

# Quantitative genetic analysis indicates natural selection on leaf phenotypes across wild tomato species (*Solanum* sect. *Lycopersicon*; Solanaceae)

Christopher D. Muir<sup>\*,†,1</sup>, James B. Pease<sup>†</sup>, and Leonie C. Moyle<sup>†</sup>

<sup>\*</sup>Biodiversity Research Centre and Botany Department, University of British Columbia, Vancouver, BC V6T 1Z4

<sup>†</sup>Department of Biology, Indiana University, Bloomington, Indiana 47405

## ABSTRACT

Adaptive evolution requires both raw genetic material and an accessible path of high fitness from one fitness peak to another. In this study, we used an introgression line (IL) population to map quantitative trait loci (QTL) for leaf traits thought to be associated with adaptation to precipitation in wild tomatoes (*Solanum* sect. *Lycopersicon*; Solanaceae). A QTL Sign Test showed that several traits likely evolved under directional natural selection. Leaf traits correlated across species do not share a common genetic basis, consistent with a scenario in which selection maintains trait covariation unconstrained by pleiotropy or linkage disequilibrium. Two large effect QTL for stomatal distribution colocalized with key genes in the stomatal development pathway, suggesting promising candidates for the molecular bases of adaptation in these species. Furthermore, macroevolutionary transitions between vastly different stomatal distributions may not be constrained when such large effect mutations are available. Finally, genetic correlations between stomatal traits measured in this study and data on carbon isotope discrimination from the same ILs, support a functional hypothesis that the distribution of stomata affects the resistance to CO<sub>2</sub> diffusion inside the leaf, a trait implicated in climatic adaptation in wild tomatoes. Along with evidence from previous comparative and experimental studies, this analysis indicates that leaf traits are an important component of climatic niche adaptation in wild tomatoes, and demonstrates that some trait transitions between species could have involved few, large effect genetic changes, allowing rapid responses to new environmental conditions.

Keywords: QTL, QTL Sign Test, *Solanum*, adaptation, leaf

## INTRODUCTION

The course of phenotypic evolution depends upon both the genetic basis of functionally important

traits and the mechanistic relationship between traits. All else being equal, traits governed by mutations of large phenotypic effect with low pleiotropy can respond to selection more readily

than traits that are complex, highly pleiotropic, and/or strongly correlated with other traits. Hence, the genetic basis of trait differences and covariation influences which phenotypic axes are most likely to respond to selection in new environmental conditions; understanding this underlying architecture therefore provides insight into the genetic mechanisms constraining or facilitating phenotypic evolution (LEE *et al.* 2014). It can also indicate which evolutionary forces are responsible for trait differences within and between species. When adaptation to a complex ecological niche involves many traits, for example, genetic analysis can determine whether correlations between traits are caused by a shared genetic basis (pleiotropy) or whether natural selection favors trait combinations because they function well together (coselection). The magnitude and direction of allelic effects between phenotypically divergent genotypes can also be used to infer whether this divergence is consistent with directional natural selection (ORR 1998b; RIESEBERG *et al.* 2002), and thus can provide evidence for adaptation, independent of inference from other approaches, such as comparative methods. The genetic architecture of putatively adaptive traits can therefore reveal why some traits evolve rapidly and others more gradually over micro- and macroevolutionary timescales.

With these goals, the genetics of adaptive traits has been successfully examined in a number of cases, most notably of qualitative transitions in pigmentation in response to variation in habitat coloration (e.g., NACHMAN *et al.* (2003); HOEKSTRA *et al.* (2006)) or pollinators (e.g., SCHEMSKE and BRADSHAW JR. (1999); STREISFELD *et al.* (2013)). In comparison, the genetics of adaptive structural (morphological) traits is less well understood, but has been described in the context of, for example, body arma-

Our second goal was to use these data to evaluate evidence for the role of natural selection in shaping leaf trait variation between *Solanum* species. By revealing the direction of allelic effects underlying functionally important traits,

ture (skeletal plates) in sticklebacks (COLOSIMO *et al.* 2005) and leaf shape evolution in *Solanum* (KIMURA *et al.* 2008). Despite these important advances, we still know little about the genetic basis and evolutionary forces underlying physiological and morphological evolution to complex environmental variation (DES MARAIS *et al.* 2014).

Here we examine the genetic architecture, evolutionary forces, and physiological mechanisms that contribute to divergence in leaf traits connected to adaptive differentiation. Leaf traits resemble other functionally-important quantitative traits in that they face numerous selective pressures and functional constraints. As the primary photosynthetic organs in terrestrial plants, leaves mediate several fundamental fitness trade-offs, including water lost through transpiration and carbon (CO<sub>2</sub>) gained through photosynthesis. Quantitative traits such as leaf size, shape, and anatomy affect these important physiological functions (Table 1). However, although these traits represent important axes of phenotypic variation across all land plants, respond to natural selection, and appear to vary adaptively in nature (SMITH and HUGHES 2009; NICOTRA *et al.* 2011; VOGEL 2012), relatively little is known about their genetic architecture. Our first goal in this study was to understand the genetic basis of variation in a set of these leaf traits. Specifically, we map QTL underlying interspecific divergence between two closely related tomatoes, the domesticated tomato (*Solanum lycopersicum* var. *esculentum*) and a wild relative, *S. pennellii*. Our focal traits were chosen based on *a priori* functional considerations, their observed associations with climatic variation, and/or their known ecological significance in wild tomatoes or more generally (Table 1).

QTL analysis can provide evidence that traits evolved under directional natural selection rather than genetic drift. In particular, ORR (1998b) showed that it is unlikely that many QTL affecting a trait in the same direction (e.g. smaller

**Table 1** Key to leaf traits examined in this study, the abbreviations used throughout this paper, units, and a nonexhaustive summary of their ecological significance.

Trait	Abbreviation	Units	Ecological significance	References	Evidence in tomatoes
Leaf area	LA	cm <sup>2</sup>	Thermoregulation	GATES (1968) PARKHURST and LOUCKS (1972) GIVNISH and VERMEIJ (1976) NICOTRA <i>et al.</i> (2011)	Positive correlation between precipitation and leaf size
Leaf mass per area	LMA	g m <sup>-2</sup>	Growth strategy Stress tolerance Leaf longevity Mechanical defense	WRIGHT <i>et al.</i> (2004); ONODA <i>et al.</i> (2011), but see BARTLETT <i>et al.</i> (2012)	Positive correlation between precipitation and LMA (EASLON and RICHARDS (2009); C.D. Muir, unpub. data) LMA decreases internal CO <sub>2</sub> diffusion (MUIR <i>et al.</i> 2014)
Leaf thickness	T	μm	Sun/shade Succulence	TERASHIMA <i>et al.</i> (2006); OGBURN and EDWARDS (2010); SMITH <i>et al.</i> (1997)	Unknown
Stomatal density	SD	no. per 3 fields	Gas exchange	COWAN (1977); FARQUHAR and SHARKEY (1982)	No correlation between stomatal density and climate
Stomatal ratio	SR	–	CO <sub>2</sub> supply	PARKHURST (1978); MOTT <i>et al.</i> (1982)	Higher stomatal ratio (more amphistomatous) in drier habitats (C.D. Muir, unpub. data)
Trichome density	TD	no. per 3 fields	Reflectance Biotic defense	LEVIN (1973); EHLERINGER <i>et al.</i> (1976)	Unknown
Trichome ratio	TR	–	Unknown	–	Unknown

leaf size) will fix in the absence of directional selection. His proposed statistical test of phenotypic selection – the QTL Sign Test (QTLST) – has since been used to show that the distribution of QTL effects for many trait differences between taxa are consistent with the action of selection (RIESEBERG *et al.* 2002). We applied this test to assess the evidence that natural selection was involved in fixing leaf trait differences between tomato species. We also used our genetic analysis to assess whether observed trait correlations are more likely due to genetic associations or to the action of natural selection. Several of our leaf traits covary among wild tomato species: species from drier habitats generally have smaller leaf size, lower leaf mass per area, and greater stomatal ratio (more even distribution of stomata on upper and lower leaf surfaces) (C. D. Muir, unpub. data). This covariation could be the product of coselection, whereby each trait improves thermoregulation and photosynthetic efficiency in dry environments. Alternatively, trait covariation across species could be due to a shared genetic basis; traits are constrained to evolve in concert because of underlying molecular/developmental pleiotropy. Thus, we also aimed to determine whether genetic correlations caused by pleiotropy or linkage recapitulate, and therefore could explain, phenotypic correlations across species. One caveat is that increased linkage itself can be the product of selection to keep locally adapted alleles together in disequilibrium (RIESEBERG 2001; NOOR *et al.* 2001; KIRKPATRICK and BARTON 2006), as suggested in some species (LOWRY and WILLIS 2010; JONES *et al.* 2012). However, if traits are not genetically correlated, this supports an inference that they covary across species because of natural selection.

Of the leaf traits examined in this study, stomatal density and stomatal ratio (Table 1) show the strongest evidence for the action of natural selection (Results). Our third goal was to more directly dissect the mechanistic significance of variation in these stomatal traits by evaluating their association with variation in carbon isotope discrimination ( $\delta^{13}\text{C}$ ) – a widely used indicator

of integrated water-use efficiency in quantitative genetic studies (MCKAY *et al.* 2003; JUENGER *et al.* 2005; MCKAY *et al.* 2008). Both traits potentially affect water-use efficiency and carbon isotope discrimination; however, because each trait influences different steps in the  $\text{CO}_2$  diffusion pathway (atmosphere  $\rightarrow$  inside of leaf vs. inside of leaf  $\rightarrow$  chloroplasts; see Methods and Discussion), the correlation between these different stomatal traits and  $\delta^{13}\text{C}$  can be used to infer which portion of this diffusion pathway is more functionally significant in these species. We aimed to use associations between these structural traits and integrated water-use efficiency (previously measured on the same lines (XU *et al.* 2008)) to more precisely pinpoint the physiological significance of putatively adaptive leaf trait variation in these species.

Our final goal was to identify candidate genes involved in leaf and stomatal development that colocalize with QTL for our stomatal traits. Identifying specific candidates underlying quantitative trait loci generates targets for future functional analysis (DALZIEL *et al.* 2009) but can also potentially indicate specific developmental and physiological mechanisms underlying observed trait variation. This is especially fruitful for traits associated with a relatively discrete and well-characterized developmental network, such as for stomatal development (PILLITTERI and TORII 2012). Patterns of molecular evolution in candidate genes, especially those consistent with positive selection, can also provide an additional method for narrowing specific functional candidates for specific QTL.

To address these four goals, we measured leaf phenotypes (Table 1) on an extensively examined set of introgression lines (ILs) consisting of homozygous introgressions from donor parent *S. pennellii* (*Spen*) in the genetic background of domestic tomato, *S. lycopersicum* var. *es-culentum* (*Slyc*). Previous studies using this IL population have identified numerous QTL (LIPPMAN *et al.* 2007) for traits ranging from fruit metabolite concentrations (SCHAUER *et al.* 2006), yield (SEMEL *et al.* 2006), reproductive isolation (MOYLE and NAKAZATO 2010), and

leaf traits (HOLTAN and HAKE 2003; CHITWOOD *et al.* 2013). With these data, we confirm evidence in support of adaptive evolution of leaf traits, examine the physiological and ecological significance of trait variation in several clearly adaptive traits, and identify potential molecular developmental mechanisms responsible for this trait variation. In doing so, we demonstrate the power of genetic analyses of adaptation, especially in combination with detailed physiological, ecological, and genomic data, to address the likely evolutionary dynamics of adaptive trait evolution.

## METHODS

### Plant material and cultivation

For our QTL analysis, we used previously developed introgression line library (ESHED and ZAMIR 1994), consisting of short known segments of the wild tomato species, *S. pennellii* (donor) serially introgressed into the genetic background of the domesticated tomato, *S. lycopersicum* var. *esculentum* (recipient). Donor introgressions are homozygous in a homozygous recipient background. Development of these ILs has been described elsewhere (ESHED and ZAMIR 1994). Briefly, all lines are the advanced-generation backcross progeny of a single F1 plant produced by crossing *Spen* as the male parent to *Slyc*. Original backcross lines were genotyped using 350 RFLP markers (ESHED and ZAMIR 1994), several subsequent analyses increased marker density and refined the location of introgression breakpoints (LIPPMAN *et al.* 2007), and these were recently confirmed using whole-genome resequencing (CHITWOOD *et al.* 2013). We grew 70 out of 76 introgression lines (ILs) as well as the recipient and donor species from seeds obtained from the Tomato Genetics Resource Center (TGRC) at UC Davis (<http://tgrc.ucdavis.edu>). Between 9 – 13 January 2010, seeds were soaked in 50% household bleach for 30 minutes, rinsed thoroughly, and placed on moist paper in plastic boxes to germinate in a growth chamber. Half of the seeds were

cold treated as part of a separate experiment on germination response. We did not attempt to characterize the effect of seed cold treatment on adult plant traits, which were minor, however we describe the treatment for completeness. For the cold treatment, seeds were placed on wet filter paper and placed in the cold room ( $-4^{\circ}\text{C}$ ) in dark to mimic winter for three weeks. Control seeds were placed on dry filter paper, stored in the dark, and hydrated three weeks later. We included seed treatment (Cold vs. Control) in statistical analyses to account for any plasticity caused by this manipulation. Following germination, all seedlings were transplanted to 7.56 L pots, and grown on benches in a fully randomized design at the Indiana University greenhouse with supplemental lighting to maintain a constant 16:8 h light:dark cycle. Plants were irrigated to field capacity daily to prevent drought stress and fertilized weekly.

### Trait measurements

We measured three leaf morphological traits – leaf area (LA), leaf mass per area (LMA) and leaflet dry matter content (LDMC) – and four leaf epidermal traits – stomatal density (SD), stomatal ratio (SR), trichome density (TD), and trichome ratio (TR). LA was measured on all available plants (*Slyc* sample size  $n_{SL} = 51$ , *Spen* sample size  $n_{SP} = 18$ , mean IL sample size  $\bar{n}_{IL} = 16.4$ ), whereas other traits were measured on a subset of these. Sample sizes for LMA and LDMC were:  $n_{SL} = 50$ ,  $n_{SP} = 18$ ,  $\bar{n}_{IL} = 7.9$ . Sample sizes for stomatal and trichome traits were:  $n_{SL} = 48$ ,  $n_{SP} = 7$ ,  $\bar{n}_{IL} = 4.0$ . For all traits, two leaves per plant were collected at two developmental time points: ‘Early’ (63-65 days after germination) and ‘Late’ (77-82 days after germination). Leaves were marked early in their development ( $< 2$  cm in length) and measured only after they had fully expanded. The mean leaf age at time of measurement was 33.1 days (range 24-46 days) and 40.3 days (range 21-69 days) for Early and Late leaves, respectively. LMA increased slightly with leaf age, therefore we accounted for leaf age statistically while es-

timating genotypic values. Leaf morphological traits LA, LMA, and LDMC were measured as described in MUIR and MOYLE (2009). Briefly, LDMC is the dry mass divided by the fresh mass; LMA is the dry mass divided by the projected area of fresh leaf tissue. Individual leaves were detached and scanned using an Epson GT-20000 flat bed scanner. From scanned leaves, we measured the area of the entire compound leaf using custom macros in ImageJ (ABRAMOFF *et al.* 2004). After scanning, we measured the fresh mass of only the terminal leaflet using a fine balance. Whole leaves were dried in a desiccating oven at (60 °C) and weighed to determine dry mass. Thus, we measured LDMC on the terminal leaflet while LMA is for the entire leaf. Some leaves developed mold in the drying oven because of overcrowding. We statistically removed its effect before estimating the phenotype of a given genotype. LDMC and LMA were used to generate a proxy for leaf thickness ( $T$ ), as defined by VILE *et al.* (2005):

$$T = \frac{\text{LMA}}{\text{LDMC}} \quad (1)$$

$T$  was not measured directly because a thickness gauge is unreliable for wild tomatoes (C.D. Muir, pers. obs.) and sectioning samples to accurately measure  $T$  was not feasible for a project of this scale. However, in wild tomatoes equation 1 is highly correlated with  $T$  ( $r^2 = 0.67$ ) measured directly from sections (MUIR *et al.* 2014). For simplicity, we hereafter refer to leaf thickness ( $T$ ) even though we are actually reporting a proxy of  $T$ .

Stomatal and trichome traits were measured on a subset of the same leaves as morphological traits, on all ILs. Leaf surface impressions were made from fresh tissue by applying a thin layer of nail polish to a medial portion of the terminal leaflet away from major veins. Nail polish layers were removed using adhesive tape and mounted on a microscope slide. Stomata were counted from three microscope fields each of the abaxial ('lower') and adaxial ('upper') leaf surfaces. The stomatal ratio (SR) was calculated as ratio

of the stomatal densities (SD) on the adaxial and abaxial side:

$$\text{SR} = \frac{\text{SD}_{\text{adaxial}}}{\text{SD}_{\text{abaxial}}} \quad (2)$$

Trichome traits were measured by direct counts on the same three microscope fields, and similarly used to calculate trichome density (TD) and trichome ratio (TR). These traits were included to evaluate the strength of general associations between leaf epidermal traits, and to assess an hypothesis that changes in SR could be a byproduct of selection on other developmentally integrated epidermal traits, such as trichomes. Tomato leaves are bifacial, with distinct internal (mesophyll) layers (KEBEDE *et al.* 1994; GALMÉS *et al.* 2013) and epidermal anatomies on each surface. However, if general developmental pleiotropy determines the anatomy of abaxial (lower) and adaxial (upper) epidermises, then species with high SR (i.e. more even distribution of stomata between upper and lower leaf surfaces) should show similar patterns in other epidermal traits such as trichomes.

## QTL analysis

We determined QTL using (generalized) linear models. Where appropriate, we transformed variables or used models with non-Gaussian residual distributions. In particular, we log-transformed leaf morphological traits (LA, LMA, and  $T$ ) to remove heteroscedasticity and normalize residuals. Therefore we used linear models assuming normally distributed residuals on log-transformed traits. We modelled counts (SD, TD) and ratios (SR, TR) residuals as Poisson and binomial, respectively. In our data, SD, SR, and TD residuals were overdispersed, so we used quasipoisson and quasibinomial distributions that include a dispersion factor. Using a quaslikelihood method reduced the number of statistically significant QTL compared to using standard likelihood, but did not change estimated effect sizes. We fit the data on a log-link

and logit-link scale for Poisson and binomial families, respectively. In addition to genotype, we estimated the effects of seedling treatment (Control vs. Cold), developmental stage (Early vs. Late), and leaf age (continuous). For traits based on dry mass (LMA and  $T$ ), we also accounted for effects of mold on dry mass by including a factor with three levels ('no mold', 'slight mold', and 'mold'). We calculated IL effect size in terms of percent phenotypic variance explained using Type II sum of squares, percent interspecific difference, and standard deviations of the *Slyc* parent. For non-Gaussian traits, we calculated percent deviance accounted for, the analog of percent variance explained. Analyses were conducted using the `lm` or `glm` function in R (R CORE TEAM 2012).

Coefficients of the (G)LM provide estimates of the effect of an introgression on the trait compared to *Slyc*. We determined statistical significance using the  $t$ -statistic. Because of multiple comparisons (70 IL v. *Slyc* comparisons per trait) involved in this analysis, we used strong control of false-discovery rate (BENJAMINI and HOCHBERG 1995) as implemented in the R package `multtest` (POLLARD *et al.* 2013) to calculate adjusted  $P$ -values. Only ILs that were statistically significant (adjusted  $p < 0.05$ ) were used to identify QTL. QTL were identified as the minimum number of chromosomal regions needed to account for the empirical pattern of IL phenotypes, taking into account that some introgressed regions are shared between different ILs. When two ILs with overlapping introgressions both have a significant trait effect, then a parsimonious explanation is that a single QTL located in the overlapping region is responsible (also called 'bin-mapping' (PAN *et al.* 2000)). Following the criteria of HOLTAN and HAKE (2003) who used this logic to define 107 nonoverlapping bins in the *Slyc* genome, we used an analogous procedure to define bins for our measured ILs, using IL boundaries obtained from the Sol Genomics Network (BOMBARELY *et al.* 2011). This procedure is conservative, since it identifies the minimum number of QTL needed to account for ILs with significant phenotypes.

We also calculated broad sense heritability

( $H^2$ ) as the proportion of phenotypic variance attributable among ILs after accounting for seedling treatment, developmental stage, leaf age, and mold. Variances were estimated using (generalized) linear mixed models ((G)LMMs) treating IL as a random effect. We used the same distributions to model residuals as in the QTL analysis (Gaussian for LA, LMA, and  $T$ ; Poisson for SD and TD; binomial for SR and TR). Models were fit using a Bayesian MCMC method implemented in the package `MCMCglmm` (HADFIELD 2010). We used default priors for all traits except TR, where a more diffuse prior yielded reasonable estimates and better mixing. Posterior distributions were estimated from 5000 samples taken every 10 steps in a 50 000 step chain following a 5000 step burn in.

## QTL Sign Test

The QTL Sign Test (QTLST) calculates the probability that the observed distribution of effect sizes and directions would have arisen in the absence of directional selection (ORR 1998b). We implemented QTLST rather than the more commonly used QTLEE (QTL Equal Effects) method (e.g. (RIESEBERG *et al.* 2002)) because we had quantitative estimates of the effect size distribution (see below) and QTLST is less susceptible to ascertainment bias than QTLEE (ANDERSON and SLATKIN 2003). For comparison, we also report probabilities from QTLEE since most studies use this method. We first fit an exponential effect size distribution using maximum likelihood to all significant QTL for each trait separately. An exponential distribution is predicted by theory and often approximates real data well (ORR 1998a, but see ROCKMAN 2012). For some traits, the exponential distribution did not closely fit the observed distribution of effect sizes, likely because we had insufficient power to detect small effect QTL. However, QTLST assuming a log-normal rather than exponential distribution of effect sizes yielded very similar probabilities (data not shown), indicating that our results are not sensitive to a specific distribution. Calculating the critical probability for

the QTLST with exponentially distributed effect sizes requires integrating the cumulative distribution of the gamma distribution (ORR 1998b), for which there is no closed form solution. We therefore numerically integrated probability densities using the `distr` package (RUCKDESCHEL *et al.* 2006) and integrate function in R (R CORE TEAM 2012). This code is available upon request from the authors.

## Genetic correlations

We estimated genetic covariation between traits by regressing IL means. Nonindependence between ILs due to overlapping introgression segments should increase spurious covariation between traits. However, this bias toward increased genetic covariation goes against our hypothesis that trait correlations are caused by natural selection rather than pleiotropy or linkage. Genetic correlations might also be evolutionarily relevant if one or a few loci affect multiple traits. Therefore, we also tested for significant colocalization of QTL for different traits using a one-tailed Fisher’s Exact Test (FISHER 1922). For both tests we adjusted  $P$ -values to correct for multiple comparisons using the same BENJAMINI and HOCHBERG (1995) procedure described above. For carbon isotope discrimination ( $\delta^{13}\text{C}$ ), we used previously published data from 49 of the same ILs discussed here (XU *et al.* 2008). We assessed the correlation between SD, SR, and  $\delta^{13}\text{C}$  using linear regression of IL means, as with other traits. The correlation between these different stomatal traits and  $\delta^{13}\text{C}$  can be used to infer which portion of the  $\text{CO}_2$  diffusion pathway is more functionally significant in these species. Although it is generally assumed that carbon isotope discrimination varies with stomatal conductance, which is proportional to the density and aperture of stomata (FRANKS and FARQUHAR 2001; SACK *et al.* 2003), many other traits can also influence  $\delta^{13}\text{C}$  (FARQUHAR *et al.* 1982; EVANS *et al.* 1986; FARQUHAR *et al.* 1989). Stomatal density (which we use as a proxy for stomatal conductance) affects the change in  $\text{CO}_2$  concentration from the atmosphere to the inside

of the leaf. Stomatal ratio affects the  $\text{CO}_2$  path length from stomata to chloroplasts (greater SR reduces path length). Therefore, both SR and SD potentially affect water-use efficiency and carbon isotope discrimination, but lead to different resulting correlations between water-use efficiency (WUE) and  $\delta^{13}\text{C}$ . Lower SD increases WUE and reduces discrimination, whereas greater SR increases WUE but also increases discrimination (see Discussion). Hence, the correlation between these different stomatal traits and  $\delta^{13}\text{C}$  can be used to infer whether diffusion from atmosphere  $\rightarrow$  inside of leaf (SD) vs. from inside of leaf  $\rightarrow$  chloroplasts (SR) is more functionally significant in these species.

## Stomatal Development Candidate Gene Analysis: Colocalization with QTL and patterns of molecular evolution

Focusing on traits whose QTL show the strongest evidence for the action of natural selection, we looked for colocalization between stomatal trait QTL and stomatal development candidate genes that were identified without prior knowledge of QTL location. Stomatal development candidate genes were identified from the well-characterized pathway in *Arabidopsis* (PILLITTERI and TORII 2012) supplemented with additional select candidate genes from sterol and abscisic acid (ABA) biosynthesis pathways. In *Arabidopsis*, sterol biosynthesis affects stomatal development (QIAN *et al.* 2013) though BIN2, which responds to brassinosteroid and phosphorylates multiple stomatal development genes (Gudesblat *et al.* 2012; Kim *et al.* 2012); therefore we included BIN2 orthologs in our analysis. ABA is a stress response hormone that regulates stomatal aperture, but might also regulate stomatal development (Tanaka *et al.* 2013), therefore we also investigated ABA biosynthetic and catabolic enzymes (NAMBARA and MARION-POLL 2005). We considered 25 candidate genes in total (Table 4).

We determined orthologs in tomato for the *Arabidopsis* stomatal genes by a BLASTn search

of the tomato reference transcripts (ITAG v2.4, [www.solgenomics.net](http://www.solgenomics.net)). The top match for each gene was selected as the ortholog with a minimum e-value of  $1 \times 10^{-20}$ . Genes where several top matches were equally likely (often the case in large gene families) were removed from consideration. *S.lycopersicum* reference orthologs determined for each candidate stomatal gene were located on the tomato physical map using the SGN browser ([http://solgenomics.net/gb2/gbrowse/ITAG2.4\\_genomic](http://solgenomics.net/gb2/gbrowse/ITAG2.4_genomic)). Based on known breakpoints for each of our introgression lines, we determined whether the physical position of each candidate fell anywhere within the introgressed *Spn* region of each IL that had significant effects on our stomatal traits, regardless of physical overlap with other significant ILs. We used this more liberal threshold for calling QTL boundaries than used for counting QTL (see above) to ensure that we identified all possible candidate loci and to account for the possibility that multiple separate loci fall within phenotypically significant ILs.

*S. lycopersicum* candidates for each stomatal gene were also used to search for their orthologs in other Solanum species using a BLASTn search of a combined Solanum library we constructed used previously published sequence data and/or transcripts from close relatives of *S. lycopersicum*. *S. pennellii* (LA0716) and *S. habrochaites* (LA1777) RNA-Seq reads were obtained from KOENIG *et al.* (2013). These paired-end Illumina reads were trimmed using both Scythe (<https://github.com/vsbuffalo/scythe>) to remove adapters and a custom Python quality/length FASTQ trimmer. Trimmed reads were then assembled *de novo* into transcripts using Trinity (GRABHERR *et al.* 2011) with standard parameters. The resulting set of assembled transcripts were combined with coding sequences downloaded from <ftp.solgenomics.net> for *S. corneliomulleri* (labeled *S. peruvianum*), *S. lycopersicum* (SL2.40 reference), *S. pimpinellifolium* (Reference v.1.0), *S. dulcamara*, and *S. tuberosum* (v3.4, POTATO GENOME SEQUENCING CONSORTIUM 2011) to form a working coding sequence library.

Our *S. lycopersicum* reference orthologs for each candidate stomatal gene were used as the query for a BLASTn search of this combined Solanum library. Only unambiguous orthologs with e-value  $< 1 \times 10^{-50}$  were retained for further analysis. In two cases (NCED1 and AO3), *S. tuberosum* sequences were not annotated and were derived from a strong BLAST match to unannotated chromosomal sequences in the potato reference. Coding sequences for each candidate gene were aligned separately using PRANK (codon mode, default settings) with minor manual corrections (LÖYTYNOJA and GOLDMAN 2008). The low divergence between these Solanum sequences ensured confident and high-quality coding sequence alignments. We then analyzed these alignments using the codeml package in PAML (v4.7; YANG 2007) with the independent branches model (all branches dN/dS independent), frame-specific codon frequencies, fixed tree topology (Figure S1) with initial branch lengths equal to 1. Since the sequences have extremely low divergence, we also used codeml ancestral sequence reconstructions to count non-synonymous and synonymous changes on each branch of the tree. Collation of sequences and PAML results were carried out with custom Python scripts. Alignments are available from Dryad (MUIR *et al.* 2014).

## RESULTS

### Numerous QTL underlie species differences in heritable leaf traits

The focal species (*Slyc* and *Spn*) differed significantly (nonoverlapping 95% confidence intervals) in most traits (Figure 1A). *Spn* had smaller, thicker leaves, and greater leaf mass per area. *Spn* also had slightly higher stomatal density, but a much greater stomatal ratio, meaning that stomata were distributed evenly across both abaxial and adaxial surfaces compared to *Slyc* where most stomata are on the abaxial (lower) surface (Figure 1). Differences in trichome traits were less pronounced, although *Spn* had a slightly greater trichome ratio than *Slyc*. Observed dif-

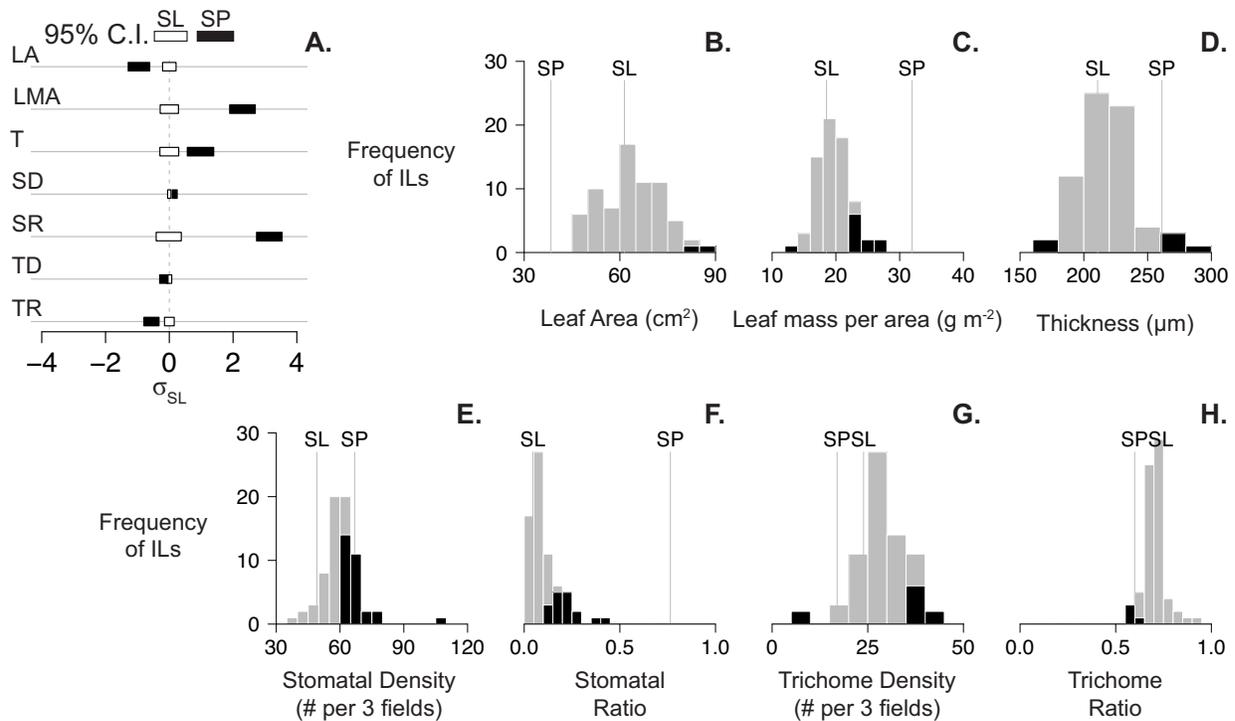
ferences between *Spem* and *Slyc* in these traits are consistent with other studies from our lab and others (KEBEDE *et al.* 1994). In contrast, some of our detected trait differences between *Slyc* and *Spem* might reflect trait sensitivity (plasticity) to the specific growth conditions in our experiment. In particular, we observed that *Spem* had greater LMA than *Slyc*, which is inconsistent with some other studies from our lab. The discrepancy is probably a result of different light intensities during experimental cultivation; LMA typically responds very strongly to irradiance (POORTER *et al.* 2010), and we consistently find that *Slyc* has lower LMA compared to *Spem* in our greenhouse (where irradiance is 200–500 PPFD), but not in experimental fields (where irradiance is 1500–2000 PPFD) (C.D. Muir pers. obs.). Because more trait plasticity is observed in the *Slyc* genotype – LMA in *Spem* is less variable across cultivation conditions, but *Slyc* has low LMA under low light, and high LMA under high light – we infer that LMA is more plastic with respect to irradiance in *Slyc* than *Spem*.

For our introgression lines,  $H^2$ , the number of QTL, the distribution of effect sizes, and the amount of transgressive segregation varied considerably across traits. Leaf traits had low to moderate broad sense heritability (LA: 0.08; LMA: 0.17;  $T$ : 0.12; SD: 0.24; SR: 0.27; TD: 0.39; TR: 0.53) but all were significantly different from zero based on 95% highest posterior density intervals. Between 2 and 30 ILs per trait were significantly different from *Slyc* after correcting for multiple comparisons. A detailed list of statistically significant ILs, their genomic location, additive genetic effect, and percent interspecific divergence explained are given in Table S1. From IL phenotypes, we identified between 2 and 23 QTL per trait (Table 2). A complete description of QTL, names, and locations are provided in File S1 and shown graphically in Figure 4 (SR QTL only) and Figures S1–S6 (remaining traits).

Only 2 QTL were detected for leaf area (Figure S3), and both were transgressive: they conferred increased leaf size, which is opposite to the direction of the *Spem* donor phenotype (Figure 1B). In contrast, there were a substantial

number of QTL for leaf mass per area (Figure S4) and leaf thickness (Figure S5), the majority of which did not transgress parental species’ means (Figure 1C–D). We detected a large number of QTL for stomatal density (Figure S6) and stomatal ratio (Figure 4) compared to trichome density (Figure S7) and trichome ratio (Figure S8), respectively. With one exception (*sd3.1*), stomatal density QTL were of small to moderate effect, always in the direction of *Spem*, and not highly transgressive (Figure 1E). Stomatal ratio was characterized by two large effect QTL (*sr3.1* and *sr9.1*, explaining 15.9% and 5.9% percent of the phenotypic variance, respectively; Table S1), in addition to several small effect QTL (Figure 1F).

Some observed differences in the genetic complexity of leaf traits depended on genetic and experimental factors. In particular, the number of ILs that differed from *Slyc* (and therefore the number of QTL per trait) was influenced by the heritability, the phenotypic difference between species, and the amount of variation within *Slyc*. For example, although stomatal density did not differ greatly between species, there was very little variation within *Slyc* for this trait, allowing us to detect many small effect QTL (Figure 1E and Figure S6). Compared to stomatal density, there was greater variation in stomatal ratio within *Slyc*, however because species differed by  $> 3$  standard deviations, several moderate and large effect QTL were still detected for SR (Figure 1F and Figure 4). In contrast to stomatal traits, we detected few QTL for leaf area despite the fact that LA differs significantly between *Spem* and *Slyc*. This was either because LA was determined by many small effect QTL below our detection threshold and/or because of low heritability (high variance among replicate genotypes) possibly due to environmentally induced plasticity (as discussed above, for LMA). In addition, because we used introgression lines, we were unable to measure epistasis among different chromosomal regions from the same genetic background; therefore trait differences that are determined by strong epistatic effects among unlinked loci will likely have undetected QTL under



**Figure 1** Species' differences (A) and the distribution of IL phenotypes (B-H) for seven leaf traits: Leaf area (LA), Leaf mass per area (LMA), leaf thickness (T), stomatal density (SD), stomatal ratio (SR), trichome density (TD), and trichome ratio (TR). (A) 95% confidence intervals around the mean trait value for the recipient *S. lycopersicum* var. *esculentum* (*Slyc*; open bars) and donor *S. pennellii* (*Spn*; filled bars) in units of *Slyc* standard deviations ( $\sigma_{SL}$ ) with the *Slyc* mean set to zero. (B-H) Histograms of 70 IL phenotypes for seven leaf traits. Gray bars represent ILs that were not significantly different from *Slyc* after adjusting for multiple comparisons. Black bars are lines that were significantly different. *Slyc* and *Spn* point to species' means, but these are not included in the histogram bars.

**Table 2** QTLST results calculated from the number of QTL ( $n$ ), the number of QTL that altered a trait in the direction of *Spen* ( $i$ ), the phenotypic difference between species (R), and the average absolute additive effect size of QTL ( $|\bar{a}|$ ). The  $P_{\text{QTLST}}$ -value is the probability that the empirical distribution of QTL signs would occur without directional natural selection. For comparison, we also calculated  $P_{\text{QTLEE}}$  from the commonly used QTLEE test. Note that R and  $|\bar{a}|$  are given in log-transformed units for LA, LMA, T, SD, and TD, but logit-transformed units for SR and TR.

Trait	$n$	$i$	R	$ \bar{a} $	$P_{\text{QTLST}}$	$P_{\text{QTLEE}}$
LA	2	0	-0.47	0.16	NA	NA
LMA	8	7	0.54	0.13	0.112	0.055
T	4	4	0.21	0.13	0.165	0.091
SD	23	23	0.31	0.15	$2.71 \times 10^{-7}$	$2.38 \times 10^{-7}$
SR	10	10	4.22	0.86	0.003	0.002
TD	7	1	-0.34	0.34	1	1
TR	4	4	-0.63	0.36	0.168	0.091

this experimental design.

### QTL Sign Test indicates directional selection on stomatal traits

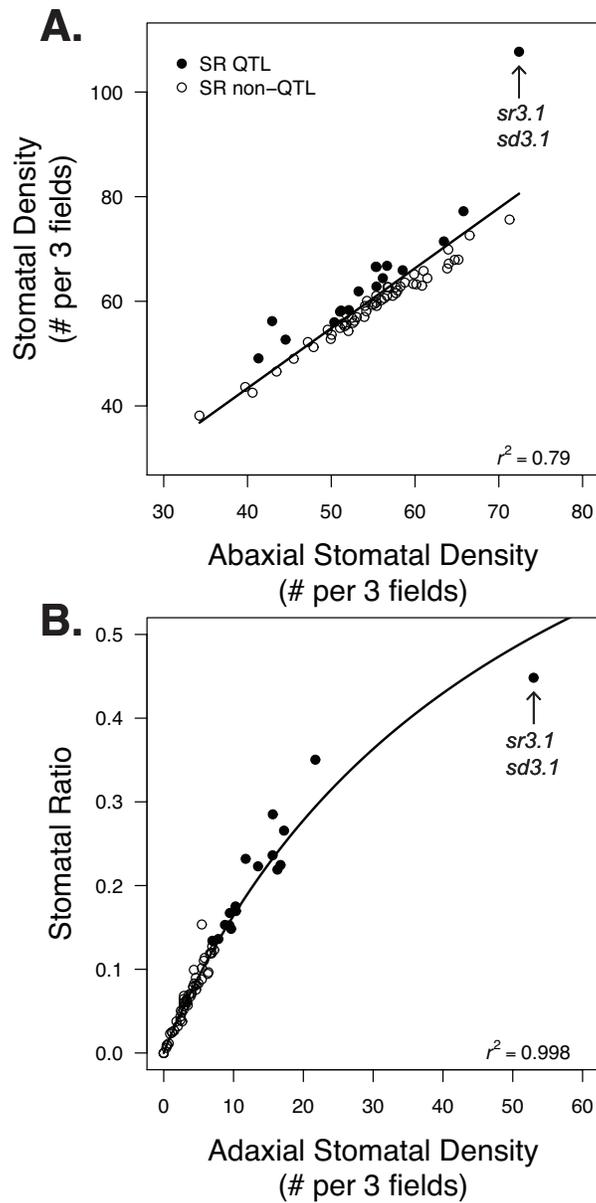
For 5 of 7 traits, all or almost all QTL effects were in the direction of the donor parent *Spen* (Table 2). A QTL Sign Test showed that the distribution of QTL effect sizes for stomatal traits SD and SR were unlikely to have arisen in the absence of directional selection (Table 2). We could not reject a neutral model for LA, LMA, T, TD, and TR, although using less stringent criteria for counting QTL would have made LMA and T significant (data not shown); thus a more powerful study might have rejected the neutral hypothesis for these traits. The more commonly used QTLEE gave qualitatively similar results to QTLST, but  $P$ -values were  $\approx 2 \times$  lower (Table 2), indicating that QTLST is more conservative than QTLEE (unlike stated in ORR 1998b).

### Most leaf traits are not genetically correlated

There was little evidence for genetic correlations between traits (Table 3). Among our focal traits (LA, LMA, T, SD, and SR), only two correlations were significant. Leaf mass per area and leaf thickness were strongly positively correlated, which is expected because LMA is by definition the product of leaf thickness and bulk tissue density (WITKOWSKI and LAMONT 1991). Stomatal

density and stomatal ratio were also positively correlated, but this finding was strongly influenced by a single pleiotropic QTL, *sr3.1*, that had a normal abaxial (lower) stomatal density, but high adaxial (upper) stomatal density, and thus high SR. The correlation between SD and SR is non-significant if this QTL is removed from the analysis ( $r = 0.04$ ,  $P = 0.38$ ). Interestingly, with the exception of *sr3.1*, abaxial and adaxial stomatal density appear to be genetically decoupled from one another. Across ILs, there was no correlation between ab- and adaxial SD ( $r = 0.18$ ,  $P = 0.13$ ). Because most stomata were on the abaxial surface (similar to *Slyc*) genetic variation in SD was determined primarily by variation in stomatal density on this lower leaf surface (Figure 2A). In contrast, variation in SR was determined almost entirely by variation in stomatal density on the upper (adaxial) leaf surface (Figure 2B).

Several observations indicate that stomatal patterning is genetically independent of trichome patterning. First, there was no correlation between stomatal and trichome traits (Table 3). Second, the relationship between trichome density and trichome ratio was very different from that between stomatal density and stomatal ratio: The strong correlation between TD and TR was not driven by a few influential QTL (data not shown) and ab- and adaxial TD were highly positively correlated ( $r = 0.83$ ,  $P < 0.001$ ). Although we had no *a priori* prediction, trichome ratio was negatively correlated with both leaf



**Figure 2** (A) Abaxial ('lower') stomatal density is the most important determinant of stomatal density (SD) of the entire leaf, but (B) adaxial ('upper') stomatal density determines stomatal ratio (SR). Each point is the phenotype of one IL. In both (A) and (B) filled points are ILs that had significant greater SR than *Slyc* after adjusting for multiple comparisons. A major QTL for both SR (*sr3.1*) and SD (*sd3.1*) found on IL3-2 is labeled to show that the same locus has a large effect on both traits. In (A) the line was fit and  $r^2$  calculated using linear regression on untransformed stomatal counts. In (B) the line was fit and  $r^2$  calculated using linear regression between untransformed stomatal counts and SR on a logit scale, but data are plotted on an untransformed scale.

**Table 3** Trait correlations across ILs. Full trait names are used in the rows; abbreviations in the columns.

	LA	LMA	T	SD	SR	TD	TR
Leaf Area	-						
Leaf Mass per Area	0.05	-					
Thickness	0.15	0.71***	-				
Stomatal Density	-0.04	0.01	-0.06	-			
Stomatal Ratio	-0.24	0.12	0.05	0.34*	-		
Trichome Density	-0.11	0.3*	0.23	0.24	0.13	-	
Trichome Ratio	-0.25	-0.37**	-0.38**	-0.02	-0.15	-0.46***	-

\* $P < 0.05$  \*\* $P < 0.01$  \*\*\* $P < 0.001$

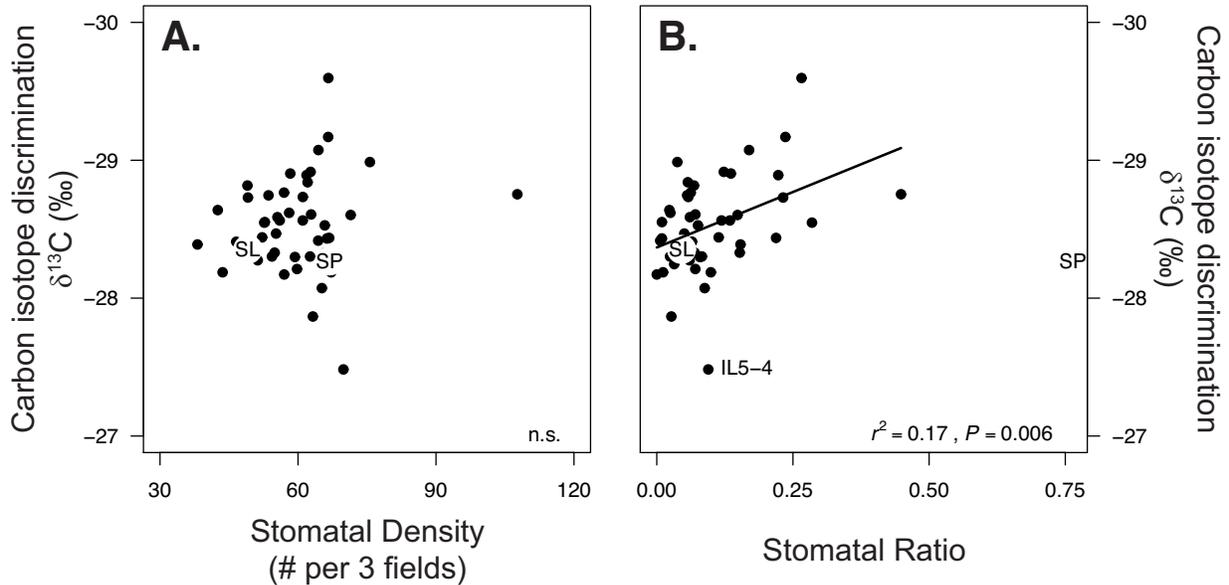
mass per area and thickness (Table 3), which could reflect an unknown connection between trichome patterning and other aspects of leaf development, or could be a false positive. The results from Fisher’s Exact Tests to examine evidence of QTL colocalization closely mirrored genetic correlations, except that there were no cases of significant colocalization after adjustment for multiple comparisons (Table S2). We also did not find evidence that any particular IL harbored a significant excess or deficit of QTL; given the number of QTLs and ILs, the distribution of QTL across ILs did not depart from chance ( $\chi^2_{69} = 80, P = 0.17$ ).

### Stomatal ratio, not stomatal density is correlated with carbon isotope discrimination

We found that stomatal ratio, but not stomatal density, was significantly correlated with carbon isotope discrimination ( $\delta^{13}\text{C}$ ) in these ILs (Figure 3). Our data indicate that for these species, when grown under well-watered greenhouse conditions, the  $\text{CO}_2$  diffusion path length (mediated by SR) has a significant effect on  $\delta^{13}\text{C}$ , but variation in stomatal conductance (using SD as a proxy) does not. In particular, leaves with greater SR exhibited greater discrimination (i.e. more negative  $\delta^{13}\text{C}$ ; Figure 3B), indicating that having stomata evenly distributed on both leaf surfaces significantly affects  $\delta^{13}\text{C}$  by shortening the average distance between stomata and chloroplasts. As the diffusion pathway shortens, more of the fractionation occurs during carboxy-

lation by Rubisco rather than during gas diffusion. Since Rubisco discriminates against  $^{13}\text{C}$  much more than does gaseous diffusion (27–30‰ vs. 4‰) (FARQUHAR *et al.* 1989), leaves with shorter path lengths should have a more negative  $\delta^{13}\text{C}$ , as we observe. One exception to the overall pattern between stomatal traits and  $\delta^{13}\text{C}$  is IL5-4, which had much lower discrimination than expected based on the stomatal ratio observed in this genotype (Figure 3B); the  $\delta^{13}\text{C}$  estimates in this particular genotype might be due instead to lower stomatal conductance, as concluded by XU *et al.* (2008).

We are uncertain why stomatal density did not generally affect  $\delta^{13}\text{C}$ , but two possible explanations are that SD was not a good proxy for stomatal conductance, which is also affected by stomatal aperture, or that the range of variation in stomatal conductance among lines was too small to have an appreciable effect on  $\delta^{13}\text{C}$ . When there are large differences in average stomatal conductance between plants (e.g. when drought induces stomatal closure), variation in  $\delta^{13}\text{C}$  correlates strongly with WUE. However, when plants are grown under non-stressed conditions stomatal conductance is uniformly high; therefore differences in  $\delta^{13}\text{C}$  of a few ‰ between genotypes can be affected by several traits other than stomatal conductance. As with our analysis here, in their study XU *et al.* (2008) measured  $\delta^{13}\text{C}$  under non-stressed conditions.



**Figure 3** Carbon isotope data suggest that amphistomy (greater stomatal ratio) decreases the distance between stomata and chloroplast. (A) Carbon isotope discrimination is uncorrelated with stomatal density (SD), but (B) positively correlated with stomatal ratio (SR) across ILs (more discrimination equals more negative  $\delta^{13}\text{C}$ ). Each point is an IL. *Slyc* and *Spen* means are indicated for comparison, but these were not used in the regression. Carbon isotope data were obtained from (XU *et al.* 2008); stomatal data are from this study.

### Multiple candidate genes co-localize with stomatal QTL

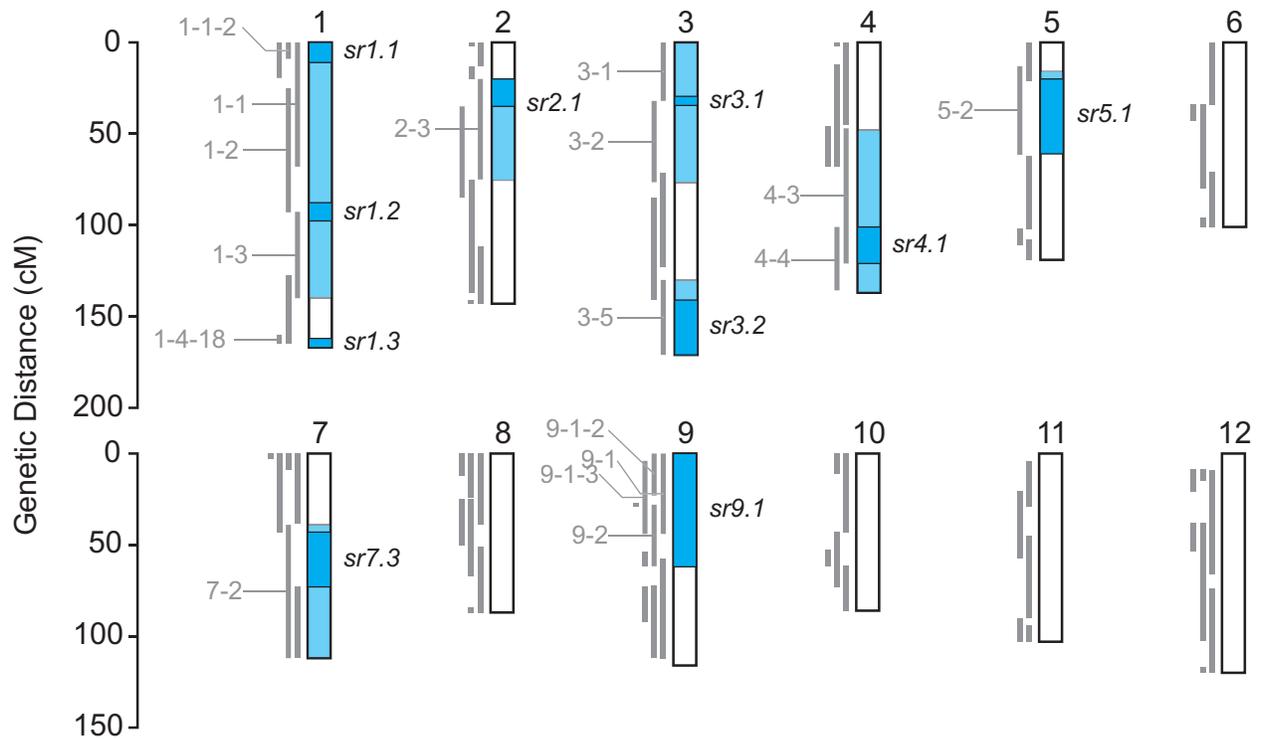
Of the candidate genes drawn from the stomatal developmental biology literature (PILLITTERI and TORII 2012), several colocalized with stomatal QTL (Table 4), including QTL with the largest effect sizes (*sr3.1*, *sr9.2*, *sd3.1*). In addition, we observed elevated rates of nonsynonymous compared to synonymous substitution

( $dN/dS > 1$ ) in two of our candidate genes (ER and STOMAGEN) specifically on the branches leading to our focal species, *Slyc* and *Spen*, perhaps indicating a history of positive selection. Interestingly, neither of these genes colocalized with the largest effect QTL. We found no colocalization between QTL and ABA biosynthesis protein genes, suggesting that constitutively altered ABA levels are not responsible for stomatal trait evolution.

## DISCUSSION

It is tempting to ascribe the phenotypic differences between species to the action of natural selection, but mutation and drift can also cause phenotypic divergence without adaptation. In this study, we looked at several leaf traits that are believed to be involved in climatic adaptation and ecological divergence between species. In particular, we considered two adaptive hypotheses: first, that phenotypes diverged because of directional natural selection; second, that traits

are correlated across species because of selection rather than genetic correlations caused by pleiotropy or linkage. We used QTL mapping to identify the genetic architecture of phenotypic divergence, and found evidence supporting our adaptive hypotheses, especially for stomatal traits. In addition, by combining our trait variation data with information on physiological variation and genomic position, we were able to directly assess the physiological significance of stomatal trait variation, and uncover candidate genes that point toward particular developmen-



**Figure 4** Stomatal ratio QTL positions and ILs observed. To the left of each chromosome is the position of *S. pennellii* introgressions in this IL mapping population. Only ILs had were significantly different than *S. lycopersicum* var. *esculentum* after adjusting *P*-values for multiple comparisons are labeled (e.g. 'IL1-1'). Narrow regions (dark boxes) were determined using bin-mapping; broad regions (light boxes) show the extent of all contiguous ILs that significantly affected the trait (see Methods). QTL names are given on the right side of chromosomes. Similar maps for other traits can be found in Figures S1-6.

**Table 4** Colocalization between stomatal trait QTL with stomatal development candidate genes and patterns of molecular evolution. Several candidate genes are located near QTL for stomatal ratio (SR) and/or stomatal density (SD). The last column indicates which candidate genes had more nonsynonymous substitutions (dN) relative to synonymous substitutions (dS) on either the *S. lycopersicon* (Slyc) or *S. pennellii* (Spen) branches.

Gene name	Symbol	SGN Name <sup>†</sup>	SR QTL	SD QTL	dN/dS > 1? (branch)
NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 1	NCED1	Solyc01g087260	sr1.1, sr1.2	sd1.1	
MAP KINASE 6	MPK6	Solyc02g084870			
ZEAXANTHIN EPOXIDASE	ZEP	Solyc02g090890		sd2.2	
ERECTA-LIKE1	ERL	Solyc03g007050	sr3.1	sd3.1	
SPEECHLESS	SPCH	Solyc03g007410	sr3.1	sd3.1	
MKK7/MKK9	MKK7/9	Solyc03g097920		sd3.2	
SCREAM/ICE1	SCRM	Solyc03g123800	sr3.2	sd3.2	
MKK4/MKK5	MKK4/5	Solyc04g071940		sd4.1	✓ (Slyc)
ABA DEFICIENT 2	ABA2	Solyc04g072880	sr4.1		
MPK3/MPK6	MPK3/6	Solyc04g080730	sr4.1	sd4.3	
FOUR LIPS	FLP	Solyc05g007160	sr5.1		
FAMA	FAMA	Solyc05g053660		sd5.1	
CHALLAH/EPF-LIKE6	CHAL/EPFL6	Solyc06g007460		sd6.1	
SCREAM2	SCRM2	Solyc06g068870			
HYDRA1	HYD1	Solyc06g082980		sd6.2	
STOMATAL DENSITY AND DISTRIBUTION1	SDD1	Solyc06g083720		sd6.2	
ERECTA	ER	Solyc08g061560		sd8.1	✓ (Slyc)
STOMAGEN/EPF-LIKE9	EPFL9	Solyc08g066610			✓ (Spen)
FAKEL	FK	Solyc09g00904 0	sr9.1		
STOMATAL DENSITY AND DISTRIBUTION1	SDD1	Solyc09g064490	sr9.1	sd9.1	
ABA DEFICIENT 3	ABA3	Solyc11g013810			
ABSCISIC ALDEHYDE OXIDASE 3	AAO3	Solyc11g065920		sd12.1	
EPIDERMAL PATTERNING FACTOR1	EPF1	Solyc12g010720			
TOO MANY MOUTHS	TMM	Solyc12g042760			
CYCLOPROPYL ISOMERASE	CPI1	Solyc12g098640		sd12.2	

<sup>†</sup>Names based on the International Tomato Annotation Group genomic annotation release 2.3 available at [http://solgenomics.net/organism/Solanum\\_lycopersicum/genome](http://solgenomics.net/organism/Solanum_lycopersicum/genome)

tal mechanisms underlying adaptation.

### **Genetic architecture of species differences suggests different evolutionary dynamics and trait lability**

We detected between 2 and 23 QTL for each of our putatively adaptive leaf traits, each of which altered the phenotype between 0.12 and 2.10 *Slyc* trait standard deviations (Table S1). This large range of genetic complexity could result from several genetic factors, including the magnitude of phenotypic difference between species, the amount of variation within *Slyc*, and trait differences in heritability (Results). In some cases, low heritability appeared to be affected by environmentally induced plasticity, especially for traits related to leaf area (LA and LMA; Results) which are known to vary plastically with growth environment. As such, QTL identified for these traits in our study might be associated with genetic differences in plasticity rather than constitutive differences. A better understanding of G×E interactions for these traits will clarify whether these QTL also underlie species differences in nature.

The different genetic architectures we observe suggest that certain traits might evolve more readily in response to new environmental conditions. Many traits in tomato and other species, including some traits in this study, are highly polygenic (LIPPMAN *et al.* 2007), which could be consistent with either weak phenotypic selection, significant pleiotropy, and/or lack of large effect mutations. In contrast, the two large effect loci for one of our traits (stomatal ratio) more closely resemble the genetic architecture of traits that are known to have evolved under strong selection with limited pleiotropy such as flower color in *Mimulus* (YUP and MYB transcription factors; SCHEMSKE and BRADSHAW JR. (1999); STREISFELD *et al.* (2013); YUAN *et al.* (2013)), armor plating in stickleback (EDA; COLOSIMO *et al.* (2005)), and coat/skin color in mammals (MC1R; NACHMAN *et al.* (2003); HOEKSTRA *et al.* (2006)). Like these traits, few genetic changes appear necessary to achieve large pheno-

typic transitions in SR; together, our two large effect SR loci account for 98% of the phenotypic difference between our two parental species (assuming they act additively). The similarity in genetic architecture suggests that SR is likewise an ecologically important trait under divergent selection in tomatoes, although we have not yet identified the specific selective agent.

Another inference from the striking genetic architecture of SR is that the availability of large effect loci might make this trait more labile in response to changes in selection. There is some qualitative support for this hypothesis. Variation in SR among wild tomato species spans the entire range of variation observed across the Solanaceae, which contains numerous hypo- and amphistomatous species (C.D. Muir, unpub. data). In comparison, variation in LA among wild tomatoes, for which we found no large effect loci, comprises a small fraction of the total variation in *Solanum* and has high phylogenetic signal (C.D. Muir, unpub. data). In the future, this kind of hypothesis can be tested quantitatively by examining macroevolutionary patterns of trait lability across broader taxonomic groups, such as all *Solanum* (~1500 species), in response to environmental niche transitions. In wild tomatoes, the evolution of amphistomy is closely correlated with transitions to drier environments, an important component of niche evolution between sister species (NAKAZATO *et al.* 2010). Although the ancestral SR of all wild tomatoes is ambiguous (outgroups are both hypo- and amphistomatous [C.D. Muir, unpub. data]), amphistomy likely evolved in *Spina* as it colonized desert habitats; *Spina* has the highest SR of any tomato species and its sister species (*S. habrochaites*) is hypostomatous (MUIR *et al.* 2014 and unpub. data), indicating that a trait shift to more equal distribution of stomata on both leaf surfaces occurred specifically within the *Spina* lineage.

## Genetic architecture reveals evidence for natural selection on individual traits and trait combinations

A clear preponderance of QTL had allelic effects in the direction of the *Spen* donor phenotype (Table 2), and a model assuming random, nondirectional fixation was strongly rejected for stomatal traits (SD and SR) consistent with trait differentiation due to divergent natural selection. Because the number and size of detected QTL limits the power of the QTLST test, failure to reject a model of random fixation for several of our other traits does not rule out the action of natural selection. For example, smaller leaf area correlates with precipitation specifically in wild tomatoes, across the entire genus *Solanum* (C.D. Muir unpub. data) and in many other clades (M<sup>c</sup>DONALD *et al.* 2003). However, because we did not detect QTL that reduced LA in the direction of *Spen*, QTLST could not be performed for this trait. In such cases, especially where there is substantial independent evidence that a trait is adaptive, alternative methods such as selection gradient analysis will be needed (DUDLEY 1996). Regardless, our analysis clearly demonstrated selection on stomatal traits, either because selection on stomatal traits has been stronger than that on leaf morphology or because the larger effect sizes of stomatal trait QTL made them easier to detect and hence increased our power to reject the random fixation model.

Comparison of genetic architecture across traits also provided evidence that trait correlations across species are due to selection rather than pleiotropy or linkage. In wild tomatoes, leaf area, leaf mass per area, and stomatal ratio are correlated with each other and with climate, especially precipitation (C.D. Muir, unpub. data). While one explanation for these correlations is shared underlying mechanisms, we found no evidence that leaf traits are correlated across species because of genetic correlations. The only evidence for pleiotropy/linkage came from a single major QTL on chromosome 3 that affected both stomatal density and stomatal ratio (Figure 2). In the absence of this QTL, however, the remain-

ing SD and SR QTL are uncorrelated in our analysis. Similarly, across wild tomatoes in general, SD and SR are uncorrelated (C.D. Muir unpub. data). In fact, the wild tomato species with the highest stomatal density (*S. habrochaites*) has very low stomatal ratio (most of the stomata are on the abaxial surface). Overall, from these data we infer that leaf traits phenotypically covary across species because they function well together in certain ecological contexts; that is, these suites of covarying traits are the product of selection. In particular, small leaves, low LMA, and high SR are likely more common in wild tomato species from drier habitats because they confer greater photosynthetic efficiency and reduce water lost relative to carbon gain.

Finally, we also examined whether species differences in stomatal traits could be a pleiotropic effect of selection on other features of the plant epidermis – specifically trichomes – rather than the direct target of selection. We found that the density and distribution of stomata is independent of the density and distribution of trichomes. Although we did not measure additional epidermal traits, such as cell size or shape, these data suggest that stomatal traits did not evolve as a pleiotropic byproduct of changes in other traits, providing a second piece of evidence that stomatal traits *per se* are the target of divergent selection.

## Structure-function relationship reveals the mechanistic significance of adaptive stomatal trait variation

By examining the association between variation in our stomatal traits and a previously measured metric of whole plant water use efficiency (XU *et al.* 2008), we were able to address the precise physiological significance of leaf trait variation under our experimental conditions. The positive correlation we observed between stomatal ratio and carbon isotope discrimination suggests both physiological and ecological inferences: first, that having stomata on both sides of the leaf shortens the CO<sub>2</sub> diffusion path length inside the leaf

– thereby potentially influencing water-use efficiency; and second, that interspecific variation in SR is ecologically significant in wild tomatoes.

The physiological inference, that SR decreases the diffusion path length, differs from that typically made using  $\delta^{13}\text{C}$  in quantitative genetic studies of water-use efficiency. Generally, lower stomatal conductance increases water-use efficiency and reduces discrimination against the heavier isotope  $^{13}\text{C}$ , which is why carbon isotope discrimination is often used as a proxy for WUE. In contrast to this general expectation, here we found that variation in stomatal ratio, but not stomatal density (a proxy for conductance), predicted carbon isotope discrimination among ILs. This is surprising because it suggests that the  $\text{CO}_2$  diffusion path length from stomata to the chloroplast, rather than stomatal conductance, determines  $\delta^{13}\text{C}$  in these ILs.

The ecological inference is that selection has acted specifically on photosynthetic efficiency (rather than solely water conservation) during adaptation to low precipitation across wild tomato species. In wild tomatoes, stomatal ratio is typically greater in species native to drier climates, such as *Spn* (C. D. Muir, unpub. data). By reducing the distance between stomata and chloroplasts, greater SR can increase WUE by increasing the concentration of  $\text{CO}_2$  around the chloroplasts (and therefore the rate of photosynthesis) without incurring greater evaporative water loss. However, under this mechanism, greater discrimination would be indicative of greater WUE, which is exactly the opposite of how  $\delta^{13}\text{C}$  is interpreted when stomatal conductance is assumed to be the dominant mechanism affecting both  $\delta^{13}\text{C}$  and WUE. Our finding stresses the importance of identifying the mechanism underlying variation in  $\delta^{13}\text{C}$  in quantitative genetic studies (JUENGER *et al.* 2005), when interpreting the ecological significance of this variation.

### **Candidate genes co-localized with stomatal trait QTL suggest specific molecular mechanisms of trait variation**

Overall, our stomatal traits were most clearly and consistently associated with both ecological and physiological roles in climatic adaptation of wild tomatoes. To begin assessing the molecular mechanisms responsible for stomatal trait evolution, we identified candidate genes that co-localize with quantitative trait loci these traits, especially for QTL of large effect for stomatal ratio (*sr3.1*, *sr9.1*) and stomatal density (*sd3.1*) (Table 4). Our phenotypic data indicate that *sr3.1* and *sr9.1* both changed SR specifically by greatly increasing adaxial stomatal density (2 vs. 53 and 22 per field, respectively) without a concomitant reduction in abaxial density. These phenotypes imply a particular developmental mechanism underlies trait variation at both these loci, wherein *Spn* alleles confer adaxial expression of the entire stomatal developmental pathway but this expression is nearly absent in *Slyc*. This adaxial-specific change could arise from structural changes in proteins regulating stomatal development, *cis*-regulatory changes altering binding by ab-adaxial polarity factors, and/or altered sensitivity to hormonal regulators of stomatal development in the adaxial epidermis. Based on the identity and molecular evolution of our colocalized candidates, we find evidence in support of the latter two mechanisms only. In the first case, none of the candidate genes colocalized with our large effect QTL had a significant signature of adaptive evolution in either of the branches leading to *Slyc* or *Spn*, arguing against structural protein changes, although the analysis of selection used here (dN/dS) will not detect adaptive differences due to single or few nonsynonymous substitutions. In contrast, the specific molecular identity of some our candidates suggest the second alternative—*cis*-regulatory changes—could underlie our observed phenotypic differences. In particular, we find that the tomato homolog of SPCH resides within *sr3.1*, our largest effect SR locus (*sr3.1*). In *Arabidopsis*, SPCH activity initiates stomatal devel-

opment; therefore loss (gain) of adaxial stomata could be as simple as SPCH losing (gaining) regulation by a factor responsible ab-adaxial polarity. Moreover, several described mutations in the cis-regulatory region of SPCH in *Arabidopsis* can completely eliminate stomatal development specifically on one side of the leaf, but not the other, indicating that SPCH interacts with ab-adaxial polarity factors (Dow *et al.* 2014). We also found that SDD1 and FACKEL colocalized with *sr9.1*. The products of these genes modulate stomatal density, and hence altered expression in adaxial tissue could alter stomatal ratio.

Therefore, based on current functional information on colocalized stomatal development genes, we find that mutation(s) in cis-regulatory elements are the most likely molecular mechanism underlying the trait change associated with our largest effect SR QTL. These hypotheses will require future functional analyses, which are well-developed in tomato (GUPTA *et al.* 2009). Regardless, despite intense interest in stomata because of their role in adaptation to climate change, ours is among the first studies on the genetics of natural variation in stomata density and distribution (HALL *et al.* 2005; RAE *et al.* 2006; GAILING *et al.* 2008), and the first study of stomatal ratio genetics.

## Conclusions

Based on fundamental biophysical principles and empirical trait-environment correlations, we hypothesized that interspecific variation in several ecologically important leaf traits reflects adaptation to climate in wild tomatoes. In all leaf traits there was significant heritability and we identified QTL underlying phenotypic differences between two tomato species. Most QTL affected

traits in the direction of the donor parent, the wild species *S. pennellii*, suggesting that loci were fixed by natural selection rather than genetic drift, and this pattern was strongly supported for stomatal traits. We found no evidence that focal leaf traits that correlate across species are genetically correlated, or that stomatal traits were shaped by selection on other epidermal features. We infer from this that association between traits in nature likely reflects natural selection. We also found evidence that variation in the distribution of stomata is functionally significant and could affect ecologically important traits like water-use efficiency. Collectively, these data along with ecophysiological and comparative data on variation in leaf traits, make a compelling argument for adaptation between recently diverged species in this ecologically and physiological diverse clade. For key traits such as SD and SR, our analysis also suggests that these ecologically important transitions can involve relatively few changes of large phenotypic effect, that allow rapid responses to new environmental conditions. Because this leaf trait variation also captures important axes of phenotypic variation across all land plants, these results also suggest mechanisms that might underlie the evolution of these traits across much broader macroevolutionary scales.

## ACKNOWLEDGEMENTS

M. Yakub and S. Vosters assisted with germinating, transplanting, and maintaining plants in the greenhouse. E. Josephs, S. Josway, and E. Levin assisted with phenotyping. This research was supported by a NSF Graduate Research Fellowship to CDM and NSF grants DEB-0841957 and DEB-1135707 to LCM.

## LITERATURE CITED

- ABRÀMOFF, M. D., P. J. MAGALHÃES, and S. J. RAM, 2004 Image processing with ImageJ. *Biophotonics International* 11(7): 36–42.
- ANDERSON, E. C. and M. SLATKIN, 2003 Orr’s quantitative trait loci sign test under conditions of trait ascertainment. *Genetics* 165(1): 445–446.
- BARTLETT, M. K., C. SCOFFONI, and L. SACK, 2012 The determinants of leaf turgor loss point and prediction of drought tolerance of species and biomes: a global meta-analysis. *Ecology Letters* 15(5): 393–405.
- BENJAMINI, Y. and Y. HOCHBERG, 1995 Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* 57(1): 289–300.
- BOMBARELY, A., N. MENDA, I. Y. TECLE, R. M. BUELS, S. STRICKLER, T. FISCHER-YORK, A. PUJAR, J. LETO, J. GOSSELIN, and L. A. MUELLER, 2011 The Sol Genomics Network (solgenomics.net): growing tomatoes using Perl. *Nucleic acids research* 39(suppl 1): D1149–D1155.
- CHITWOOD, D. H., R. KUMAR, L. R. HEADLAND, A. RANJAN, M. F. COVINGTON, Y. ICHIHASHI, D. FULOP, J. M. JIMÉNEZ-GÓMEZ, J. PENG, J. N. MALOOF, and N. R. SINHA, 2013 A quantitative genetic basis for leaf morphology in a set of precisely defined tomato introgression lines. *The Plant Cell* 25(7): 2465–2481.
- COLOSIMO, P. F., K. E. HOSEMAN, S. BALABHADRA, G. VILLARREAL, M. DICKSON, J. GRIMWOOD, J. SCHMUTZ, R. M. MYERS, D. SCHLUTER, and D. M. KINGSLEY, 2005 Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *Science* 307(5717): 1928–1933.
- COWAN, I., 1977 Stomatal behaviour and environment. *Advances in botanical research* 4: 117–228.
- DALZIEL, A. C., S. M. ROGERS, and P. M. SCHULTE, 2009 Linking genotypes to phenotypes and fitness: how mechanistic biology can inform molecular ecology. *Molecular Ecology* 18(24): 4997–5017.
- DES MARAIS, D. L., L. C. AUCHINCLOSS, E. SUKAMTOH, J. K. MCKAY, T. LOGAN, J. H. RICHARDS, and T. E. JUENGER, 2014 Variation in *MPK12* affects water use efficiency in *Arabidopsis* and reveals a pleiotropic link between guard cell size and ABA response. *Proceedings of the National Academy of Sciences* 111(7): 2836–2841.
- DOW, G. J., D. C. BERGMANN, and J. A. BERRY, 2014 An integrated model of stomatal development and leaf physiology. *New Phytologist* 201(4): 1218–1226.
- DUDLEY, S. A., 1996 Differing selection on plant physiological traits in response to environmental water availability: a test of adaptive hypotheses. *Evolution* 50(1): 92–102.
- EASLON, H. M. and J. H. RICHARDS, 2009 Drought response in self-compatible species of tomato (Solanaceae). *American Journal of Botany* 96(3): 605–611.
- EHLERINGER, J., O. BJÖRKMAN, and H. A. MOONEY, 1976 Leaf pubescence: effects on absorbance and photosynthesis in a desert shrub. *Science* 192(4237): 376–377.
- ESHED, Y. and D. ZAMIR, 1994 A genomic library of *Lycopersicon pennellii* in *L. esculentum*: a tool for fine mapping of genes. *Euphytica* 79(3): 175–179.

- EVANS, J. R., G. D. FARQUHAR, T. D. SHARKEY, and J. BERRY, 1986 Carbon isotope discrimination measured concurrently with gas exchange to investigate CO<sub>2</sub> diffusion in leaves of higher plants. *Australian Journal of Plant Physiology* *13*(2): 281–292.
- FARQUHAR, G. D., J. R. EHLERINGER, and K. T. HUBICK, 1989 Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 503–537.
- FARQUHAR, G. D., M. O’LEARY, and B. JA, 1982 On the relationship between carbon isotope discrimination and the inter-cellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* *9*(2): 121–137.
- FARQUHAR, G. D. and T. D. SHARKEY, 1982 Stomatal conductance and photosynthesis. *Annual review of plant physiology* *33*(1): 317–345.
- FISHER, R. A., 1922 On the interpretation of  $\chi^2$  from contingency tables, and the calculation of P. *Journal of the Royal Statistical Society* *85*(1): 87–94.
- FRANKS, P. J. and G. D. FARQUHAR, 2001 The effect of exogenous abscisic acid on stomatal development, stomatal mechanics, and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiology* *125*(2): 935–942.
- GAILING, O., R. LANGENFELD-HEYSER, A. POLLE, and R. FINKELDEY, 2008 Quantitative trait loci affecting stomatal density and growth in a *Quercus robur* progeny: implications for the adaptation to changing environments. *Global Change Biology* *14*(8): 1934–1946.
- GALMÉS, J., J. OCHOGAVÍA, J. GAGO, E. J. ROLDÁN, J. CIFRE, and M. À. CONESA, 2013 Leaf responses to drought stress in Mediterranean accessions of *Solanum lycopersicum*: anatomical adaptations in relation to gas exchange parameters. *Plant, Cell & Environment* *36*(5): 920–935.
- GATES, D. M., 1968 Transpiration and leaf temperature. *Annual Review of Plant Physiology* *19*(1): 211–238.
- GIVNISH, T. J. and G. J. VERMEIJ, 1976 Sizes and shapes of liane leaves. *American Naturalist* *110*(975): 743–778.
- GRABHERR, M. G., B. J. HAAS, M. YASSOUR, J. Z. LEVIN, D. A. THOMPSON, I. AMIT, X. ADICONIS, L. FAN, R. RAYCHOWDHURY, Q. ZENG, Z. CHEN, E. MAUCELI, N. HACHEN, A. GNIRKE, N. RHIND, F. DI PALMA, B. W. BIRREN, C. NUSBAUM, K. LINDBLAD-TOH, N. FRIEDMAN, and A. REGEV, 2011 Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotech* **29**: 644–652.
- GUPTA, V., S. MATHUR, A. U. SOLANKE, M. K. SHARMA, R. KUMAR, S. VYAS, P. KHURANA, J. P. KHURANA, A. K. TYAGI, and A. K. SHARMA, 2009 Genome analysis and genetic enhancement of tomato. *Critical Reviews in Biotechnology* *29*(2): 152–181.
- HADFIELD, J. D., 2010 MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm R Package. *Journal of Statistical Software* *33*(2): 1–22.
- HALL, N., H. GRIFFITHS, J. CORLETT, H. JONES, J. LYNN, and G. KING, 2005 Relationships between water-use traits and photosynthesis in *Brassica oleracea* resolved by quantitative genetic analysis. *Plant Breeding* *124*(6): 557–564.
- HOEKSTRA, H. E., R. J. HIRSCHMANN, R. A. BUNDEY, P. A. INSEL, and J. P. CROSSLAND, 2006 A single amino acid mutation contributes to adaptive beach mouse color pattern. *Sci-*

- ence *313*(5783): 101–104.
- HOLTAN, H. E. and S. HAKE, 2003 Quantitative trait locus analysis of leaf dissection in tomato using *Lycopersicon pennellii* segmental introgression lines. *Genetics* *165*(3): 1541–1550.
- JONES, F. C., M. G. GRABHERR, Y. F. CHAN, P. RUSSELL, E. MAUCELI, J. JOHNSON, R. SWOFFORD, M. PIRUN, M. C. ZODY, S. WHITE, E. BIRNEY, S. SEARLE, J. SCHMUTZ, J. GRIMWOOD, M. C. DICKSON, R. M. MYERS, C. T. MILLER1, B. R. SUMMERS, A. K. KNECHT, S. D. BRADY, H. ZHANG, A. A. POLLEN, T. HOWES, C. AMEMIYA, BROAD INSTITUTE GENOME SEQUENCING PLATFORM & WHOLE GENOME ASSEMBLY TEAM, E. S. LANDER, F. D. PALMA, K. LINDBLAD-TOH, and D. M. KINGSLEY, 2012 The genomic basis of adaptive evolution in threespine sticklebacks. *Nature* *484*(7392): 55–61.
- JUENGER, T. E., J. K. MCKAY, N. HAUSMANN, J. J. KEURENTJES, S. SEN, K. A. STOWE, T. E. DAWSON, E. L. SIMMS, and J. H. RICHARDS, 2005 Identification and characterization of QTL underlying whole-plant physiology in *Arabidopsis thaliana*:  $\delta^{13}C$ , stomatal conductance and transpiration efficiency. *Plant, Cell & Environment* *28*(6): 697–708.
- KEBEDE, H., B. MARTIN, J. NIENHUIS, and G. KING, 1994 Leaf anatomy of two *Lycopersicon* species with contrasting gas exchange properties. *Crop Science* *34*(1): 108–113.
- KIMURA, S., D. KOENIG, J. KANG, F. Y. YOONG, and N. SINHA, 2008 Natural variation in leaf morphology results from mutation of a novel *KNOX* gene. *Current Biology* *18*(9): 672–677.
- KIRKPATRICK, M. and N. H. BARTON, 2006 Chromosome inversions, local adaptation and speciation. *Genetics* *173*(1): 419–434.
- KOENIG, D., J. M. JIMÁLNEZ-GÁŞMEZ, S. KIMURA, D. FULOP, D. H. CHITWOOD, L. R. HEADLAND, R. KUMAR, M. F. COVINGTON, U. K. DEVISSETY, A. V. TAT, T. TOHGE, A. BOLGER, K. SCHNEEBERGER, S. OSSOWSKI, C. LANZ, G. XIONG, M. TAYLOR-TEEPLES, S. M. BRADY, M. PAULY, D. WEIGEL, B. USADEL, A. R. FERNIE, J. PENG, N. R. SINHA, and J. N. MALOOF, 2013 Comparative transcriptomics reveals patterns of selection in domesticated and wild tomato. *Proceedings of the National Academy of Sciences* *110*(28): E2655–E2662.
- LEE, Y. W., B. A. GOULD, and J. R. STINCHCOMBE, 2014 Identifying the genes underlying quantitative traits: a rationale for the QTN programme. *AOB PLANTS* **6**: plu004.
- LEVIN, D. A., 1973 The role of trichomes in plant defense. *Quarterly Review of Biology*: 3–15.
- LIPPMAN, Z. B., Y. SEMEL, and D. ZAMIR, 2007 An integrated view of quantitative trait variation using tomato interspecific introgression lines. *Current Opinion in Genetics & Development* *17*(6): 545–552.
- LOWRY, D. B. and J. H. WILLIS, 2010 A widespread chromosomal inversion polymorphism contributes to a major life-history transition, local adaptation, and reproductive isolation. *PLoS Biology* *8*(9): e1000500.
- LÖYTYNOJA, A. and N. GOLDMAN, 2008 Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. *Science* *320*(5883): 1632–1635.
- MCKAY, J., J. RICHARDS, and T. MITCHELL-OLDS, 2003 Genetics of drought adaptation in *Arabidopsis thaliana*: I. Pleiotropy contributes to genetic correlations among ecological traits. *Molecular Ecology* *12*(5): 1137–1151.
- MCKAY, J. K., J. H. RICHARDS, K. S. NEMALI, S. SEN, T. MITCHELL-OLDS, S. BOLES, E. A.

- STAHL, T. WAYNE, and T. E. JUENGER, 2008 Genetics of drought adaptation in *Arabidopsis thaliana* II. QTL analysis of a new mapping population, Kas-1 × Tsu-1. *Evolution* 62(12): 3014–3026.
- MOTT, K. A., A. C. GIBSON, and J. W. O’LEARY, 1982 The adaptive significance of amphistomatic leaves. *Plant, Cell & Environment* 5(6): 455–460.
- MOYLE, L. C. and T. NAKAZATO, 2010 Hybrid incompatibility “snowballs” between *Solanum* species. *Science* 329(5998): 1521–1523.
- MCDONALD, P., C. FONSECA, J. OVERTON, and M. WESTOBY, 2003 Leaf-size divergence along rainfall and soil-nutrient gradients: is the method of size reduction common among clades? *Functional Ecology* 17(1): 50–57.
- MUIR, C. D., R. P. HANGARTER, L. C. MOYLE, and P. A. DAVIS, 2014 Morphological and anatomical determinants of mesophyll conductance in wild relatives of tomato (*Solanum* sect. *Lycopersicon*, sect. *Lycopersicoides*; Solanaceae). *Plant, Cell & Environment* 37(6): 1415–1426.
- MUIR, C. D. and L. C. MOYLE, 2009 Antagonistic epistasis for ecophysiological trait differences between *Solanum* species. *New Phytologist* 183(3): 789–802.
- MUIR, C. D., J. B. PEASE, and L. C. MOYLE, 2014 Data from: Quantitative genetic analysis indicates natural selection on leaf phenotypes across wild tomato species (*Solanum* sect. *Lycopersicon*; Solanaceae). *Genetics*, Dryad Digital Repository.
- NACHMAN, M. W., H. E. HOEKSTRA, and S. L. D’AGOSTINO, 2003 The genetic basis of adaptive melanism in pocket mice. *Proceedings of the National Academy of Sciences* 100(9): 5268–5273.
- NAKAZATO, T., D. L. WARREN, and L. C. MOYLE, 2010 Ecological and geographic modes of species divergence in wild tomatoes. *American Journal of Botany* 97(4): 680–693.
- NAMBARA, E. and A. MARION-POLL, 2005 Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology* 56: 165–185.
- NICOTRA, A. B., A. LEIGH, C. K. BOYCE, C. S. JONES, K. J. NIKLAS, D. L. ROYER, and H. TSUKAYA, 2011 The evolution and functional significance of leaf shape in the angiosperms. *Functional Plant Biology* 38(7): 535–552.
- NOOR, M. A. F., K. L. GRAMS, L. A. BERTUCCI, and J. REILAND, 2001 Chromosomal inversions and the reproductive isolation of species. *Proceedings of the National Academy of Sciences* 98(21): 12084–12088.
- OGBURN, R. M. and E. J. EDWARDS, 2010 The ecological water-use strategies of succulent plants. *Advances in Botanical Research* 55: 179–225.
- ONODA, Y., M. WESTOBY, P. B. ADLER, A. M. CHOONG, F. J. CLISSOLD, J. H. CORNELISSEN, S. DÍAZ, N. J. DOMINY, A. ELGART, L. ENRICO, P. V. A. FINE, J. J. HOWARD, A. JALILI, K. KITAJIMA, H. KUROKAWA, C. MCARTHUR, P. W. LUCAS, L. MARKESTEIJN, N. PÁLREZ-HARGUINDEGUY, L. POORTER, L. RICHARDS, L. S. SANTIAGO, E. E. S. JR., S. A. V. BAELE, D. I. WARTON, I. J. WRIGHT, S. J. WRIGHT, and N. YAMASHITA, 2011 Global patterns of leaf mechanical properties. *Ecology letters* 14(3): 301–312.
- ORR, H. A., 1998a The population genetics of adaptation: the distribution of factors fixed during adaptive evolution. *Evolution* 52(4): 935–949.
- ORR, H. A., 1998b Testing natural selection vs. genetic drift in phenotypic evolution using quan-

- titative trait locus data. *Genetics* *149*(4): 2099–2104.
- PAN, Q., Y.-S. LIU, O. BUDAI-HADRIAN, M. SELA, L. CARMEL-GOREN, D. ZAMIR, and R. FLUHR, 2000 Comparative genetics of nucleotide binding site-leucine rich repeat resistance gene homologues in the genomes of two dicotyledons: tomato and *Arabidopsis*. *Genetics* *155*(1): 309–322.
- PARKHURST, D. F., 1978 The adaptive significance of stomatal occurrence on one or both surfaces of leaves. *The Journal of Ecology* *66*(2): 367–383.
- PARKHURST, D. F. and O. LOUCKS, 1972 Optimal leaf size in relation to environment. *The Journal of Ecology* *60*(2): 505–537.
- PILLITTERI, L. J. and K. U. TORII, 2012 Mechanisms of stomatal development. *Annual Review of Plant Biology* **63**: 591–614.
- POLLARD, K. S., H. N. GILBERT, Y. GE, S. TAYLOR, and S. DUDOIT, 2013 multtest: Resampling-based multiple hypothesis testing. R package version 2.12.0.
- POORTER, H., Ü. NIINEMETS, A. WALTER, F. FIORANI, and U. SCHURR, 2010 A method to construct dose–response curves for a wide range of environmental factors and plant traits by means of a meta-analysis of phenotypic data. *Journal of Experimental Botany* *61*(8): 2043–2055.
- POTATO GENOME SEQUENCING CONSORTIUM, 2011 Genome sequence and analysis of the tuber crop potato. *Nature* *475*(7355): 189–195.
- QIAN, P., B. HAN, E. FORESTIER, Z. HU, N. GAO, W. LU, H. SCHALLER, J. LI, and S. HOU, 2013 Sterols are required for cell-fate commitment and maintenance of the stomatal lineage in *Arabidopsis*. *The Plant Journal* *74*(6): 1029–1044.
- R CORE TEAM, 2012 R: A Language and Environment for Statistical Computing. ISBN 3-900051-07-0.
- RAE, A. M., R. FERRIS, M. J. TALLIS, and G. TAYLOR, 2006 Elucidating genomic regions determining enhanced leaf growth and delayed senescence in elevated CO<sub>2</sub>. *Plant, Cell & Environment* *29*(9): 1730–1741.
- RIESEBERG, L. H., 2001 Chromosomal rearrangements and speciation. *Trends in Ecology & Evolution* *16*(7): 351–358.
- RIESEBERG, L. H., A. WIDMER, A. M. ARNTZ, and J. M. BURKE, 2002 Directional selection is the primary cause of phenotypic diversification. *Proceedings of the National Academy of Sciences* *99*(19): 12242–12245.
- RUCKDESCHEL, P., M. KOHL, T. STABLA, and F. CAMPHAUSEN, 2006 S4 Classes for Distributions. *R News* *6*(2): 2–6.
- SACK, L., P. COWAN, N. JAIKUMAR, and N. HOLBROOK, 2003 The ‘hydrology’ of leaves: co-ordination of structure and function in temperate woody species. *Plant, Cell & Environment* *26*(8): 1343–1356.
- SCHAUER, N., Y. SEMEL, U. ROESSNER, A. GUR, I. BALBO, F. CARRARI, T. PLEBAN, A. PEREZ-MELIS, C. BRUEDIGAM, J. KOPKA, L. WILLMITZER, D. ZAMIR, and A. R. FERNIE, 2006 Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. *Nature Biotechnology* *24*(4): 447–454.

- SCHEMSKE, D. W. and H. BRADSHAW JR., 1999 Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences* *96*(21): 11910–11915.
- SEMEL, Y., J. NISSENBAUM, N. MENDA, M. ZINDER, U. KRIEGER, N. ISSMAN, T. PLEBAN, Z. LIPPMAN, A. GUR, and D. ZAMIR, 2006 Overdominant quantitative trait loci for yield and fitness in tomato. *Proceedings of the National Academy of Sciences* *103*(35): 12981–12986.
- SMITH, W. K. and N. M. HUGHES, 2009 Progress in coupling plant form and photosynthetic function. *Castanea* *74*(1): 1–26.
- SMITH, W. K., T. C. VOGELMANN, E. H. DELUCIA, D. T. BELL, and K. A. SHEPHERD, 1997 Leaf form and photosynthesis. *BioScience* *47*(11): 785–793.
- STREISFELD, M. A., W. N. YOUNG, and J. M. SOBEL, 2013 Divergent selection drives genetic differentiation in an R2R3-Myb transcription factor that contributes to incipient speciation in *Mimulus aurantiacus*. *PLoS genetics* *9*(3): e1003385.
- TERASHIMA, I., S.-I. MIYAZAWA, and Y. T. HANBA, 2006 Why are Sun Leaves Thicker than Shade Leaves? - Consideration based on Analyses of CO<sub>2</sub> Diffusion in the Leaf. *Journal of Experimental Botany* *57*(2): 343–354.
- VILE, D., E. GARNIER, B. SHIPLEY, G. LAURENT, M.-L. NAVAS, C. ROUMET, S. LAVOREL, S. DIAZ, J. G. HODGSON, F. LLORET, G. F. MIDGLEY, H. POORTER, M. C. RUTHERFORD, P. J. WILSON, and I. J. WRIGHT, 2005 Specific leaf area and dry matter content estimate thickness in laminar leaves. *Annals of Botany* *96*(6): 1129–1136.
- VOGEL, S., 2012 *The Life of a Leaf*. University of Chicago Press.
- WITKOWSKI, E. and B. B. LAMONT, 1991 Leaf specific mass confounds leaf density and thickness. *Oecologia* *88*(4): 486–493.
- WRIGHT, I. J., P. B. REICH, M. WESTOBY, D. D. ACKERLY, Z. BARUCH, F. BONGERS, J. CAVENDER-BARES, T. CHAPIN, J. H. CORNELISSEN, M. DIEMER, J. FLEXAS, E. GARNIER, P. K. GROOM, J. GULIAS, K. HIKASAKA, B. B. LAMONT, T. LEE, W. LEE, C. LUSK, J. J. MIDGELEY, M.-L. NAVAS, Ü. NIINEMETS, J. OLEKSYN, N. OSADA, H. POORTER, P. POOT, L. PRIOR, V. I. PYANKOV, C. ROUMET, S. C. THOMAS, M. G. TJOELKER, E. J. VENEKLAAS, and R. VILLAR, 2004 The worldwide leaf economics spectrum. *Nature* *428*(6985): 821–827.
- XU, X., B. MARTIN, J. P. COMSTOCK, T. J. VISION, C. G. TAUER, B. ZHAO, R. C. PAUSCH, and S. KNAPP, 2008 Fine mapping a QTL for carbon isotope composition in tomato. *Theoretical and Applied Genetics* *117*(2): 221–233.
- YANG, Z., 2007 PAML 4: phylogenetic analysis by maximum likelihood. *Molecular Biology and Evolution* *24*(8): 1586–1591.
- YUAN, Y.-W., K. J. R. P. BYERS, and H. D. BRADSHAW JR., 2013 The genetic control of flower-pollinator specificity. *Current Opinion in Plant Biology* **16**: 422–428.