

1 **Surprisingly weak coordination between leaf structure and function among closely-related**
2 **tomato species**

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14

15 **Abstract**

16 Natural selection may often favor coordination between different traits, or phenotypic
17 integration, in order to most efficiently acquire and deploy scarce resources. As leaves are the
18 primary photosynthetic organ in plants, many have proposed that leaf physiology, biochemistry,
19 and anatomical structure are coordinated along a functional trait spectrum from fast, resource-
20 acquisitive syndromes to slow, resource-conservative syndromes. However, the coordination
21 hypothesis has rarely been tested at a phylogenetic scale most relevant for understanding rapid
22 adaptation in the recent past or predicting evolutionary trajectories in response to climate change.
23 To that end, we used a common garden to examine genetically-based coordination between leaf
24 traits across 19 wild and cultivated tomato taxa. We found surprisingly weak integration between
25 photosynthetic rate, leaf structure, biochemical capacity, and CO₂ diffusion, even though all were
26 arrayed in the predicted direction along a ‘fast-slow’ spectrum. This suggests considerable scope
27 for unique trait combinations to evolve in response to new environments or in crop breeding. In
28 particular, we find that partially independent variation in stomatal and mesophyll conductance
29 may allow a plant to improve water-use efficiency without necessarily sacrificing maximum
30 photosynthetic rates. Our study does not imply that functional trait spectra or tradeoffs are
31 unimportant, but that the many important axes of variation within a taxonomic group may be
32 unique and not generalizable to other taxa.
33

34 **Introduction**

35 The ecology of organisms critically depends on their ability to obtain energy for growth and
36 reproduction. In C₃ plants, both passive diffusion of CO₂ from the atmosphere to chloroplasts,
37 via stomata and the leaf mesophyll, and biochemical capacity limit photosynthetic rates
38 (Farquhar & Sharkey 1982; Parkhurst 1994; Evans *et al.* 2009; Flexas *et al.* 2015). Optimization
39 theory predicts that these functions should be tightly coordinated: leaf anatomies that allow rapid
40 diffusion of CO₂ should also have the biochemical capacity to take advantage of it and *vice versa*
41 (Givnish 1986; Wright *et al.* 2004; Reich 2014). Natural selection on trait coordination, also
42 known as phenotypic integration (Pigliucci 2003), should thus generate major functional trait
43 spectra associated with different ecological strategies (Grime 1977; Westoby *et al.* 2002; Chave
44 *et al.* 2009; Reich 2014). Indeed, intrinsic photosynthetic capacity varies widely between species,
45 indicating that leaf-level CO₂ diffusion and biochemistry are major levers through which natural
46 selection and crop breeders can alter plant performance and fitness in different environments. For
47 example, selection might favor leaf traits that inhibit rapid CO₂ diffusion (e.g. lower stomatal
48 density or thicker leaves) if doing so has an adaptive benefit, bringing about a tradeoff (e.g.
49 sclerophyll leaves of species from water-limited environments [Medrano *et al.* 2009]). It is not
50 clear whether most functional trait variation between species lies along a single major axis (e.g.
51 fast-slow continuum [Reich 2014]) or orthogonally along multiple independent trait axes.

52 In particular, the presence of functional trait spectra has rarely been examined among
53 closely-related species in a phylogenetic context (Edwards *et al.* 2014; Mason & Donovan 2015).
54 However, for certain ecological questions, data on genetically-based variation among closely-
55 related species, rather than broad comparisons across disparate families and plant functional
56 types, are most appropriate (Donovan *et al.* 2014). Specifically, ecological diversification is not

57 always predictable because different groups of organisms find unique adaptive solutions, leading
58 to multiple trait spectra. For example, the leaf economics spectrum (Wright *et al.* 2004) is not a
59 one-size-fits-all trait axis, but rather a set of axes that vary between communities (Funk &
60 Cornwell 2013) and taxa (Edwards *et al.* 2014; Mason & Donovan 2015). Second, the
61 evolutionary routes (not) taken in the recent past among closely-related species may help predict
62 how species will respond to natural and anthropogenic climate change (Kellerman *et al.* 2012;
63 Donovan *et al.* 2014). Finally, crop breeders can take advantage of existing trait variation in
64 crop-wild relatives to develop new varieties for sustainable agriculture (Moyle & Muir 2010;
65 Dennison 2012; Giuliani *et al.* 2013). Hence, if coordination among leaf traits is limited among
66 closely-related species, then broad-scale or global trait spectra may be of little use in addressing
67 fundamental challenges like the ecology of local adaptation (Kawecki & Ebert 2004), near-term
68 responses to climate change, and crop breeding. Conversely, weak coordination may indicate
69 significant opportunities for natural and artificial selection to fine-tune leaf traits in response to
70 different ecological circumstances.

71 We used a common garden experiment to measure coordination between leaf traits,
72 specifically diffusive and biochemical limitations on photosynthesis and water-use efficiency as
73 well as leaf structure. We considered these traits in relation to climate of origin among tomato
74 species (*Solanum* sect. *Lycopersicon*) and their relatives (*Solanum* sect. *Juglandifolia* and
75 *Lycopersicoides*). These species are closely related herbaceous perennials: fossil-calibrated
76 molecular clocks put section *Lycopersicon* at approximately 2.48 my old, and the split between
77 *Lycopersicon* and *Lycopersicoides* at approximately 4.7 my ago (Särkinen *et al.* 2013). Despite
78 sharing a recent common ancestor, tomato species are ecologically and phenotypically diverse
79 (Moyle 2008; Peralta *et al.* 2008; Nakazato *et al.* 2008, 2012; Chitwood *et al.* 2012; Haak *et al.*

80 2014; Muir & Thomas-Huebner 2015). Adaptation to different climates, especially along
81 temperature and precipitation gradients, may have played an important role in diversification
82 (Nakazato *et al.* 2010). Most wild tomato species are interfertile (e.g. Baek *et al.* 2015) with the
83 domesticated tomato (*S. lycopersicum* var. *esculentum*), providing a valuable source of
84 germplasm for crop improvement. In particular, altering leaf CO₂ diffusion properties may
85 enhance photosynthetic rate and/or water-use efficiency (Tholen *et al.* 2012; Flexas *et al.* 2013;
86 Gago *et al.* 2014; Buckley & Warren 2014; Flexas *et al.* 2015). Diffusion limits photosynthesis
87 in some wild tomato species (Muir *et al.* 2014) and cultivars (Galmés *et al.* 2011, 2013), but we
88 know little about the ecological and evolutionary significance of this variation.

89 More generally, we know relatively more about the mechanistic basis of variation in leaf
90 photosynthesis than its ecological or adaptive significance. Stomatal and mesophyll CO₂
91 diffusion are strongly affected by leaf anatomy (Brown & Escombe 1900; Franks & Farquhar
92 2001; Nobel 2009; Tholen and Zhu 2011; Tomás *et al.* 2013); biochemical limitations depend on
93 the amount of Rubisco, its activation and catalytic rates (Farquhar *et al.* 1980; von Caemmerer
94 2000; Galmés *et al.* 2014). Maximum photosynthetic rates are generally higher among certain
95 plant functional types (herbs, C₃ graminoids, and crop plants) than others (trees, sclerophylls,
96 and succulents) (Körner *et al.* 1979; Wullschleger 1993; Flexas *et al.* 2008). These broad-scale
97 correlations suggest that slow growing plants tradeoff high photosynthetic rate in favor of leaf
98 traits that may have other fitness benefits, such as durability, defense against herbivores, or
99 increased resource-use efficiency (Turner 1994; Aerts 1995; Onoda *et al.* 2011).

100 We found surprisingly weak coordination among CO₂ diffusion, biochemical capacity,
101 photosynthesis, water-use efficiency, and leaf structure among 19 wild and cultivated tomato
102 taxa. The principal axis of variation indicates an expected tradeoff between robust leaf structure

103 and photosynthesis. However, there was substantial variation orthogonal to this axis that allowed
104 some species simultaneously achieve relatively high photosynthetic rates and intrinsic water-use
105 efficiency. Also, contrary to expectations from the literature, there was no indication that more
106 conservative leaf traits were associated with drier or hotter environments. If anything, there was
107 a signature of less robust leaf structure among species from the driest habitats. Thus, our data
108 suggest that functional trait spectra inferred from global comparisons across plant families and
109 functional types may be of limited utility for predicting short-term evolutionary responses.

110

111 **Methods**

112 *Plant materials and cultivation*

113 We obtained seeds of 19 taxa, 16 wild species (Table S1) from the Tomato Genetics Resource
114 Center at UC Davis (TGRC; <http://tgrc.ucdavis.edu>) as well as 3 cultivated accessions of *S.*
115 *lycopersicum* var. *esculentum*, cv. ‘Roma VF’ (Batlle S.A.) and two Mediterranean ‘Tomàtiga de
116 Ramellet’ accessions from the University of the Balearic Islands seedbank collection (UIB1-30
117 and UIB1-48). To even out plant size during measurements, and following TGRC indications,
118 slower growing species were germinated two weeks ahead of faster growing species. Seeds were
119 soaked in 2.5% sodium hypochlorite (household bleach) for 30 or 60 minutes (following TGRC
120 instructions for each species), rinsed thoroughly, and placed on moist paper to germinate. After
121 one week, seedlings were transplanted to cell-pack flats. Two weeks later, five plants of each
122 taxa were transplanted to 19 L pots where the experiment was performed. Pots contained a
123 mixture of standard horticultural substrate mixed with perlite in a 4:1 proportion v/v. Plants
124 were grown outdoors in an open experimental field under typical Mediterranean conditions at the
125 University of the Balearic Islands (39° 38’ 14.9’’ N, 2° 38’ 51.5’’ E) during spring-summer

126 season. Plants were irrigated to field capacity daily to prevent drought stress and fertilized
127 weekly with an NPK solution.

128

129 *Diffusional and biochemical constraints on photosynthesis and water-use efficiency*

130 We measured stomatal (g_s), mesophyll (g_m) conductance to CO_2 , net CO_2 assimilation rate (A_N)
131 at ambient CO_2 concentrations, and intrinsic water-use efficiency ($iWUE = A_N / g_{sw}$) using an
132 open-path infrared gas exchange analyzer (LI-6400 or LI-6400XT, LI-COR Inc., Lincoln, NE,
133 USA) with a 2-cm² leaf chamber fluorometer. Note that g_s and g_{sw} refer to stomatal conductance
134 to CO_2 and H_2O , respectively. Each leaf acclimatized in the chamber until steady state (usually
135 15-30 min) under standardized conditions: ambient CO_2 ($C_a = 400$ ppm); constant leaf
136 temperature ($T_{\text{leaf}} = 25^\circ \text{C}$), saturating irradiance (photosynthetically active radiation, PAR =
137 $1500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$), and moderate humidity (relative humidity = 40-60%). We took point
138 measurements of all traits under these steady-state conditions. Additionally, we calculated the
139 maximum rate of carboxylation (V_{cmax}) using $A-C_c$ curves and leaf dark respiration (R_{dark}) at
140 predawn. Estimating g_m can be particularly sensitive to assumed parameter values. To accurately
141 measure g_m we estimated species-specific parameters of leaf respiration, light
142 absorbance/photosystem partitioning, and Rubisco kinetic parameters. Further detail on
143 measuring g_m using combined gas exchange and chlorophyll fluorescence is provided in
144 Methods S1. To investigate Rubisco kinetics, we also sequenced the *rbcL* gene encoding the
145 Rubisco large subunit (LSu) from each species to identify amino acid sequence changes that
146 could alter Rubisco kinetics (see Methods S1 for further detail). Next, we characterized Rubisco
147 kinetic parameters directly from two species (*S. lycopersicum* var. *esculentum*, cv. ‘Roma VF’
148 and *S. lycopersicoides*) representing the two Rubisco LSu types identified from sequence

149 analysis (Methods S1). Kinetic properties of the two Rubisco LSu types were compared using
150 ANOVA.

151

152 *Leaf anatomical measurements*

153 We used the youngest, unshaded, fully-expanded leaf from each individual. Leaves were
154 immediately weighed and scanned to obtain fresh mass (FM) and leaf area (LA), respectively.
155 Afterwards, leaves were dried for at least 48 h in a drying oven at 60 °C to obtain dry mass
156 (DM). We report *LMA* (as DM/LA) on whole leaves, which in tomato are pinnately compound,
157 but we excluded structural, non-laminar portions (petiole, rachis, and petioles) because we were
158 particularly interested tradeoffs between leaf structure and diffusive conductance within the
159 lamina. Because leaf thickness measurements using a micrometer are unreliable in tomato leaves
160 (C.D. Muir, pers. obs), we estimated leaf thickness (*LT*) using the method of Vile *et al.* (2005):

$$LT = \frac{LMA}{LDMC} \quad (S1)$$

161 *LDMC* is the leaf dry matter content, the ratio of leaf DM to saturated FM. Leaf thickness
162 calculated using Eq. 1 is closely correlated with leaf thickness measured from sections in
163 tomatoes (Muir *et al.* 2014). We obtained leaf morphological data from 80 of 82 individuals.

164

165 *Statistical analyses*

166 We used phylogenetic linear mixed effects models ('phyloLME') to estimate key relationships
167 among traits while accounting for phylogenetic nonindependence. Specifically, we fit statistical
168 models using a Bayesian Markov chain Monte Carlo (MCMC) algorithm implemented in the R
169 package MCMCglmm version 2.21 (Hadfield 2010). For all models, we ran the MCMC chain
170 under diffuse priors for 10^6 steps after a burn-in of 10^5 steps, sampling the posterior distribution

171 10^4 times every 10^2 steps. We tested whether stomatal and mesophyll conductance were
172 significantly correlated with A_N and $iWUE$. These variables were log-transformed for linearity
173 and homoscedasticity. We estimated the effect of these diffusion traits on A_N and $iWUE$ from the
174 mode of the posterior distribution and inferred statistical significance if the 95% highest
175 posterior density (HPD) interval did not overlap zero. We simultaneously tested whether
176 phylogeny explained photosynthetic trait variation by including Species as a phylogenetically-
177 structured random effect and compared that to a model without Species using the deviance
178 information criterion (DIC), where a decrease of 2 or more is interpreted as a significant increase
179 in model fit. We used a maximum likelihood phylogenetic tree inferred from 18 genes (Figure 1;
180 Haak *et al.* 2014). Maximum likelihood analyses were conducted using RAxML version 8.1.24
181 (Stamatakis 2014). The topology of the best tree agreed with previous Bayesian estimates
182 (Rodriguez *et al.* 2009).

183 We used principal component analysis (PCA) to identify major axes of variation among
184 nine leaf traits: g_m , g_s , A_N , V_{cmax} , R_{dark} , $iWUE$, LMA , LT , $LDMC$. All traits except V_{cmax} and R_{dark}
185 were log-transformed to make the distribution approximately multivariate normal. Parallel
186 analysis of the trait correlation matrix using the ‘parallel’ function from the R package nFactors
187 version 2.3.3 (Raiche 2010) indicated that the first four principal components explained
188 significantly more variance, 90.0% cumulatively, than expected by chance from an uncorrelated
189 matrix with rank 7 (we used 9 traits, but $iWUE$ and LT are linear combinations of other traits).
190 We focus on the first principal component, denoted PC1, which explained a moderate amount of
191 variation (35.5%). For reference, PC1 loaded positively with leaf thickness and LMA , but
192 negatively with A_N and components of CO_2 diffusion (g_m , g_s).

193 To test the hypothesis that species from dry and/or hot environments tradeoff efficient
194 CO₂ diffusion for a more robust, stress-tolerant leaf anatomy, we looked at the correlation
195 between mean annual precipitation and temperature to species' average position along PC1. For
196 this analysis, we removed the three cultivars of *S. lycopersicum* var. *esculentum* and one
197 accession *S. lycopersicum* var. *cerasiforme*, an unimproved landrace. To reduce the influence of
198 a single outlier species (*S. juglandifolium*), we shrank the interspecific variance of PC1 using a
199 signed logarithm transformation:

$$PC1_T = \text{sign}(PC1) \log|PC1| \quad (S2)$$

200 We also performed analyses with and without this species. We obtained mean annual
201 precipitation and temperature at the habitat of origin for each source population from Worldclim
202 (Hijmans *et al.* 2005), which has been used previously to study climatic adaptation in wild
203 tomatoes (Chitwood *et al.* 2012; Nakazato *et al.* 2008). Phylogenetic regression was carried out
204 with the R package phylolm version 2.2 (Ho & Ané 2014) using the 'OUrandomRoot' model to
205 account for phylogenetic nonindependence. Other trait-climate relationships were tested in the
206 same way.

207

208 **Results**

209 *CO₂ diffusion and biochemistry limit photosynthesis and alter water-use efficiency*

210 Tomato species vary considerably in photosynthetic rate (A_N) and intrinsic water-use efficiency
211 ($iWUE$), driven by constraints on leaf CO₂ diffusion and the maximum rate of carboxylation
212 (V_{cmax}). Phylogenetic linear mixed effects models ('phyloLME') showed that between individual
213 plants, stomatal (g_s) and mesophyll (g_m) conductance increased A_N (Table 1; Figure 2A,B), but
214 had opposing effects on $iWUE$ (Table 1). However, phylogenetic relationship explained little of

215 the trait variation, thus phylogenetic and nonphylogenetic gave nearly identical results (Table 1).
216 Increased g_s was associated with lower $iWUE$ (Figure 2C), whereas greater g_m was associated
217 with greater $iWUE$ (Figure 2D). Like g_m , greater biochemical capacity, as indicated by V_{cmax} , was
218 associated with significantly greater A_N and $iWUE$ (Figure 2). Thus, species achieved high
219 photosynthetic rates via two routes: high g_s , but lower $iWUE$ or high g_m , but relatively high
220 $iWUE$. The drawdown of CO_2 concentration from the leaf interior (C_i) to the chloroplast (C_c),
221 another indicator of diffusional constraint in the leaf mesophyll, was even more strongly
222 correlated with $iWUE$ than g_m (Figure S1), arguing that reduced internal diffusion enhances
223 $iWUE$.

224

225 *Limited variation in Rubisco biochemistry between tomato species*

226 The two Rubisco LSU types showed significant differences in some of the kinetic parameters
227 (Table 2; see Methods S1 for description of how types were identified and Results S1 for further
228 detail on molecular evolution of *rbcL* in tomatoes). Interestingly, the Rubisco LSU type 2, which
229 occurred in the domesticated clade species (*S. pimpinellifolium*, *S. cheesmaniae*, *S. galapagense*,
230 *S. lycopersicum* var. *cerasiforme*, and the cultivars; see Figure S2) and *S. habrochaites*, had a
231 higher $S_{c/o}$ value, which was due to higher Michaelis-Menten constant for CO_2 under atmospheric
232 conditions (K_c^{air}) and lower catalytic turnover rate for the oxygenase reaction (k_{cat}^o) compared to
233 the Rubisco LSU type 1. Non-significant differences were observed between the two Rubisco
234 LSU types in the remaining kinetic parameters.

235

236 *Modest coordination between leaf physiological and structural traits*

237 The first principal component (PC1) accounted for 35.5% of the variation among individual leaf
238 physiology (g_m , g_s , A_N , V_{cmax} , R_{dark} , $iWUE$) and bulk anatomy (LMA , LT , $LDMC$). On one end of
239 this axis were thin leaves with fast CO₂ diffusion and high A_N ; on the other were thick leaves
240 with slower CO₂ diffusion and lower A_N (Figure 3). This principal component indicates an axis
241 of leaf trait variation likely mediated by tradeoffs between more robust leaf structure (i.e. higher
242 LMA and LT) and CO₂ diffusion. However, the modest amount of trait variance explained
243 indicates that this tradeoff does not tightly constrain leaf trait evolution in tomatoes. The second
244 principal component (PC2; 23.8% variance explained) was most strongly associated with $iWUE$
245 and showed that greater $iWUE$ was associated with higher water content (lower $LDMC$) leaves
246 (Figure 3).

247

248 *Limited evidence leaf trait-climate associations*

249 Contrary to the hypothesis that dry, hot environments select for a stress-tolerant, robust leaf
250 structure, thinner leaves and more rapid CO₂ diffusion (i.e. higher values of PC1) was associated
251 with drier habitats (PC1-Precip, $P = 0.012$), but not temperature (PC1-Temp, $P = 0.069$).
252 However, this correlation between PC1 and precipitation was strongly influenced by a single
253 species, *S. juglandifolium* (Figure 4), and was not significant if this species was removed (PC1-
254 Precip, $P = 0.266$). Certain traits that loaded strongly with PC1, especially LMA and LT , were
255 strongly associated with precipitation. Specifically, species from the driest habitats had the
256 thinnest leaves, even when *S. juglandifolium* was excluded (Figure S3). Our data therefore do not
257 support the hypothesis that species tradeoff slow CO₂ diffusion and lower metabolic rates for
258 robust leaf structure in stressful environments. All trends in the data are actually in the exact
259 opposite direction.

260

261 **Discussion**

262 Many ecologists seek to distill organismal variation down to a manageable number of key
263 functional traits (e.g. Perez-Harguindeguy *et al.* 2013). Leaf traits in particular, which are
264 necessarily constrained by the need to capture sunlight, CO₂, and manage nutrient/water, may
265 fall along a single ‘fast-slow’ continuum (Reich 2014 and references therein) associated with
266 resource acquisitive and resource conservative traits (Mason & Donovan 2015). The key
267 assumptions are that leaf structural traits such as leaf mass per area (*LMA*) strongly constrain
268 photosynthesis by reducing CO₂ diffusion and that there is strong selection for tight coordination
269 between CO₂ diffusion and biochemical capacity for photosynthesis. These assumptions have
270 been evaluated at broad phylogenetic scales, but rarely addressed among closely-related species.
271 This is important because patterns among distantly related species can be driven primarily by
272 disparate functional groups (e.g. deciduous versus evergreen species) that have little bearing on
273 the incremental evolutionary steps taken as species adapt to new environments.

274 In tomatoes, we found evidence for a ‘fast-slow’ spectrum mediated by
275 diffusional/biochemical constraints and leaf structure, but this spectrum explained a modest
276 amount of total leaf trait variation. There is no precise prediction for exactly how much
277 coordination we *should* have seen under the coordination hypothesis, but variation was well-
278 distributed across the first four components, indicating multiple important axes. Furthermore,
279 although thicker leaves with greater *LMA* were associated with reduced CO₂ diffusion, the
280 relationships were weak (Figure S4), demonstrating ample scope for thick and/or dense leaves to
281 have relatively high diffusion and *vice versa*. Finally, lack of coordination cannot be explained
282 by relatively little physiological variation between species. Indeed, despite sharing a recent

283 common ancestor, we observed a dramatic range in traits like A_N ($6.4 - 33.0 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) in
284 wild tomatoes. In fact, our study should have been especially able to detect coordination, if it
285 existed, because we measured plants with similar growth form and functional type in a common
286 garden at the same age, eliminating sources of variation common in other studies. The lack of
287 tight coordination between leaf structure, CO_2 diffusion, and photosynthetic biochemistry means
288 there may be multiple, loosely coordinated axes of leaf trait variation that provide a substrate for
289 labile, unconstrained evolution in response to novel selective pressures from climate change and
290 crop breeders.

291 In fact, many combinations of stomatal conductance (g_s), mesophyll conductance (g_m),
292 and V_{cmax} resulted in similar photosynthetic rates but very different water-use efficiencies (Figure
293 2). Typically, we expect that to increase photosynthetic rate, plants must increase stomatal
294 conductance, increasing transpirational loss and decreasing water-use efficiency. If mesophyll
295 conductance and biochemical capacity were closely coordinated with stomatal conductance, then
296 there would be limited opportunity to increase water-use efficiency without sacrificing
297 photosynthetic rate. However, we find that there may be substantial scope to increase
298 photosynthetic rate while maintaining high water-use efficiency. Indeed, photosynthetic rate and
299 water-use efficiency were essentially uncorrelated. This axis of variation was evident in a second
300 principal component that loaded positively with $iWUE$ but was orthogonal to A_N (Figure 3). Why
301 don't all species have high photosynthetic rate and water-use efficiency? In nature, there are
302 probably other tradeoffs, especially nitrogen limitation, that prevent species from having greater
303 mesophyll conductance and/or V_{cmax} .

304 Photosynthetic variation in tomatoes is primarily mediated by anatomical differences in
305 leaves rather than differences in Rubisco kinetics. In contrast to other clades of C_3 plants that

306 have undergone rapid diversification into different environments, evolutionary changes in
307 Rubisco kinetics (e.g. faster rates of carboxylation or greater affinity for CO₂ over O₂) do not
308 appear to play a major role in the evolution of tomatoes. This contrasts with other plant groups
309 like *Limonium* (Galmés *et al.* 2014), which underwent adaptive evolution of Rubisco kinetics
310 during their radiations into novel environments. Future work is needed to understand why some
311 clades respond to selection through changes in protein biochemistry, whereas groups like tomato
312 seem to primarily differ in anatomical traits.

313 We also find no evidence that drier or hotter environments favor robust leaf structure or
314 ‘slow’, resource conservative strategies (low conductance, low V_{cmax}) in wild tomatoes (Figure
315 4). Obviously, our study does not have the statistical power of broad comparative analyses (e.g.
316 Wright *et al.* 2005), but the trends in the data were not even in the predicted direction. If
317 anything, there was a tendency for species from the driest habitats to be on the ‘fast’ end of the
318 leaf trait spectrum (see also Easlon & Richards 2009), but this was largely influenced by a single
319 species, *S. juglandifolium*. Thus, tomato species in dry habitats may rely primarily on a form of
320 drought tolerance, growing fast when water is available and dying back or going dormant during
321 droughts. Alternatively, leaf traits may be decoupled from other traits (e.g. root:shoot ratios) that
322 confer alternative drought avoidance or tolerance mechanisms.

323 The modest coordination we observe between leaf structure and physiological function
324 indicates that bulk structural traits like leaf mass per area are probably insufficient to identify the
325 most important axes of trait variation (Figure S4). Traits like leaf thickness and leaf mass per
326 area are probably important in setting the upper bounds on maximum potential CO₂ diffusion and
327 photosynthetic rate (Flexas *et al.* 2008), but realized values depend on the precise anatomical and
328 biochemical features of the leaf that are not well-captured by bulk structural traits (Tosens *et al.*

329 2012; Tomás *et al.* 2013). This suggests the possibility that there are many unique axes that vary
330 leaf anatomy and photosynthesis without large effects on traits like *LMA*. Each of these axes may
331 be a different tradeoff that could be evolutionarily important in some taxa, but not others.

332 From the modest coordination between leaf structure and function in wild tomatoes, we
333 can make three major conclusions:

- 334 1. The leaf functional traits most commonly used comparative ecology (e.g. *LMA*) may not be
335 good proxies for leaf function, especially among closely-related species. This is not because
336 there was little variation among tomatoes. Tomato leaves varied widely in diffusional,
337 biochemical, and structural traits, but there was remarkably little covariation between bulk
338 structure and function.
- 339 2. Hence, broad-scale patterns of functional trait covariation may not be useful in predicting
340 adaptive evolution in the recent past or in response to climate change. Nor should we expect
341 that the relationship between traits and environments observed at broad phylogenetic scales
342 (e.g. thick leaves in hot, dry environments) will hold at smaller scales. If anything, we found
343 that low-*LMA*, resource acquisitive traits were associated with dry environments, the
344 opposite of what is commonly predicted (see also Mason & Donovan 2015).
- 345 3. Although our study challenges commonly-held assumptions about the relationships between
346 leaf structure and function, this does not refute that intimate structure-function relationships
347 exist. Rather, detailed anatomical traits such as guard cell dimensions, mesophyll structure,
348 and photochemical enzyme concentrations are needed to identify the most important
349 tradeoffs defining leaf anatomy and physiology.

350 Adaptive radiation of tomatoes and other plant groups undoubtedly requires evolution of new
351 morphological and physiological traits, but this study suggests that we do not yet have a general
352 explanation for variation in the most important leaf traits affecting photosynthesis.

353

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360

361 **References**

- 362 Aerts, R. (1995). The advantages of being evergreen. *TREE*, 10, 402-407.
- 363 Baek, Y. S.. *et al.* (2015). Testing the SI× SC rule: Pollen–pistil interactions in interspecific
364 crosses between members of the tomato clade (*Solanum* section *Lycopersicon*,
365 Solanaceae). *Am. J. Bot.*, 102, 302-311.
- 366 Brown, H. T. & F. Escombe. (1900). Static diffusion of gases and liquids in relation to the
367 assimilation of carbon and translocation in plants." *Proceedings of the Royal Society of*
368 *London*, 67, 124-128.
- 369 Buckley, T. N. *et al.* (2014). The role of mesophyll conductance in the economics of nitrogen
370 and water use in photosynthesis. *Photosynthesis research*, 119, 77-88.
- 371 Chave, J. *et al.* (2009). Towards a worldwide wood economic spectrum. *Ecol. Lett.*, 12, 351–366.
- 372 Chitwood, D. H. *et al.* (2012). Native environment modulates leaf size and response to simulated

- 373 foliar shade across wild tomato species. *PLoS ONE*, 7:e29570.
- 374 doi:10.1371/journal.pone.0029570.
- 375 Donovan, L.A., *et al.* (2014). Ecological and evolutionary lability of plant traits affecting carbon
376 and nutrient cycling. *J. Ecol.*, 102, 302-314.
- 377 Denison, R. F. (2012). *Darwinian agriculture: how understanding evolution can improve*
378 *agriculture*. Princeton University Press.
- 379 Easlon, H. M. & J.H. Richards. (2009). Drought response in self-compatible species of tomato
380 (Solanaceae)." *American Journal of Botany*, 96, 605-611.
- 381 Edwards, E. J. *et al.* (2014). Leaf life span and the leaf economic spectrum in the context of
382 whole plant architecture. *J. Ecol.*, 102, 328–336.
- 383 Evans, J. R. *et al.* (2009). Resistances along the CO₂ diffusion pathway inside leaves. *J. Exp.*
384 *Bot.*, 60, 2235-2248.
- 385 Farquhar, G. D. *et al.* (1980). A biochemical model of photosynthetic CO₂ assimilation in leaves
386 of C₃ species. *Planta* 149, 78-90.
- 387 Farquhar, G. D. *et al.* (1982). Stomatal conductance and photosynthesis. *Annu Rev. Plant*
388 *Physiol.*, 33, 317-345.
- 389 Flexas, J. *et al.* (2013) Leaf mesophyll conductance and leaf hydraulic conductance: an
390 introduction to their measurement and coordination. *J. Exp. Bot.*, 64, 3965-3981.
- 391 Flexas, J., *et al.* (2015). Mesophyll conductance to CO₂ and Rubisco as targets for improving
392 intrinsic water use efficiency in C₃ plants. *Plant Cell Environ.* doi: [10.1111/pce.12622](https://doi.org/10.1111/pce.12622).
- 393 Flexas, J. *et al.* (2008). Mesophyll conductance to CO₂: current knowledge and future prospects.
394 *Plant Cell Environ.*, 31, 602-621.
- 395 Franks, P.J. & G.D. Farquhar. (2001). The effect of exogenous abscisic acid on stomatal

- 396 development, stomatal mechanics, and leaf gas exchange in *Tradescantia virginiana*.
397 *Plant Physiology*, 125, 935-942.
- 398 Funk, J. L. *et al.* (2013). Leaf traits within communities: context may affect the mapping of traits
399 to function. *Ecology*, 94, 1893-1897.
- 400 Gago, J. *et al.* (2014). Opportunities for improving leaf water use efficiency under climate
401 change conditions. *Plant Science* 226, 108-119.
- 402 Galmés, J. *et al.* (2011). Physiological and morphological adaptations in relation to water use
403 efficiency in Mediterranean accessions of *Solanum lycopersicum*. *Plant Cell Environ.*,
404 34, 245-260.
- 405 Galmés J. *et al.* (2013). Leaf responses to drought stress in Mediterranean accessions of *Solanum*
406 *lycopersicum*: anatomical adaptations in relation to gas exchange parameters. *Plant Cell*
407 *Environ.*, 36, 920–935.
- 408 Galmés, J. *et al.* (2014). Environmentally driven evolution of Rubisco and improved
409 photosynthesis and growth within the C₃ genus *Limonium* (Plumbaginaceae). *New Phyto.*,
410 203, 989-999.
- 411 Giuliani, R. *et al.* (2013). Coordination of leaf photosynthesis, transpiration, and structural traits
412 in rice and wild relatives (genus *Oryza*). *Plant Physiol.*, 162, 1632-1651.
- 413 Givnish, T. J. (1986). Optimal stomatal conductance, allocation of energy between leaves and
414 roots, and the marginal cost of transpiration. In: *On the economy of plant form and*
415 *function* (ed. Givnish, T.J.). Cambridge University Press, 171-213
- 416 Grime, J. P. (1977). Evidence for the existence of three primary strategies in plants and its
417 relevance to ecological and evolutionary theory. *Am. Nat.*, 111, 1169-1194.
- 418 Haak, D. C. *et al.* (2014). No evidence for phylogenetic constraint on natural defense evolution

- 419 among wild tomatoes. *Ecology*, 95,1633–1641.
- 420 Hadfield, J.D. (2010). MCMC methods for multi-response generalized linear mixed models: The
421 MCMCglmm R Package. *J. Stat. Soft.*, 33, 1-22.
- 422 Hijmans, R. J., S. E. Cameron, J. L. Parra, P. G. Jones, and A. Jarvis. (2005). Very high
423 resolution interpolated climate surfaces for global land areas. *International Journal of*
424 *Climatology*, 25, 1965-1978.
- 425 Ho, L. S. T. & Ane, C. (2014) A linear-time algorithm for Gaussian and non-Gaussian trait
426 evolution models. *Syst. Biol.*, 63, 397-408.
- 427 Kawecki, T.J. & D. Ebert. (2004). Conceptual issues in local adaptation. *Ecol. Lett.*, 7, 1225-
428 1241.
- 429 Kellermann, V. *et al.* (2012). Upper thermal limits of *Drosophila* are linked to species
430 distributions and strongly constrained phylogenetically. *Proc. Natl. Acad. Sci. U.S.A.*,
431 109, 16228-16233.
- 432 Mason, C. M. & L.A. Donovan. (2015). Evolution of the leaf economics spectrum in herbs:
433 Evidence from environmental divergences in leaf physiology across *Helianthus*
434 (Asteraceae). *Evolution*, 69, 2705-2720.
- 435 Medrano, H. *et al.* (2009). Variability in water use efficiency at the leaf level among
436 Mediterranean plants with different growth forms. *Plant and Soil*, 317, 17-29.
- 437 Moyle, L. C. (2008). Ecological and evolutionary genomics in the wild tomatoes (*Solanum* sect.
438 *Lycopersicon*). *Evolution*, 62, 2995-3013.
- 439 Moyle, L. C. *et al.* (2010). Reciprocal insights into adaptation from agricultural and evolutionary
440 studies in tomato. *Evol. App.*, 3, 409-421.

- 441 Muir, C. D. *et al.* (2015). Constraint around Quarter-Power Allometric Scaling in Wild Tomatoes
442 (*Solanum* sect. *Lycopersicon*; Solanaceae). *Am. Nat.*, 186, 421-433.
- 443 Muir, C. D. *et al.* (2014). Morphological and anatomical determinants of mesophyll conductance
444 in wild relatives of tomato (*Solanum* sect. *Lycopersicon*, sect. *Lycopersicoides*;
445 Solanaceae). *Plant Cell Environ.*, 37, 1415-1426.
- 446 Nakazato, T. *et al.* (2008). Environmental factors predict adaptive phenotypic differentiation
447 within and between two wild Andean tomatoes. *Evolution*, 62, 774–792.
- 448 Nobel P.S. (2009) *Physicochemical and Environmental Plant Physiology* 4th edn. Academic
449 Press, London, UK.
- 450 Ogburn R. M. & E.J. Edwards (2010). The Ecological Water-Use Strategies of Succulent Plants.
451 *Adv. Bot. Res.*, 55, 179-225.
- 452 Onoda, Y. *et al.* (2011). Global patterns of leaf mechanical properties. *Ecol. Lett.*, 14, 301-312.
- 453 Parkhurst, D. F. (1994). Diffusion of CO₂ and other gases inside leaves. *New Phytol.*, 126, 449-
454 479.
- 455 Peralta, I. E. *et al.* (2008). The taxonomy of tomatoes: a revision of wild tomatoes (*Solanum*
456 section *Lycopersicon*) and their outgroup relatives in sections *Juglandifolium* and
457 *Lycopersicoides*. *Syst. Bot. Monogr.*, 84, 1-186.
- 458 Perez-Harguindeguy, N. *et al.* (2013). New handbook for standardized measurement of plant
459 functional traits worldwide. *Australian Journal of Botany*. 61, 167-234.
- 460 Pigliucci, M. *et al.* (2003). Phenotypic integration: studying the ecology and evolution of
461 complex phenotypes. *Ecol. Lett.*, 6, 265-272.
- 462 Raiche, G. (2010). nFactors: an R package for parallel analysis and nongraphical solutions to the
463 Cattell scree test. R package version 2.3.3.

- 464 Reich, P. B. (2014). The world-wide ‘fast-slow’ plant economics spectrum: a traits manifesto. *J.*
465 *Ecol.*, 102, 275-301.
- 466 Sack L. *et al.* (2003). The ‘hydrology’ of leaves: co-ordination of structure and function in
467 temperate woody species. *Plant Cell Environ.*, 26, 1343–1356.
- 468 Särkinen, T. *et al.* (2013). A phylogenetic framework for evolutionary study of the nightshades
469 (Solanaceae): a dated 1000-tip tree. *BMC Evol. Biol.*, 13, 214.
- 470 Tholen, D. *et al.* (2012). Opinion: Prospects for improving photosynthesis by altering leaf
471 anatomy. *Plant Sci.*, 197, 92-101.
- 472 Tholen, D. *et al.* (2011). The mechanistic basis of internal conductance: a theoretical analysis of
473 mesophyll cell photosynthesis and CO₂ diffusion. *Plant Physiol.*, 156, 90-105.
- 474 Tomás, M. *et al.* (2013). Importance of leaf anatomy in determining mesophyll diffusion
475 conductance to CO₂ across species: quantitative limitations and scaling up by models. *J.*
476 *Exp. Bot.*, 64, 2269-2281.
- 477 Tosens, T., Ü. Niinemets, V. Vislap, H. Eichelmann & P. C. Díez. (2012) Developmental
478 changes in mesophyll diffusion conductance and photosynthetic capacity under different
479 light and water availabilities in *Populus tremula*: how structure constrains function.
480 *Plant, Cell & Environment*, 35, 839–856.
- 481 Turner, I.M. (1994). Sclerophylly: primarily protective? *Funct. Ecol.*, 8, 669-675.
- 482 Vile, D., E. Garnier, B. Shipley, G. Laurent, M.-L. Navas *et al.*, (2005) Specific leaf area and dry
483 matter content estimate thickness in laminar leaves. *Ann. Bot.*, 96, 1129–1136.
- 484 von Caemmerer, S. (2000). *Biochemical models of leaf photosynthesis*. CSIRO Publishing,
485 Collingwood, Australia.

- 486 Westoby, M. *et al.* (2002). Plant ecological strategies: Some leading dimensions of variation 652
487 between species. *Annu. Rev. Ecol. Syst.*, 33, 125–159.
- 488 Wright, I. J. *et al.* (2004). The worldwide leaf economics spectrum. *Nature*, 428, 821-827.
- 489 Wullschleger, S. D. (1993). Biochemical limitations to carbon assimilation in C₃ plants—a
490 retrospective analysis of the A/Ci curves from 109 species. *J. Exp. Bot.*, 44, 907-920.
- 491

492 **Table 1:** Diffusional and biochemical constraints drive variation in photosynthetic rate (A_N) and
 493 intrinsic water-use efficiency ($iWUE$) among tomato species. For both A_N and $iWUE$,
 494 phylogenetic linear mixed effects models including ‘Species’ as a random effect improved model
 495 fit (lower Deviance Information Criterion [DIC]) over nonphylogenetic linear models, indicating
 496 some effect of phylogenetic relatedness. Greater stomatal (g_s) and mesophyll (g_m) conductance
 497 significantly increased A_N but had opposing effects on $iWUE$. Likewise, greater maximum
 498 carboxylation rates (V_{cmax}) increased both A_N and $iWUE$. Parameters were estimated from the
 499 mode of 10^4 samples drawn from the posterior distribution using MCMCglmm. The effects of g_s ,
 500 g_m , and V_{cmax} are highly significant (95% high posterior density [HPD] intervals do not overlap
 501 zero) in all models.

Response	Fixed	Random		Parameter	95% HPD	
Variable	Effects	Effects	DIC	estimate	Interval	
$\log(A_N)$	$\log(g_s)$			$\log(g_s)$	0.39	0.35 – 0.43
	$\log(g_m)$	(none)	-233.4	$\log(g_m)$	0.31	0.28 – 0.34
	V_{cmax}			V_{cmax}	9.7×10^{-3}	$7.2 - 12.5 \times 10^{-3}$
	$\log(g_s)$			$\log(g_s)$	0.36	0.32 – 0.40
	$\log(g_m)$	Species	-250.5	$\log(g_m)$	0.32	0.28 – 0.35
	V_{cmax}			V_{cmax}	9.2×10^{-3}	$6.4 - 12.0 \times 10^{-3}$
$\log(iWUE)$	$\log(g_s)$			$\log(g_s)$	-0.61	-0.64 – -0.57
	$\log(g_m)$	(none)	-233.1	$\log(g_m)$	0.31	0.28 – 0.34
	V_{cmax}			V_{cmax}	9.7×10^{-3}	$7.1 - 12.3 \times 10^{-3}$
	$\log(g_s)$			$\log(g_s)$	-0.64	-0.67 – -0.60
	$\log(g_m)$	Species	-250.5	$\log(g_m)$	0.32	0.28 – 0.35

V_{cmax}

V_{cmax}

9.2×10^{-3}

$6.3 - 12.0 \times 10^{-3}$

502

503 **Table 2.** Comparison of the Rubisco kinetics at 25°C for the two Rubisco LSu types detected

504 among wild and domesticated tomatoes based on the amino acid sequence, namely LSu types 1

505 and 2. Parameters, measured in a representative species of each Rubisco type, describe the

506 Michaelis-Menten constant for CO₂ under 0% O₂ (K_c) and 21% O₂ (K_c^{air}), the maximum rates of

507 carboxylation (k_{cat}^c) and oxygenation (k_{cat}^o), carboxylation catalytic efficiency (k_{cat}^c/K_c), and

508 specificity factor ($S_{c/o}$). Data are means \pm SE of four replicates per Rubisco type. Significant

509 differences between both Rubisco types are indicated by the ANOVA P -value, * <0.05; **

510 <0.01.

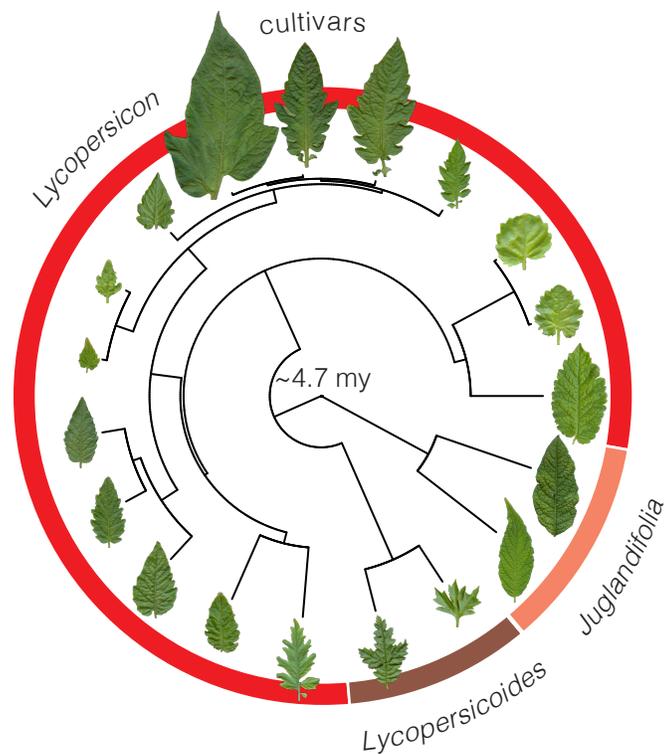
511

Rubisco LSu type	1	2	P value
Species measured	<i>S. lycopersicoides</i>	<i>S. lycopersicum</i>	
K_c (mM)	9.3 ± 0.9	8.2 ± 0.7	0.308
K_c^{air} (mM)	16.6 ± 0.8	13.5 ± 0.8	0.020*
k_{cat}^c (s ⁻¹)	2.430 ± 0.138	3.142 ± 0.518	0.176
k_{cat}^o (s ⁻¹)	0.892 ± 0.099	1.359 ± 0.108	0.010**
k_{cat}^c/K_c (s ⁻¹ mM ⁻¹)	0.261 ± 0.026	0.391 ± 0.075	0.127
$S_{c/o}$ (mol mol ⁻¹)	99.4 ± 3.5	115.0 ± 2.1	0.007**

512

513

514 **Figures**



515

516 **Figure 1:** Wild tomatoes and cultivars are closely-related, yet phenotypically-diverse taxa.

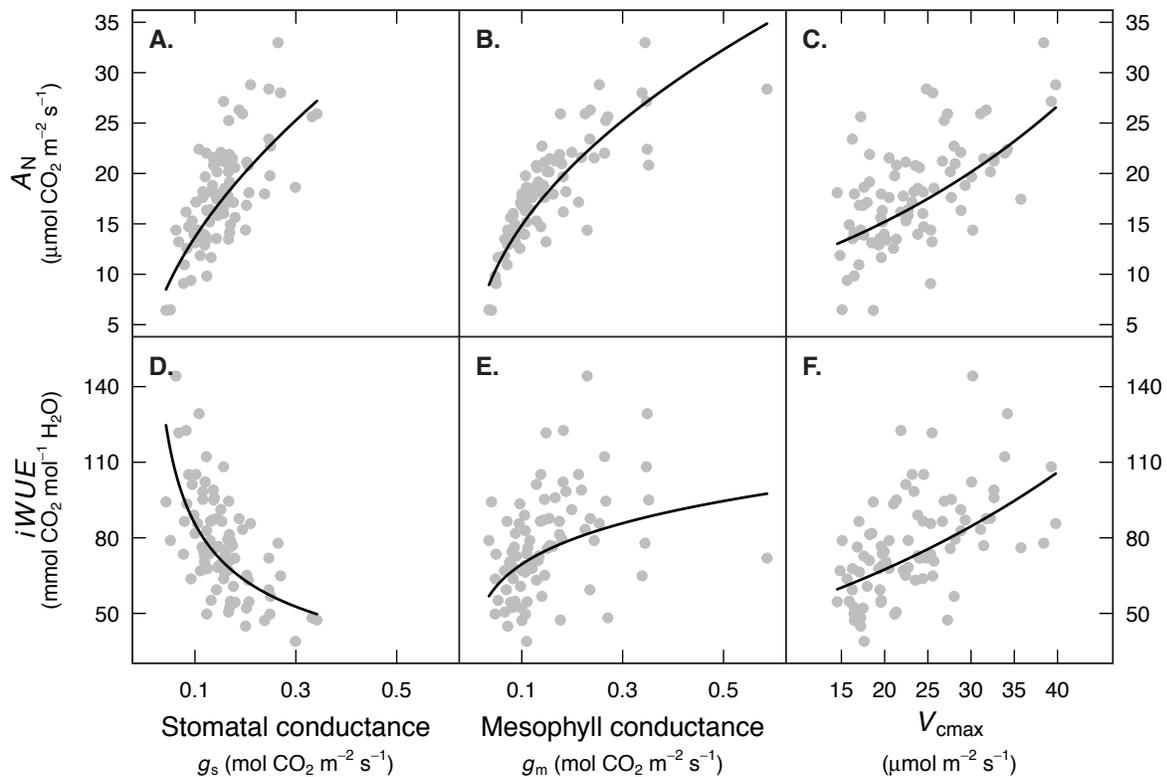
517 Among species investigated here, the oldest split is approximately 4.7 my based on a fossil-

518 calibrated phylogeny (Särkinen *et al.* 2013). Functional traits like terminal leaflet size, depicted

519 for each species at the tips of the phylogeny, as well as leaf physiological and structural traits

520 used in this study vary widely among species.

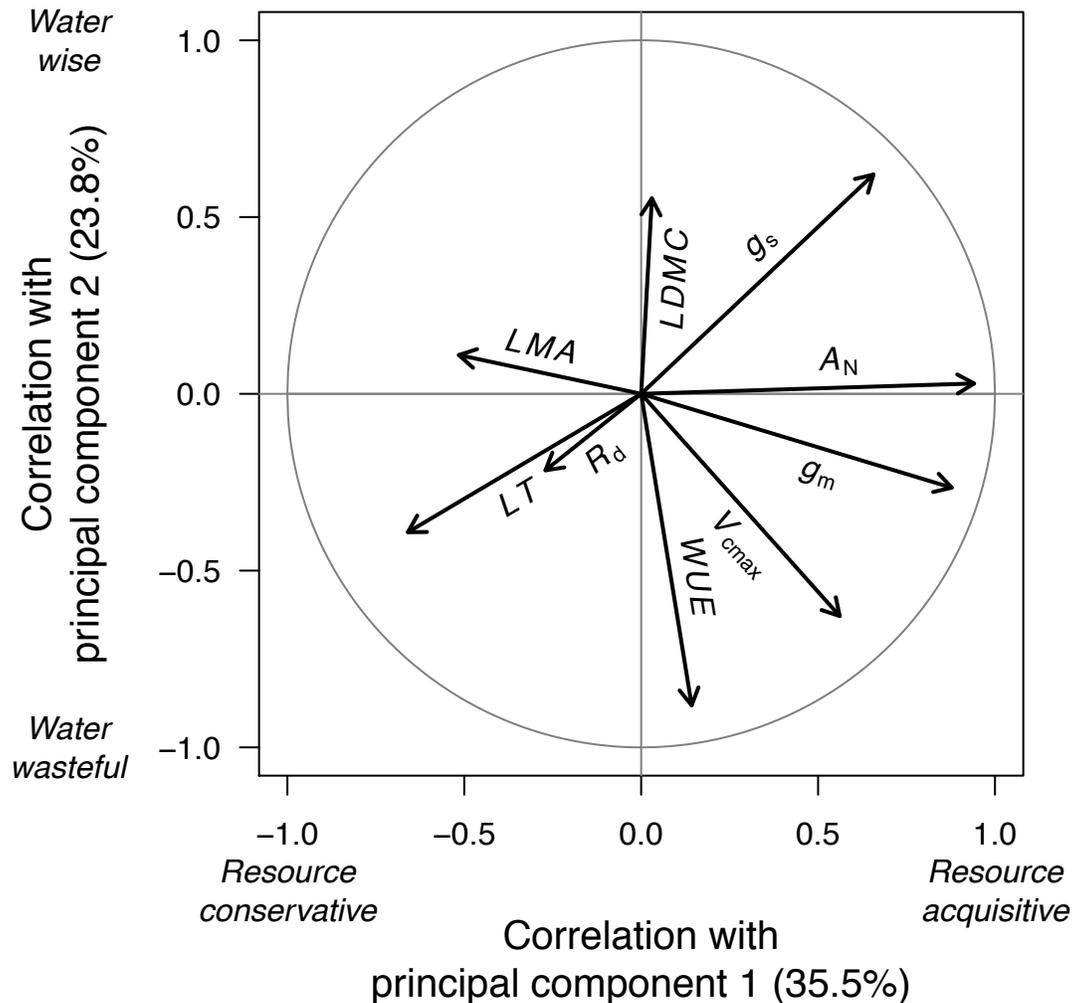
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523 **Figure 2:** Leaf CO₂ diffusion and biochemistry limit net CO₂ assimilation rates (A_N) and alter
 524 intrinsic water-use efficiency ($iWUE$). Each point is data from one of 82 individual plants across
 525 19 wild and cultivated tomato taxa. Panels A. and B. show that faster diffusion through stomata
 526 (g_s) and mesophyll (g_m) resulted in higher A_N . However, these parameters had opposing effects
 527 on $iWUE$. Greater g_s lowered $iWUE$ (panel D.), whereas greater g_m increased $iWUE$ (panel E.).
 528 Leaf biochemistry, specifically the maximum rate of carboxylation (V_{cmax}) also increased A_N
 529 (panel C.) and $iWUE$ (panel F.). Fitted lines are based on a Bayesian phylogenetic mixed models
 530 treating Species as a random effect: $\log(A_N) \sim \log(g_s) + \log(g_m) + V_{\text{cmax}} + (1|\text{Species})$ and
 531 $\log(iWUE) \sim \log(g_s) + \log(g_m) + V_{\text{cmax}} + (1|\text{Species})$. For visual aid, lines were drawn to
 532 account for positive covariation between g_s , g_m , and V_{cmax} (data not shown). For example, panel

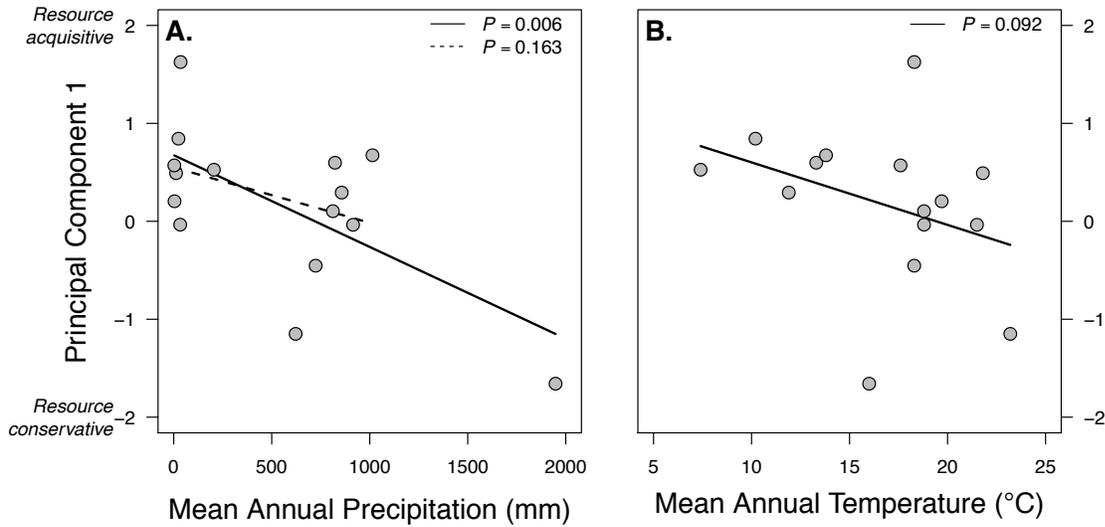
533 A. shows the predicted A_N at a given g_s with g_m and V_{cmax} set to the predicted value at a given g_s .
534 All slopes were significantly different than zero ($P < 0.0001$) based on 10^4 MCMC samples from
535 the posterior distribution of the model.
536



537

538 **Figure 3:** Major axes of leaf trait variation in tomato. The first axis, principal component 1,
539 explains 35.5% of the total variation. It delineates plants along a continuum from resource
540 acquisitive (higher PC1, greater A_N , thinner leaves) to resource conservative (lower PC1, lower
541 A_N , thicker leaves). The second axis, principal component 2, explains 23.8% of the trait variation
542 and delineates an axis from more water wise (higher PC2, greater $iWUE$) to water wasteful
543 (lower PC2, lower $iWUE$). The arrows are vectors showing the correlation across individual
544 plants between a trait and both principal components. For example, A_N is positively correlated

545 with PC1, but uncorrelated with PC2. The grey circle shows the outer possible set of correlation
546 combinations. Abbreviations: R_{dark} = dark respiration rate; $iWUE$ = intrinsic water-use efficiency;
547 V_{cmax} = maximum rate of carboxylation; g_m = mesophyll conductance; A_N = net CO₂ assimilation
548 rate; g_s = stomatal conductance; $LDMC$ = leaf dry matter content; LMA = leaf mass per area; LT
549 = leaf thickness.
550



551

552 **Figure 4:** The resource conservative to acquisitive axis of leaf trait variation was uncorrelated

553 with mean annual precipitation (A.) and temperature (B.). Each point shows the climate at

554 habitat of origin for a different wild tomato taxon and its position, averaged from multiple

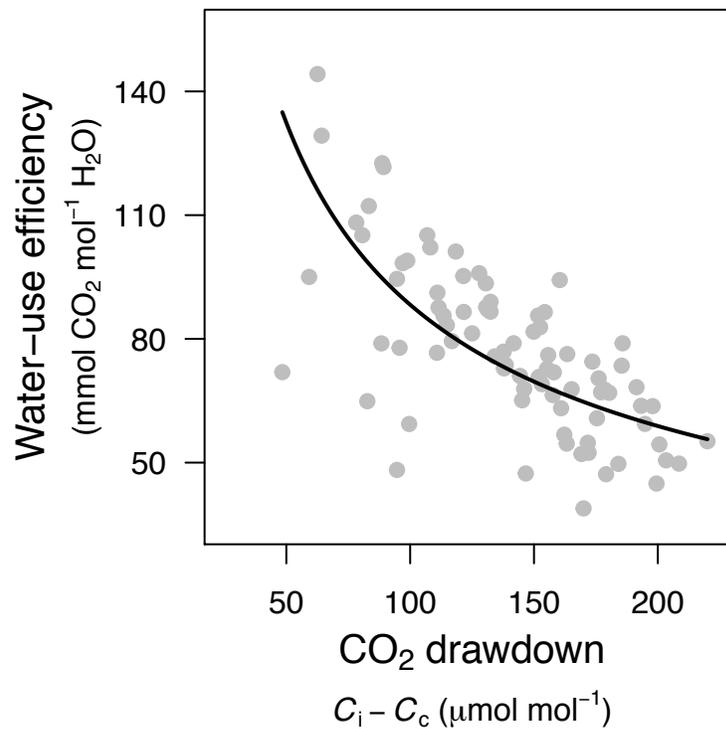
555 individuals in the experiment, along the resource acquisitive to conservative axis. There was a

556 significant positive correlation between PC1 and precipitation, but this was driven by a single

557 influential species, *S. juglandifolium*; the correlation was not significant once this species was

558 removed. All P -values are from phylogenetic linear regression.

559



560

561 **Figure S1:** CO₂ drawdown from the leaf interior (C_i) to the chloroplast (C_c) is negatively
562 associated with $iWUE$ in tomatoes, indicating that reduced diffusional constraints (lower $C_i - C_c$)
563 leads to increased $iWUE$. Fitted line is based on a Bayesian phylogenetic mixed models treating
564 Species as a random effect: $\log(iWUE) \sim \log(C_i - C_c) + (1|Species)$. The slope is significantly
565 different than zero ($P < 0.0001$) based on 10^4 MCMC samples from the posterior distribution of
566 the model.

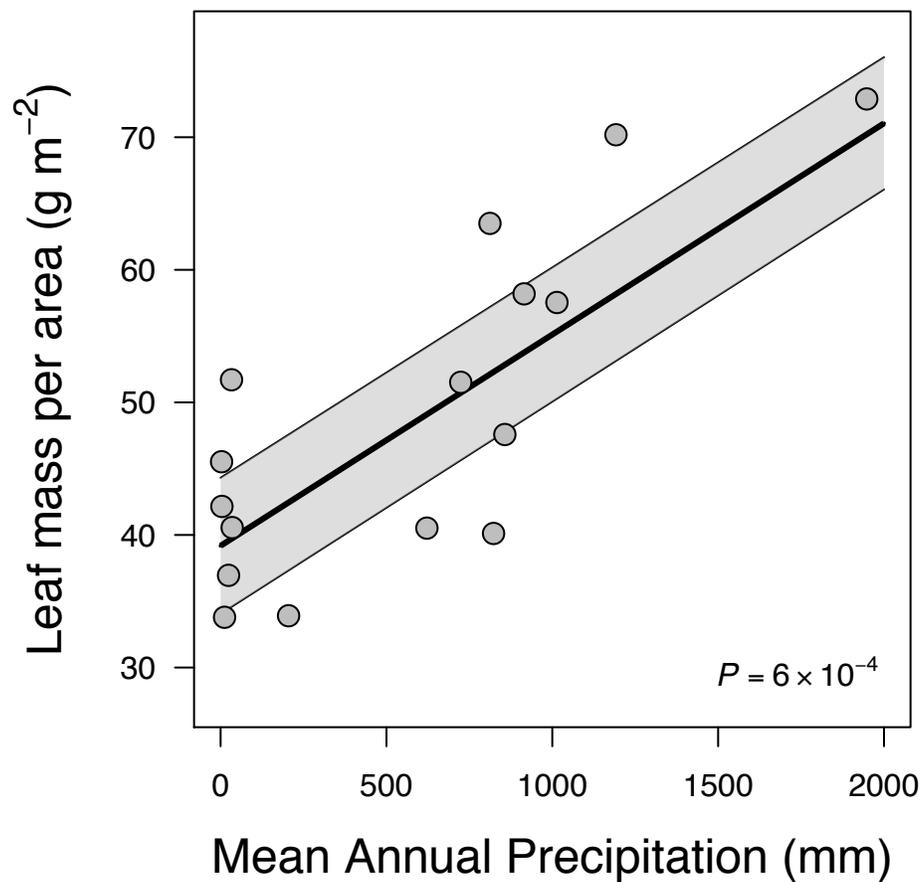
567

Taxon	Nucleotide					Amino acid		
	81	255	573	688	822	Haplotype	230	LSu type
<i>S. lycopersicoides</i>	T	C	A	G	C	1	A	1
<i>S. sitiens</i>	T	C	A	G	C	1	A	1
<i>S. ochranthum</i>	G	T	A	G	C	2	A	1
<i>S. juglandifolium</i>	G	T	G	G	C	3	A	1
<i>S. pennellii</i>	T	C	A	G	C	1	A	1
<i>S. pennellii</i> var. <i>puberulum</i>	T	C	A	G	C	1	A	1
<i>S. habrochaites</i>	T	C	A	A	C	5	T	2
<i>S. chilense</i>	T	C	A	G	C	1	A	1
<i>S. peruvianum</i>	T	C	A	G	C	1	A	1
<i>S. arcanum</i>	T	C	A	G	C	1	A	1
<i>S. neorickii</i>	T	C	A	G	C	1	A	1
<i>S. chmielewskii</i>	T	C	A	G	C	1	A	1
<i>S. pimpinellifolium</i>	T	C	A	A	T	4	T	2
<i>S. cheesmaniae</i>	T	C	A	A	T	4	T	2
<i>S. galapagense</i>	T	C	A	A	T	4	T	2
<i>S. lycopersicum</i> var. <i>cerasiforme</i>	T	C	A	A	T	4	T	2
Ramellet tomato 1-48	T	C	A	A	T	4	T	2
Ramellet tomato 1-30	T	C	A	A	T	4	T	2
Commercial tomato 'Roma VF'	T	C	A	A	T	4	T	2

568

569 **Figure S2:** Evolutionary patterns of the Rubisco large subunit (LSu) among wild and
 570 domesticated tomato species. The species relationships shown are based on an 18-gene
 571 phylogeny from Haak *et al.* (2014; see Methods). Nucleotide differences in the *rbcL* gene are
 572 indicated, giving rise to five different haplotypes. Those mutations result in a single difference at
 573 amino acid level and thus two Rubisco LSu types within tomatoes.

574



575

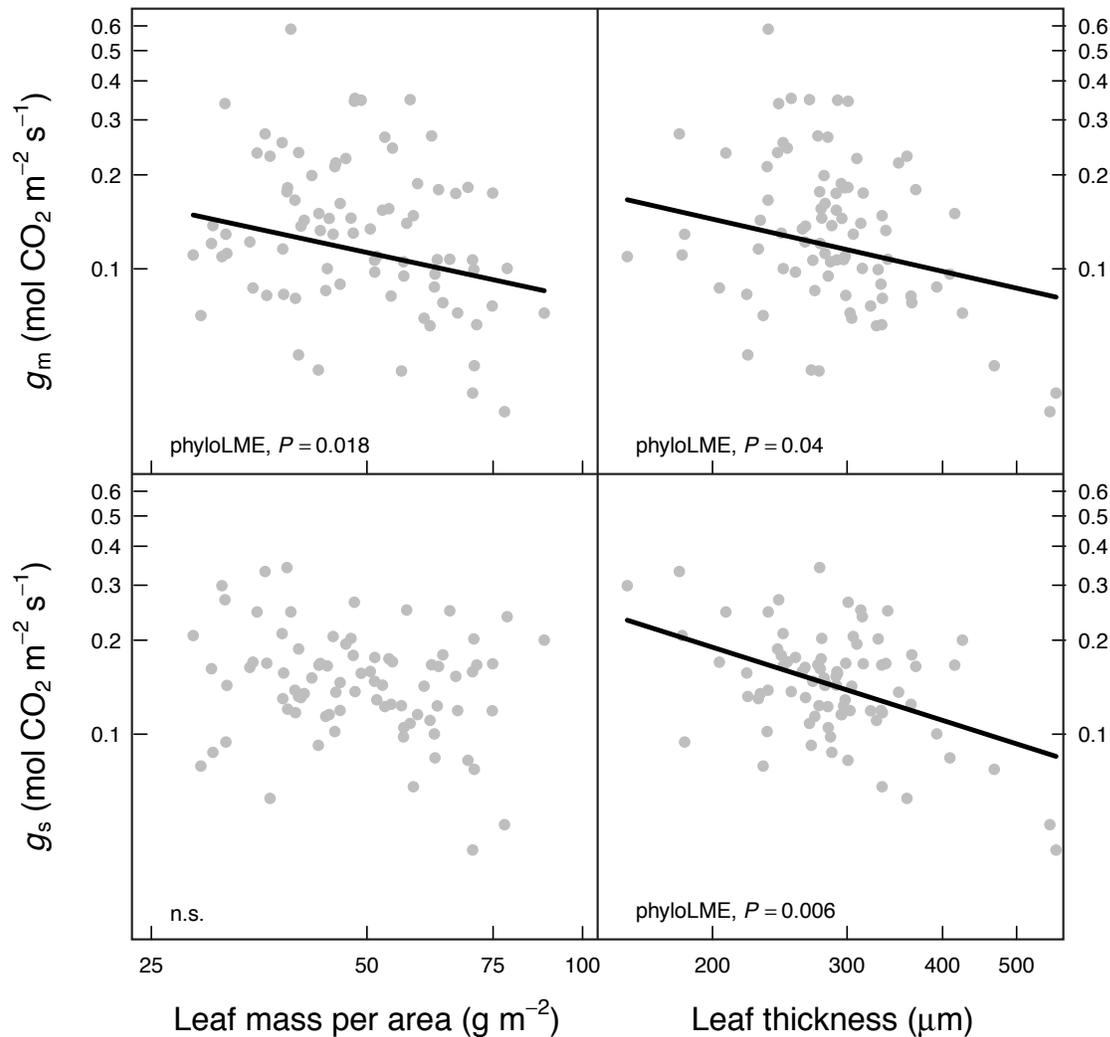
576 **Figure S3:** Leaf mass per area (LMA) was positively correlated with mean annual precipitation.

577 Each point shows the climate at habitat of origin for a different wild tomato taxon and its LMA ,

578 averaged from multiple individuals in the experiment. There was a significant positive

579 correlation between PC1 and precipitation. All P -values are from phylogenetic linear regression.

580



581

582 **Figure S4:** Bulk leaf structure (leaf mass per area [*LMA*] and leaf thickness [*LT*]) weakly
583 constrain leaf CO₂ diffusive conductance through stomata (*g_s*) and mesophyll (*g_m*). Each point is
584 data from one of 80 individual plants across 19 wild and cultivated tomato taxa. Panels A. and B.
585 show that diffusion through mesophyll (*g_m*, mesophyll conductance) was limited by higher *LMA*
586 and *LT*. Additionally, *LT* (panel D.), but not *LMA* (Panel C.) were associated with decreased
587 stomatal conductance (*g_s*) parameters. Fitted lines are based on Bayesian phylogenetic mixed

588 models treating Species as a random effect. Mixed models with *LT* also included *LDMC*, but this
589 did not have a significant effect (results not shown). All *P*-values are based on 10^4 MCMC
590 samples from the posterior distribution of the model.
591

592 **Methods S1**

593 *Gas exchange*

594 We used an open path infrared gas exchange analyzer with a 2-cm² leaf chamber fluorometer
595 (LI-6400-40, LI-COR Inc., Lincoln, NE, USA) to simultaneously measure leaf gas exchange and
596 chlorophyll *a* fluorescence. To minimize leaf position and age effects, all measurements were
597 made on young, fully-expanded leaves ($\bar{n} = 4.3$, range = [3, 5]). The ambient CO₂ concentration
598 in the chamber (C_a) was 400 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ air, leaf temperature was 25° C, photosynthetic
599 photon flux density (PPFD) was 1500 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ with 90:10 red:blue light, and relative
600 humidity was between 40 – 60%. Once a leaf reached steady-state photosynthesis (A) and
601 stomatal conductance (g_s), usually after ~30 min, we measured the response to changing
602 substomatal CO₂ concentrations (C_i) by adjusting the ambient CO₂ concentration in the leaf
603 chamber (C_a). We used 11 concentrations between 0 and 1750 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ air. The flow rate
604 was 300 $\mu\text{mol s}^{-1}$. Diffusional leaks for CO₂ were corrected for using the methods of Rodeghiero
605 *et al.* (2007). We observed no differences between diffusion coefficients calculated for leaves of
606 different species or an empty chamber (data not shown).

607 From fluorescence measurements and $A - C_i$ curves, we determined the net CO₂
608 assimilation rate at $C_a = 400 \mu\text{mol CO}_2 \text{ mol}^{-1}$ air (A_N), stomatal conductance (g_s), mesophyll
609 conductance to CO₂ (g_m), and the maximum rate of carboxylation (V_{cmax}). For all analyses, we
610 used g_s and g_m at ambient C_a (400 +/- 5 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ air) because these values are most
611 ecologically relevant and allowed us to directly analyze how g_s and g_m limited A_N . Photosynthetic
612 rate and stomatal conductance are estimated directly from gas exchange measurements. We
613 estimated mesophyll conductance using from the equation (Harley *et al.* 1992):

$$g_m = \frac{A}{C_i - \frac{\Gamma^*[J+8(A+R_d)]}{J-4(A+R_d)}} \quad (\text{S1})$$

614 We measured the mitochondrial respiration rate (R_{dark}) for each plant at predawn and used the
615 common assumption $R_d = R_{\text{dark}} / 2$. As described below, we measured the chloroplastic CO_2
616 compensation point (Γ^*) *in vitro* from two species, *S. lycopersicoides* and *S. lycopersicum* var.
617 *esculentum* cv. ‘Roma’, one each from the two LSu amino acid sequences among tomato species
618 (see Results). For *S. lycopersicoides* (Rubisco LSu Type 1, Figure S2), we estimated $\Gamma^* = 40.46$
619 $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$; for *S. lycopersicum* (Rubisco LSu Type 2, Figure S2) we estimated $\Gamma^* =$
620 $35.39 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$. These values were applied to the other species with the same Rubisco
621 LSu Type (Figure S2).

622 The electron transport rate of photosystem II (J_f) was calculated from fluorescence
623 measurements as $J_f = \Phi_{\text{PSII}} I \alpha \beta$, where Φ_{PSII} is the quantum yield (moles of CO_2 fixed per mole
624 of quanta absorbed) of photosystem II, I is irradiance, α is the leaf light absorptance, and β is the
625 photosystem partitioning factor. Φ_{PSII} was estimated from the chlorophyll fluorescence data. We
626 estimated the product $\alpha\beta$ separately for each species using the relationship $\Phi_{\text{PSII}} = \frac{\alpha\beta\Phi_{\text{CO}_2}}{4}$
627 under nonphotorespiratory conditions (Genty, Briantis & Baker, 1989). To vary the quantum
628 yield, we measured photosynthetic light response curves under 2% O_2 . In summary, we
629 incorporated data on species-specific respiration (R_d), Rubisco kinetics (Γ^*), and light
630 absorptance/photosystem partitioning ($\alpha\beta$) to improve the accuracy of g_m estimates.

631 Using the calculated values of g_m , we used the relationship:

$$C_c = C_i - A/g_m \quad (\text{S2})$$

632 to determine $A - C_c$ curves to estimate the maximum velocity of carboxylation (V_{cmax} [$\mu\text{mol m}^{-2}$
633 s^{-1}]), which is proportional to the concentration of activated Rubisco. We estimated V_{cmax}

634 following Long & Bernacchi (2003) from the linear, Rubisco limited portion of the $A - C_c$ curve,
635 empirically determined to be $C_i < 200 \text{ mol CO}_2 \text{ mol}^{-1} \text{ air}$. We used kinetic values from Table 2
636 (methods described below) to calculate effective Michaelis-Menten constants for each LSu type.

637

638 *Sequencing the Rubisco large subunit*

639 Total DNA was extracted from fresh leaf tissue using the DNeasy Plant MiniKit (Qiagen). The
640 *rbcL* gene coding for the Rubisco large subunit (LSu) was amplified using primers specifically
641 designed for *Solanum*, the forward primer SoSpaF (5'-ATGAGTTCTAGGGAGGGAT-3') and
642 the reverse primer So1462R: (5'-GCAGGAAATAAAGAAGGATAAGG-3'). The BioMix Red
643 reaction mix (Bioline Ltd., London, UK) was used to carry out the polymerase chain reaction
644 (PCR) with the following conditions: an initial cycle of 95 °C, 2 min; 55 °C, 30 s; 72 °C, 4 min,
645 followed by 36 cycles of 93 °C, 30 s; 53 °C, 30 s; 72 °C, 3.5 min. PCR products were visualized
646 on 1% agarose gels, purified using the High Pure PCR Product Purification Kit (Roche,
647 Germany) and sequenced with an ABI 3130 Genetic analyzer using the ABI BigDye™
648 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California).
649 Due to the *rbcL* length, sequences were obtained in a two-fragment fashion, using the primer
650 So1462R and the primer *rbcL*638R (5'-CGCATAAATGGTTGGGAATTC-3') (Chen 1998).
651 Sequence chromatograms were checked and corrected with the Chromas software and the
652 contigs were assembled using BioEdit v7.1.3 software (Hall 1999). MEGA 5 (Tamura *et al.*
653 2011) was used to convert DNA to amino acid, and to align both sequence types.

654

655 *Rubisco catalytic characterization*

656 Based on the two different amino acid sequences detected, a representative species for each type
657 was selected, *S. lycopersicoides* for Rubisco LSu Type 1, and a domesticated accession *S.*
658 *lycopersicum* var. *esculentum* cv. ‘Roma’ for the Rubisco LSu Type 2. We characterized Rubisco
659 kinetics following Galmés *et al.* (2014). Rates of Rubisco $^{14}\text{CO}_2$ fixation using fresh leaf protein
660 extract were measured in 7 ml septum-capped scintillation vials, containing reaction buffer
661 (yielding final concentrations of 100 mM Bicine-NaOH, pH 8.0, 20 mM MgCl_2 , 0.4 mM RuBP
662 and ca. 100 W-A units of carbonic anhydrase) and one of nine different concentrations of CO_2
663 (0-80 μM , each with a specific radioactivity of 3.7×10^{10} Bq mol^{-1}), each at two concentrations
664 of O_2 (0 and 21% v/v), as described previously (Parry *et al.* 2007). Assays (1.0 ml total volume)
665 were started by the addition of activated leaf extract, and the maximum velocity of carboxylase
666 activity (V_{max}) together with the Michaelis-Menten constant (K_m) for CO_2 (K_c) determined from
667 the fitted data. The K_m for the oxygenase activity was calculated from the relationship $K_{c,(21\%\text{O}_2)}$
668 $= K_{c,(0\%\text{O}_2)} \cdot (1 + [\text{O}_2]/K_o)$. The $[\text{O}_2]$ was assumed to be 265 μM , but corrected for partial pressure
669 by taking account of the atmospheric pressure and water saturated vapor pressure. Replicate
670 measurements ($n = 4$) were made using protein preparations from different leaves of different
671 individuals. For each sample, the maximum rate of carboxylation (k_{cat}^c) was extrapolated from
672 the corresponding V_{max} value after allowance was made for the Rubisco active site concentration,
673 as determined by ^{14}C CPBP binding (Yokota & Canvin 1985). Rubisco CO_2/O_2 specificity ($S_{c/o}$)
674 was measured as described previously (Galmés *et al.* 2005) using enzyme purified by
675 polyethylene glycol (PEG) precipitation and ion exchange chromatography, and the values given
676 for each species were the mean of 7-8 repeated determinations. The maximum oxygenation rate
677 (k_{cat}^o) was calculated using the equation $S_{c/o} = (k_{\text{cat}}^c / K_c) / (k_{\text{cat}}^o / K_o)$. All kinetic measurements
678 were performed at 25°C.

679

680 **Results S1: Sequence comparisons reveal two Rubisco LSu types in tomatoes**

681

682 We identified five mutations in the *rbcL* gene sequence (Figure S2), encoding for the Rubisco
683 LSu. However, only one of these five mutations altered the amino acid sequence. The limited
684 variability in LSu among tomato species contrasts with previous studies reporting large
685 variability among closely related taxa in *Limonium* (Galmés *et al.* 2014), *Schiedea* (Kapralov &
686 Filatov 2006), *Amanthaceae* (Kapralov *et al.* 2012), and *Quercus* (Hermida-Carrera *et al.* unpub.
687 data). The single mutation leading to a different amino acid sequence for the LSu at position 230
688 (Figure S2) resulted in improved $S_{c/o}$ (i.e., higher specificity of the enzyme for CO₂), but did not
689 reduce the enzyme velocity (k_{cat}^c ; Table 2). The same amino acid replacement A230 to T230 was
690 observed in the Hawaiian endemic genus *Schiedea* (Kapralov & Filatov 2006). *Schiedea*
691 represents one of the largest plant adaptive radiations on Hawaii and, similarly to the wild
692 species of tomato in the present study, comprises closely related species with a broad range of
693 morphological and ecological forms, inhabiting diverse environments from rainforest to desert-
694 like conditions. According to Kellogg & Juliano (1997), residue 230 interacts with the βA - βB
695 loop of small subunit, and replacement A230 to T230 causes a decrease in the hydrophobicity of
696 the residue with effects in the stability at this position.

697 We designate the sequence associated with lower $S_{c/o}$ ‘Rubisco LSu Type 1’ and that with
698 higher $S_{c/o}$ ‘Rubisco LSu Type 2’. Interestingly, the Rubisco LSu Type 2 occurs in the closest
699 wild relatives of the domesticated tomato (i.e., from here on “the domesticated clade”: *S.*
700 *pimpinellifolium*, *S. cheesmaniae*, *S. galapagense*, *S. lycopersicum* var. *cerasiforme*, and the
701 domesticated accessions; see Figure S2), maybe pointing to this biochemical improvement at

702 Rubisco level to one of the reasons triggering the diversification of the most recent tomato
703 species, and from which the domesticated tomato was originated. Also, the lack of DNA
704 mutations in the *rbcL* among those species could reflect a very recent occurrence of this Rubisco
705 LSU type.

706 The phylogenetic relationship among sections *Lycopersicoides*, *Juglandifolia* and
707 *Lycopersicon* is consistent both based on nuclear markers and cpDNA markers (Peralta *et al.*
708 2008; Robertson *et al.* 2011), and indicates that sect. *Lycopersicoides* diverged before sect.
709 *Juglandifolia* (Figure 1). Therefore, since the cpDNA of sect. *Lycopersicon* is identical to that of
710 sect. *Lycopersicoides* but different to that of sect. *Juglandifolia*, mutations in the latter section
711 may have occurred after the split from sect. *Lycopersicon*, which maintained the original sect.
712 *Lycopersicoides* cpDNA. Therefore, sect. *Juglandifolia* shows two exclusive mutations (in
713 positions 81 and 255), and within *S. juglandifolium* has a third one (in position 573; Figure S2),
714 which shows that this section has the most divergent *rbcL* sequences among tomatoes. However,
715 these mutations did not alter the amino acid sequence of the LSU, suggesting that negative
716 selection on the majority of nonsynonymous substitutions.

717 As an exception to the above is *S. habrochaites*, which diverged early on in the sect.
718 *Lycopersicon* clade with *S. pennellii*. This species has the Rubisco LSU Type 2, but a different,
719 exclusive haplotype as compared to that in the domesticated clade. Therefore, it appears that the
720 mutation in the DNA position 688, resulting in the amino acid change leading to Rubisco LSU
721 Type 2, may have occurred independently in *S. habrochaites* and in the domesticated clade
722 species. Evidence for convergence is reinforced by the mutation in the DNA position 822, which
723 occurs exclusively in all the domesticated clade species, whereas the *S. habrochaites* haplotype
724 resembles other species in the Rubisco LSU Type 1 at this position. This apparently convergent

725 origin of the mutation leading to an improved Rubisco would reflect 1) the importance of
726 maintaining the ancestral (i.e., Type 1, sect. *Lycopersicoides*) Rubisco LSU across the tomato
727 radiation, because it has been maintained in most species, and 2) little positive selection on the
728 LSU, because only a single amino acid change was detected, in the amino acid position 230, and
729 moreover, it may have occurred twice.

730

731 **References S1**

- 732 Galmés J., J. Flexas, A.J. Keys, J. Cifre, R.A.C. Mitchell, P.J. Madgwick, R.P. Haslam, H.
733 Medrano, M.A.J. Parry. (2005). Rubisco specificity factor tends to be larger in plant
734 species from drier habitats and in species with persistent leaves. *Plant, Cell &*
735 *Environment*, 28, 571–579.
- 736 Galmés, J. *et al.* (2014). Environmentally driven evolution of Rubisco and improved
737 photosynthesis and growth within the C₃ genus *Limonium* (Plumbaginaceae). *New Phyto.*,
738 203, 989-999.
- 739 Genty B., J.M. Briantais, N.R. Baker. (1989). The relationship between the quantum yield of
740 photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica*
741 *et Biophysica Acta* 990, 87–92.
- 742 Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis
743 program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.
- 744 Harley, P.C., F. Loreto, G. Di Marco, T.D. Sharkey. (1992). Theoretical considerations when
745 estimating the mesophyll conductance to CO₂ flux by analysis of the response of
746 photosynthesis to CO₂. *Plant Physiology*, 98, 1429–1436.

- 747 Kellogg E.A. & N.D. Juliano. (1997). The structure and function of RuBisCO and their
748 implications for systematic studies. *Am J Bot*, 84, 413–428.
- 749 Kapralov, M.V. & D.A. Filatov. (2006). Molecular adaptations during adaptive radiation in the
750 Hawaiian endemic genus *Schiedea*. PLoS ONE, 1, e8.
- 751 Kapralov M.V., J.A.C. Smith, D.A. Filatov. (2012). Rubisco evolution in C₄ eudicots: an
752 analysis of Amaranthaceae *sensu lato*. PLoS ONE, 7, e52974.
- 753 Parry, M.A.J., P.J. Madgwick, J.F.C. Carvalho, P.J. Andralojc. (2007). Prospects for increasing
754 photosynthesis by overcoming the limitations of Rubisco. *Journal of Agricultural Science*
755 145, 31–43.
- 756 Peralta, I.E. *et al.* (2008). The taxonomy of tomatoes: a revision of wild tomatoes (*Solanum*
757 section *Lycopersicon*) and their outgroup relatives in sections *Juglandifolium* and
758 *Lycopersicoides*. *Syst. Bot. Monogr.*, 84, 1-186.
- 759 Robertson, K.A., E.E. Goldberg, B. Igić. (2011). Comparative evidence for the correlated
760 evolution of polyploidy and self-compatibility in Solanaceae. *Evolution* 65, 139-155.
- 761 Tamura K.D., D. Peterson, N. Peterson, G. Strecher, M. Nei, S. Kumar. (2011). MEGA5:
762 Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary
763 distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28, 2731–
764 2739.
- 765 Yokota A. & D.T. Canvin. (1985). Ribulose bisphosphate carboxylase/oxygenase content
766 determined with [¹⁴C] carboxypentitol bisphosphate in plants and algae. *Plant*
767 *Physiology*, 77, 735–739.
- 768
- 769