. Similar patterns also emerge in our data set. For example, among our 24 *S. pimpinellifolium* accessions, both mean style length and stigma exsertion decrease with increasing altitude at their geographical site of origin (Fig. S3, Supporting Information). This relationship may reflect increasing scarcity of exothermic pollinators (i.e. bees) at higher, cooler elevations; it also could reflect distance away from the preferred (most common) habitat of this species—coastal lowlands— and therefore the consequences of selection in response to reduced local density of conspecifics as they approach their range margin.

Our observed trait values in SC vs. SI accessions are also consistent with the inference that floral trait variation associated with increased selfing rates arises after the transition to self-compatibility. In our data set, mean anther (stamen) length values for SI accessions range from 7.34–9.88 mm, whereas for SC accessions these range from 4.89–10.03 mm. *In situ* field estimates of outcrossing rates in the SC species *S. pimpinellifolium* indicate that morphs with anther lengths of 7 mm or less have outcrossing rates of <10%, whereas outcrossing increases rapidly in morphs with AL above 7 mm (Rick *et al.* 1978; by scoring offspring phenotypes using a visible marker, Rick *et al.* (1978) could exclude geitonogamous selfing from their outcrossing estimates). Therefore, our morphological data indicate that SI lineages have reproductive trait values that are indicative of higher natural outcrossing rates under field conditions. In comparison, SC accessions show a much greater range of variation in phenotypes associated with outcrossing rates in the field, including populations with trait values indicative of substantial outcrossing and populations with trait values indicative of high selfing rates (Fig. 2). The trait variation relevant to increased selfing therefore arises only after the transition to SC. These findings support the expectation that there is no advantage to floral transitions that increase rates of self-pollination unless lineages have already evolved the competence to self-fertilize.

Our observations can also be used to evaluate more specific hypotheses about the timing of morphological transitions that follow the loss of SI. For example, Rick (1982) proposed that transitions from outcrossing to selfing within wild tomatoes should involve three phases: 1) loss of SI, 2) mutational change(s) that decrease stigma exsertion to assure fruitfulness in the absence of pollinators and 3) reductions in other characters such as floral display size that are only then permitted by relaxed selection on traits that attract pollinators. Under this model, SC lineages with large values of stigma exsertion should also retain other floral trait values consistent with outcrossing, whereas SC lineages with small stigma exsertion can be comparatively more variable for other floral traits like stamen length and corolla diameter, depending upon the progression of these accessions into the final phase. Our observations are inconsistent with this expectation: SC accessions that have stigma exsertion trait values that are below the median of all SC populations have significantly smaller variance in corolla diameter (Levene's test $F = 8.63$, $P < 0.005$) and marginally smaller variance in stamen length (Levene's test $F = 3.72$, $P < 0.059$), in comparison with SC accessions with stigma exsertion values above the median. The stepwise three-phase model is also inconsistently supported by intraspecific patterns of floral variation in *Solanum*, which sometimes agree (e.g. *S. pimpinellifolium*; Rick *et al.* 1977) and sometimes depart from (e.g. *S. habrochaites*; Rick *et al.* 1979) this hypothesized order.

Style2.1 regulatory deletions accompany, but are unlikely to underpin, functional variation in style length

As previously observed (Chen *et al.* 2007), we confirmed that deletion alleles at StyleD1 only occur within SC lineages (Fig. 4). We also confirmed a strong statistical association between deletion alleles at StyleD1 and natural phenotypic variation between species across the wild tomato clade. Interestingly, we also observed these associations for deletion alleles at StyleD2 (Table 4). Both observations could suggest a mechanistic connection between these deletion alleles and the functional regulatory change in style length at *style2.1*. Nonetheless, our inference is that deletion variation at StyleD1 is phylogenetically associated with shifts in style length between *Solanum* lineages, but is not mechanistically responsible for developmental differences between long- and short-styled species.

This inference is supported by both direct and indirect evidence. Most importantly, within both *S. lycopersicum* and *S. l. var. cerasiforme*, we found no association between StyleD1 variation and phenotypic variation in style length; style lengths are indistinguishable between individuals with different genotypes at StyleD1 (Fig. 5)

and, more specifically, the StyleD1-S allele is not associated with phenotypically shorter styles. In contrast, in these species, we did detect associations between StyleD1 variation and variation in other floral traits (Fig. 5 and see below), as well as between style length variation and variation across species and accession (Table 2), indicating that we have sufficient power to detect such an association if it exists.

It is important to note that although our analysis indicates that StyleD1 and StyleD2 deletion variants are not causally responsible for phenotypic differences in style length, it does not indicate that the *style2.1* regulatory region is functionally unimportant for this trait variation. Chen *et al.*'s (2007) transgenic study unambiguously demonstrates that differences within a 12-kb regulatory region of *style2.1* are sufficient to produce style length variation between long-styled *S. pennellii* and short-styled *S. lycopersicum* (Chen *et al.* 2007). Moreover, this region of chromosome 2 has the largest detected effect on style length variation between *S. lycopersicum* and several other long-styled species in this clade (Bernacchi & Tanksley 1997; Fulton *et al.* 1997; Moyle 2007). Therefore, molecular variants in this region, other than deletion variation at StyleD1, are clearly important in the transition from long to short styles.

If StyleD1 deletion variants are not functionally responsible, which variants are? A comparison of BACs from long- and short-styled species reveals many hundreds of molecular differences within the *style2.1* regulatory region (Figure S7 in Chen *et al.* 2007; and L.C. Moyle, unpublished data). Our detection of several additional deletion alleles also supports that the regulatory region of *style2.1* is highly variable within and between species. Consequently, identifying the molecular genetic basis of this regulatory difference is likely to be complex and could potentially involve more than one mutational change. Nonetheless, the abundant natural variation in this clade provides a potentially valuable resource for further refining the molecular genetics of this trait variation. For example, an association study of variants in the regulatory region of *style2.1* across our variable *S. l.* var. *cerasiforme* accessions could be helpful in clarifying which, if any, specific molecular variants could be responsible for the quantitative style length variation we detect between these populations. Such studies could be complemented with analyses of expression variation at *style2.1* in the relevant tissue (i.e. styles) to identify variable expression profiles that match those responsible for developmental differences between long- and short-styled species. Intraspecific analyses are particularly powerful for evaluating trait associations without the additional confounding effect of many other differences that can be associated with between-species comparisons. Given several independent transitions from outcrossing to inbreeding reproductive strategies, the wild tomato group also provides opportunities for gaining insight into the genetic and developmental mechanisms that underlie parallel adaptive evolution (Olsen & Wendel 2013; Stern 2013).

Finally, although our analysis excludes a functional role for StyleD1 in style length variation, it does suggest that the deletion variants assessed here might be involved in quantitative trait variation in stamen length (Fig. 5). The *se2.1* QTL region that contains *style2.1* also harbours loci affecting stamen length, and fine mapping resolved a set of very tightly linked loci for stamen length just proximate to *style2.1* (Chen & Tanksley 2004). StyleD1, or some mutation(s) in linkage disequilibrium with it, could affect regulation of the nearby genes that function in determining stamen length. Changes in stamen length are another means of reducing stigma exsertion – as we observe in *S. lycopersicum* and to a lesser extent in *S. l.* var. *cerasiforme* – and thus of shifting the mating system towards greater selfing. Interestingly, Rick *et al.*'s (1978) field study of outcrossing rate differences between *S. pimpinellifolium* genotypes found a stronger association between outcrossing rate and stamen length (AL) compared to stigma exsertion (SE). In addition, a developmental study of outcrossing and inbreeding *S. pimpinellifolium* morphs showed that anther differences are the first indication of developmental divergence between alternative flower forms (Georgiady & Lord 2002). Together, these observations suggest that future work to understand the molecular basis of shifts from outcrossing to selfing based on quantitative floral traits should also focus on the potential role of regulatory changes in genes influencing stamen length.

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L.C.M., B.K.B. and S.L.V. designed the experiments. S.L.V., C.P.J., N.A.S. and F.E. performed the experiments; L.C.M. analysed the data; and L.C.M., B.K.B. and C.P.J. wrote the paper.

Data accessibility

DNA sequences: GenBank Accession nos KJ482691-KJ482702. Accession ID and location data, trait data, genotype data and biogeographical data: Tables S1, S2, S5 and S7 (Supporting Information). Trait values and genotypes for each experimental individual: Dryad doi:10.5061/dryad.9540r alignment with StyleD1 and StyleD2 sequences: Dryad doi:10.5061/dryad.9540r

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Chen *et al.*'s 2007 hypothesis of the origin of the causal deletion mutation in the regulatory region of *style2.1*. Our alternative hypothesis, Figure 4, includes more contemporary phylogenetic (species) designations, and relationships. Figure 4 cladogram is based on a phylogenetic reconstruction using 18 unlinked loci in RAxML; nodes with less support (bootstrap <70, posterior probability <0.95) are depicted as polytomies (Haak, Ballenger and Moyle, in press).

Fig. S2 Floral measurements included as quantitative genetic traits. CD = corolla diameter, AL = stamen length, SL = style length and SE = stigma exsertion.

Fig. S3 Relationship between phenotypic variation in (A) style length and (B) stigma exsertion within *S. pimpinellifolium* (*Y*-axis) and altitude (*X*-axis) at collection site. The two accessions that have deletion variants at StyleD1 are marked with asterisks, reiterating that phenotypic variation is not associated with deletion variation. Instead, for SE there is a significant negative relationship with altitude (log-transformed) ($F = 13.24$, $P < 0.0015$) possibly due to variation in pollinator availability and/or reduced population densities at the species range margin. This relationship is marginal for SL ($F = 2.98$, $P = 0.099$) and not significant for CD or AL (data not shown). In comparison, SE is marginally correlated with latitude ($F = 3.04$, $P < 0.096$), but this relationship is stronger for the other floral traits (CD: $F = 7.73$, $P < 0.0112$; AL: $F = 15.79$, $P < 0.0007$; SL $F = 15.20$, $P < 0.0008$) as has been previously reported for size-related floral traits in this species (Caicedo & Schaal 2004).

Table S1 Species and accession IDs, and trait means for 74 accessions of Solanum.

Table S2 Genotypes at StyleDel1 and StyleDel2 for 74 accessions of Solanum.

Table S3 Principal components analysis (PCA) on four reproductive traits.

Table S4 One-way nested ANOVA results for each of four quantitative floral traits.

Table S5 Physical location of deletion breakpoints in I and S deletion alleles in StyleD1 and StyleD2.

Table S6 Within species associations (*S. lycopersicum* var. *cerasiforme* and *S. lycopersicum*) between StyleD1 alleles and phenotypic variation.

Table S7 Floral trait (SL and SE) and biogeographical data for 24 *S. pimpinellifolium* accessions.