Adaptive divergence generates distinct plastic responses in two closely related *Senecio* species

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Abstract

Organisms rely on plasticity to track environmental variation within their native range. However, it remains unclear how adaptation and plasticity interact, and how adaptive divergence affects the evolution of plasticity. To test for variation in plastic responses among two closely related but ecologically divergent ragwort species (*Senecio,* Asteraceae), we sampled c.40 genotypes of each species from natural populations. We then transplanted multiple clones of each genotype into four field sites along an elevational gradient representing each species' native range, the edge of their range, and conditions outside their native range. At each transplant site, we quantified survival, growth, leaf investment, leaf morphology, chlorophyll fluorescence and gene expression. Both species performed better at their home sites, but the high elevation species showed lower tolerance to conditions outside its range than the low elevation species, suggesting stronger specialisation to the high elevation habitat. The two species also differed substantially in the direction of phenotypic and gene expression change across elevation, suggesting that distinct plastic responses have rapidly evolved in these two species. Adaptive divergence has led to the evolution of distinct plastic responses to environmental variation with distinct genomic architectures, despite these two species having shared a recent common ancestry.

Keywords: adaptation, differential gene expression, environmental sensitivity, evolutionary history, genotype-by-environment interactions, phenotypic plasticity, physiological plasticity, specialisation

Introduction

The resilience of natural populations and communities to novel or changing environments relies on the ability of genotypes to adjust their phenotype to track local conditions (Chevin et al. 2010). Phenotypic plasticity generates different phenotypes from the same genotype depending on the environment to which it is exposed (Via et al. 1995; Ghalambor et al. 2007; Charmantier et al. 2008). The ability for plasticity to track environmental variation is shaped by selection within environments routinely experienced (Ghalambor et al. 2007). Plasticity therefore evolves to buffer populations in response only to particular environmental regimes (Bradshaw 1965; Schlichting 1986; Baythavong and Stanton 2010), which can increase ecological specialisation when populations adapt to highly predictable environments (Poisot et al. 2011). Characterising plasticity in closely related, but ecologically divergent species can test to what extent adaptive divergence and ecological speciation is also associated with divergence in phenotypic plasticity.

The effect of adaptation on the nature of plastic responses will depend on how plasticity and selection interact (de Jong 2005). Phylogenetic relatedness (Pigliucci et al. 1999; Kellermann et al. 2018), ecology (Kulkarni et al. 2011) and the predictability of the environment (Oostra et al. 2018) can determine the nature and amount of variation in plastic responses. We would predict that contrasting environments select for different forms and different magnitudes of plasticity, but it is not known to what extent a shared common ancestry will constrain such divergence in plasticity. We would also predict that plasticity can maintain fitness within a narrower range of environmental variation in species that are more specialised to a particular environment, reflecting a narrower range of ecological tolerances in such species (Lortie and Aarssen 1996; Debat and David 2001). However, few studies have assessed whether adaptation to contrasting environments causes not only adaptive divergence, but also divergence in plastic responses when exposed to the same environmental variation.

Plasticity can evolve where genotypes vary in their level of plasticity (Lande 2009; Chevin and Lande 2011). Genotypes that vary in their sensitivity to environmental variation show genotype-specific responses (reaction norms) that reflect genotype-by-environment interactions (G×E) underlying plastic responses (de Jong 2005; Pigliucci 2005; Josephs 2018). However, strong stabilising selection in highly predictable environments can lead to reduced genetic variation in plasticity (Oostra et al. 2018). By contrast, populations adapted to habitats that are more spatially or temporally variable are expected to be less predictable, which should maintain genetic variation in plasticity and increase their potential for the evolution of plasticity in response to future novel environments (Chevin et al. 2010). Characterising patterns of G×E for a range of naturally occurring genotypes can therefore predict the likely rate and trajectory of evolutionary responses in subsequent generations (Via 1993; Chevin and Hoffmann 2017).

Assaying variation in gene expression across environments can reveal key aspects of the genomic basis of plasticity. If allelic (sequence changes in regulatory genes) or epiallelic (e.g. DNA methylation, chromatin remodelling, post-transcriptional modifications) variation underlying trait plasticity becomes fixed during adaptive divergence, limits to plasticity may arise (Gibson and Wagner 2000; Shaw et al. 2014; Oostra et al. 2018). Highly predictable environments will reduce genetic variation in plasticity through stabilising selection acting either on the genetic regulators (e.g. transcription factors) or long-term epiallelic changes, such as transgenerational DNA methylation (Colicchio et al. 2015; Oostra et al. 2018). If such stabilising selection varies among environments, divergence in phenotypic plasticity will occur. Determining the gene expression profiles for closely related but ecologically divergent species can therefore reveal the effect of adaptive divergence on the genomic basis of plasticity.

In this study, we first identify physiological differences in a common garden experiment between two closely related species of *Senecio* that inhabit contrasting elevations on Mt. Etna, Sicily. We then quantify variation in environmental sensitivity and phenotypic plasticity across four field transplant sites along the elevational range of both species. *Senecio chrysanthemifolius* (**Fig. 1a**) is a short-lived perennial with highly dissected leaves that occupies disturbed habitats (e.g., vineyards, abandoned land and roadsides) in the foothills of Mt. Etna c. 400-1,000m a.s.l (above sea level) and at similar elevations throughout Sicily. By contrast, *S. aethnensis* (**Fig. 1b**) is a perennial with entire, glaucous leaves and is endemic to lava flows c. 2,000-2,600m a.s.l on Mt. Etna that are covered by snow each winter. These two species diverged recently, probably c.150,000 years before present, which was concurrent with the uplift of Mt. Etna that created the novel high elevation environment to which *S. aethnensis* is adapted (Chapman et al. 2013; Osborne et al. 2013). Their recent shared ancestry is reflected by very low genetic divergence across the genome, despite large differences in habitat, phenotype and life history (Chapman et al. 2016).

Given that *S. aethnensis* is endemic to high elevations on Mt. Etna, while *S. chrysanthemifolius* is found in a variety of lower-elevation habitats across Sicily, we predicted that: (1) Their occupation of highly contrasting habitats would be reflected by differences in physiology in a common garden, suggesting functional trait divergence. (2) As evidence of adaptive divergence, species would perform better at their native elevation, and better than the foreign species. In addition, *S. aethnensis* would be less tolerant of novel environments, providing evidence that it is more specialised to its native habitat than *S. chrysanthemifolius*. (3) Despite clear patterns of adaptive divergence, their recent common ancestry would mean that both species will show similar patterns of plasticity, and similar patterns of G×E underlying plasticity. By contrast, if ecological speciation is associated with rapid changes in plasticity, then functional traits associated with adaptive divergence would also show large differences in plasticity between the two species, and different levels of

G×E underlying plasticity. To test these hypotheses, we sampled 37 genotypes of *S. chrysanthemifolius* and 42 genotypes of *S. aethnensis*, from several natural populations of each species. We then reciprocally transplanted multiple cuttings of each genotype to four transplant sites across an elevational range that included the home range of each species, and two intermediate elevations. We quantified survival, growth, leaf gene expression, photosynthetic activity and leaf investment. Given that rapid divergence in leaf shape and dissection is key feature of speciation in this system, as well as in other *Senecio* species (Walter et al. 2018), we also quantified leaf morphology traits, which are likely to be associated with fitness at different elevations.

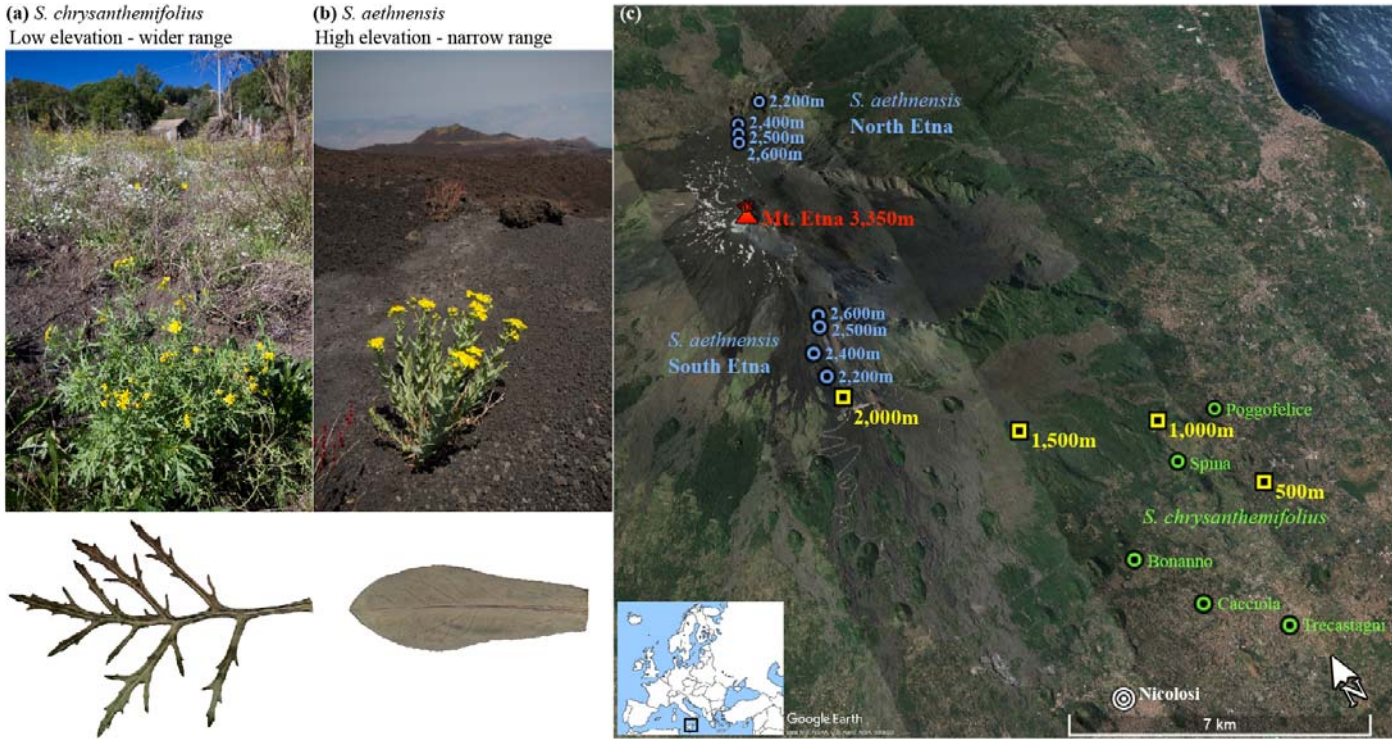

Fig. 1 (a) *Senecio chrysanthemifolius* occupies disturbed habitats below c.1,000m a.s.l, and has thin, dissected leaves. **(a)** *Senecio aethnensis* inhabits lava flows and has thicker, smooth-margined leaves with a thick waxy cuticle. **(c)** Map of sampling locations (*S. chrysanthemifolius*: green circles; *S. aethnensis*: blue circles) and transplant sites (yellow squares). Sampling locations for *S. aethnensis* and the transplant sites are labelled by their elevation. Inset map denotes the location of the study system within Europe.

Materials and methods

Sampling natural populations

We sampled seeds, and took cuttings from naturally growing individuals of both species after plants started flowering. This was conducted in May-June 2017 for *S. chrysanthemifolius* and July 2017 for *S. aethnensis* because *S. aethnensis* grows more slowly and flowers later than *S. chrysanthemifolius*, given its high

elevation habitat. For *S. chrysanthemifolius*, we sampled from 88 individuals from five sites, each a geographically separated patch of individuals representing potentially discrete populations, all below 800m.a.s.l (**Fig 1c**, **Table S1**). For *S. aethnensis*, we sampled from 87 individuals at four different elevations (2,600m, 2,500m, 2,400m and 2,300m.a.s.l) on both the North and South slopes of Mt. Etna (**Fig 1c**, **Table S1**). To minimise the risk of sampling close relatives, most plants sampled were more than 10m apart.

Physiological differences between species under common garden conditions

To assess differences in physiology between the species, we grew plants from seeds in a growth cabinet (see **Methods S1**), which represented environmental conditions intermediate between both species. Seeds were scarified mechanically and placed in petri dishes containing moist filter paper. Seedlings were transplanted into 70mm square pots with standard potting mix. From eight maternal families of *S. chrysanthemifolius* we grew 34 individuals, and from ten maternal families of *S. aethnensis* we grew 41 individuals. Seedlings were grown for two months and physiological measurements taken. With a Dualex+® instrument (ForceA, France), we measured the leaf content of chlorophyll, anthocyanin and flavonol pigments. Using an LCpro gas analyser (ADC BioScientific, UK), we measured photosynthetic gas exchange. Intrinsic water use efficiency (iWUE) was calculated as the ratio between photosynthesis and stomatal conductance. Chlorophyll fluorescence was measured using an IMAGING-PAM M-series chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Using output from the fluorometer, we quantified two mechanisms of physiological light defence of leaves (see **Methods S1**): (1) the unregulated dissipation of heat [Y(NO)], and (2) the regulated dissipation of heat [Y(NPQ)].

Field transplant experiment

In the glasshouse (Giarre, Italy), cuttings from all individuals sampled from natural populations (hereafter, genotypes) were cut into 5cm stem segments, each possessing 2-3 leaf nodes. Each smaller cutting was then dipped in rooting plant growth regulator for softwood cuttings (Germon® Bew., Der. NAA 0.5%, L. Gobbi, Italy) and placed in a compressed mix of coconut coir and perlite (1:1) in one cell of an 84-cell tray. All cuttings (i.e. clones) from each genotype were kept together in one half of a tray, with tray positions randomised regularly to prevent systematic environmental or positional effects. Trays were kept moist and checked regularly for cuttings that successfully produced roots (roots extending out of the bottom of tray). For each genotype, rooted cuttings were randomised into experimental blocks and transplanted at four field sites. From the initial genotypes sampled, we transplanted 37 *S. chrysanthemifolius* genotypes and 42 *S. aethnensis* genotypes that produced enough cuttings with roots.

Field transplant sites were at four elevations (500m, 1,000m, 1,500m and 2,000m a.s.l) along a transect on the south-eastern side of Mt. Etna (**Fig. 1c**). The 500m site was located in a garden among fruit trees and

grape vines, the 1,000m site on an abandoned vineyard among *Quercus ilex*, the 1,500m site among an apple and pear orchard, and the 2,000m site surrounded by pine trees on a lava flow from 1983. Both the native elevations (500m for *S. chrysanthemifolius* and 2,000m for *S. aethnensis*) were located less than 1km from natural populations. Furthermore, plants of both species were often observed at intermediate elevations (including close to the 1,000m and 1,500m transplant sites), but were never observed within the native range of the other species. Soil is characterised as a silty sand at elevations between 500m and 1,500m, but changes to volcanic sand at 2,000m. At each transplant site we deployed four data loggers (Tinytag Plus, Gemini Data Loggers, UK), which measured temperature hourly. We also took three soil samples at each transplant site, which were analysed for 36 variables that included nutrients, salts and ions (Nucleo Chimico Mediterraneo laboratories, Catania, Italy). To analyse the soil data, we used Multi-Dimensional Scaling (MDS) to calculate the scaled distance between the soil samples taken at all transplant sites.

We transplanted multiple cuttings of each genotype into three experimental blocks at each transplant site. Cuttings were transplanted into grids of 20×7 plants, with the position of cuttings randomised with respect to genotype, and separated from each other by 40cm (*S. chrysanthemifolius* block *n*=109; site *n*=327; total N=1,308; *S. aethnensis* block *n*=130; site *n*=390; total N=1,560). Depending on the number of cuttings that successfully produced roots, we transplanted 6-15 cuttings per genotype at each transplant site (see **Table S1**). Cuttings of *S. chrysanthemifolius* were transplanted in June-July 2017, and cuttings of *S. aethnensis* were transplanted (into adjacent experimental blocks) at the start of August 2017. The difference in timing was because seasonal constraints meant that sampling from natural populations of *S. aethnensis* was only possible after *S. chrysanthemifolius.* Following each transplant, cuttings were watered daily for three weeks to encourage establishment. To prevent death during high temperatures in July-August (consistently >35ºC), we watered cuttings daily during this period, which allowed us to assess the phenotypic responses of genotypes to what were still stressful conditions. We recorded mortality approximately every two weeks and measured the phenotypic traits of all plants at a single time point when both species showed substantial post-transplant growth (November 2017). To test whether the survival rates in this 2017 experiment were consistent across years, we conducted a similar transplant in 2018 by transplanting both species at the same time (total N=984 cuttings) in spring (April 2018) and providing less supplementary water.

Characterising leaf morphology and investment

Plasticity in leaf investment and leaf shape across elevation are often adaptive responses to changes in climate or water availability (Nicotra et al. 2008; Royer et al. 2009; Scheepens et al. 2010). To characterise leaf morphology, we sampled and pressed 3-5 young but fully expanded leaves from each cutting (five and four months after transplant for *S. chrysanthemifolius* and *S. aethnensis*, respectively). Leaves were weighed,

and then scanned and morphology quantified using the program Lamina (Bylesjo et al. 2008), which generates estimates of leaf area, perimeter and the number of indentations. To estimate the density of indentations along the leaf margin, we standardised the number of indentations by the perimeter. To capture leaf complexity we calculated perimeter^2/area, where lower numbers indicate less complex leaves, i.e. more entire leaves. As measures of leaf investment we included leaf area, and calculated Specific Leaf Area ($\mathrm{SLA} = \frac{leaf\ area}{leaf\ weight}$), where greater values represent thinner leaves.

Quantifying chlorophyll fluorescence

To quantify photosynthetic capacity for both species across the elevational gradient, at each transplant site we measured chlorophyll *a* fluorescence, which estimates the efficiency of the photosystem response to intense light. We selected five genotypes at random from each species and measured chlorophyll fluorescence on four cuttings per genotype at each transplant site (site *n*=40 plants, total N=160). We took measurements at two transplant sites each day, completing all four sites within one week in October 2017. For each cutting we measured four leaves, and to temporally replicate measurements we measured the same cuttings at each site on a second day. To take measurements, we put leaf clips on four leaves of each plant and dark-adapted the plants for 30 minutes by covering them with large black plastic containers. We then took fluorescence induction curve measurements for 2 seconds at 3,500μmol $s^{-1}m^{-2}$ photosynthetic photon flux density from each leaf (clip) using a Handy PEA instrument (Hansatech Instruments Ltd., UK). Using the JIP test (Tsimilli-Michael and Strasser 2013) we calculated PI_{total}, the total performance of photosystem I and II (see **Methods S1**).

Statistical analyses of survival, growth and plasticity

We first tested for significant differences in survival across elevation, and quantified plasticity as the change in all univariate traits across elevation (all leaf traits were first averaged for each clone). Secondly, to understand multivariate plasticity across elevation, we standardised each morphological trait to a mean of zero and standard deviation of one, and then used a Principal Components Analysis (PCA) with leaf area, complexity, SLA and number of indents. We used the first two principal components (describing 81% of total variation) to quantify how multivariate phenotypes differed between species, and changed across elevation.

To compare differences in growth, survival, leaf morphology and chlorophyll fluorescence across transplant sites and for both species, we used linear mixed models in R v3.6.1 (R Core Team 2019) within the package *lme4* v1.1-23 (Bates et al. 2015),

$$y_{ijklm} = T_i + S_j + T_i \times S_j + T_i \times G_{k(j)} + B_{l(i)} + e_{m(ijkl)}\ . \qquad (1)$$

Separate implementations of equation 1 were used for different univariate response variables of interest (y_{ijklm}). Changes in the response variable across transplant sites were modelled by the *j*th species (S_j) in the *i*th transplant site (T_i) and their interaction ($T_i \times S_j$), which were all included as fixed effects. Random effects included the interaction between transplant site and genotype $T_i \times G_{k(j)}$, and experimental block within each environment ($B_{l(i)}$). The residual error variance was captured by $e_{m(ijkl)}$.

Equation 1 was implemented as a generalised linear mixed model with a binomial error distribution for survival. The remaining phenotypic traits were normally distributed, for which we used a linear mixed model. For each implementation we tested the significance of the interaction between transplant site and species using likelihood ratio tests. To test whether differences in morphology between transplant sites were significant for each species, we used *emmeans* v1.4 (Lenth 2019) to conduct pairwise t-tests adjusted for multiple comparisons.

To test for significant G×E within each species, we applied equation 1 separately for each species on PC1 and PC2. To separate the effect of genotype from G×E, we included genotype and the genotype×elevation interaction as separate random effects. We tested the significance of all random effects using likelihood ratio tests. To identify whether G×E was created by changes in the magnitude of among-genotype variance across elevation, or by differences in reaction norms (i.e., a change in rank of genotypes across elevation), we used the parameters estimated from equation 1 to calculate

$$\sigma^2_{G\times E} = \frac{\sum_{i=1}^{h}\sum_{j=1}^{h}\left[2\sigma_i\sigma_j(1-r_{ij})+(\sigma_i-\sigma_j)^2\right]}{h(h-1)}, \qquad (2)$$

where σ represents square root of the variance among genotypes for the *i*th and *j*th transplant sites. The number of sites is represented by *h*, for which we only compared the elevational extremes (500m and 2,000m). The first term within the square brackets, $2\sigma_i\sigma_j(1-r_{ij})$, represents G×E as differences in reaction norms, with r_{ij} representing the genotypic correlation between the *i*th and *j*th habitats. The second term, $(\sigma_i-\sigma_j)^2$, represents G×E as changes in the magnitude of among genotype variance (Cockerham 1963; Johnson 2007; Friedman et al. 2019).

Sampling of plant tissue and RNA extraction

To quantify gene expression, we sampled 2-3 newly emerged leaves (15-20mm in length) from all cuttings at a single time-point following the initial transplant, which was after cuttings showed sufficient growth determined as 12-15 new, fully expanded leaves (July 2017 for *S. chrysanthemifolius*; October 2017 for *S. aethnensis*). All leaves for a cutting were placed in the same Eppendorf tube and stored in RNAlater at -20ºC.

Three genotypes of each species were then selected at random, and three clones sampled from each transplant site (36 samples in total per species). We extracted RNA from each sample using QIAgen RNeasy kits. Library preparation and RNA sequencing was performed at the Oxford Genomics Centre on an Illumina Hiseq4000 platform, producing 75bp paired-end reads.

Quantifying differential expression across transplant sites, genotypes, and species

A reference transcriptome was assembled for each species (**Methods S1**). Trimmed reads were mapped to each species' reference transcriptome using *Salmon* v0.13.1 (Patro et al. 2017). Read counts were normalised by transcript size, library size and filtered based on counts >5 across half of all samples. All estimates were repeated using *DESeq2* (Love et al. 2014) and *limma/voom* (Law et al. 2014) according to

$$\textit{Counts} \sim \textit{Species} + \textit{Transplant Site} + \textit{Species} \times \textit{Transplant Site} + \textit{Genotype}\ . \qquad (3)$$

In *limma/voom*, the genotype was modelled as a random effect. For comparisons within species, each treatment was compared with the home transplant site of each species (2,000m for *S. aethnensis* and 500m for *S. chrysanthemifolius*), with differentially expressed genes determined based on an adjusted p-value < 0.01 (Benjamini and Hochberg 1995) and a log fold change >2 for overexpression or <-2 for underexpression. In *DESeq2*, log fold changes were shrunk using the 'apeglm' method and then used to rank genes based on high overexpression and underexpression.

Annotation of differentially expressed genes and functional enrichment

Reference transcriptomes were annotated using *Trinotate* v3.2.1 (Bryant et al. 2017). Predicted amino-acid sequences were generated using *TransDecoder* v5.5 (https://transdecoder.github.io) and protein sequences were blasted against the Uniprot database. Annotation of the transcriptomes resulted in 7,579 unique GO (Gene Ontology) terms for 14,701 transcripts (mean of 7.2 GO terms/transcript).

To test for significant representation of functional categories among differentially expressed genes, gene ontology enrichment analyses were performed using *topGO* v2.3.6 (Alexa and Rahnenfuhrer 2019). Enrichment was determined using genes that were significantly differentially expressed (adjusted p values < 0.01) between the native transplant site and the furthest transplant site and Kolmogarov-Smirnoff (KS) test using the 'weight' algorithm.

Weighted network construction of differentially expressed genes

Weighted Gene Coexpression Network Analysis (WGCNA) identifies correlations of expression among all genes and then forms modules of coexpressed genes within that network. Consensus modules were constructed for each species (**Methods S1**). Each module was then summarized using its first principal component as a module eigengene, which represents the expression profile of the module. We tested for

a correlation between each module eigengene in each species and transplant elevation. Each module was tested for gene ontology enrichment in *topGO*, using Fisher's exact test.

Results

Physiological differences between species under common garden conditions

Under common garden conditions, *S. chrysanthemifolius* and *S. aethnensis* showed substantial differences in physiology. Both species had similar values for their ability to regulate heat dissipation from their leaves [Y(NPQ)] (**Fig. 2a**; *S.ae* 0.60±0.008 [one standard error], *S.ch* 0.61±0.010, t(65)=0.0116, P=0.3760). However, *S. aethnensis* showed greater values than *S. chrysanthemifolius* for non-regulated heat dissipation [Y(NO)] (**Fig. 2b**; *S.ae* 0.20±0.003, *S.ch* 0.18±0.004, t(65)=2.351, P=0.0217), which indicates that photochemical energy conversion and protective regulatory mechanisms are less efficient in *S. aethnensis.* *Senecio chrysanthemifolius* showed evidence of higher intrinsic water use efficiency than *S. aethnensis* (**Fig. 2c**; *S.ae* 62.11±3.30, *S.ch* 81.76±3.85, t(69)=3.875, P=0.0002), suggesting that leaves of *S. chrysanthemifolius* conserve water more effectively. *Senecio aethnensis* showed greater leaf concentrations of chlorophyll (**Fig. 2d**; *S.ae* 38.16±1.29, *S.ch* 34.55±1.50, t(143.8)=2.085, P=0.0388) and flavonols (**Fig. 2e**; *S.ae* 0.88±0.03, *S.ch* 0.73±0.03, t(200.9)=4.399, P<0.0001).

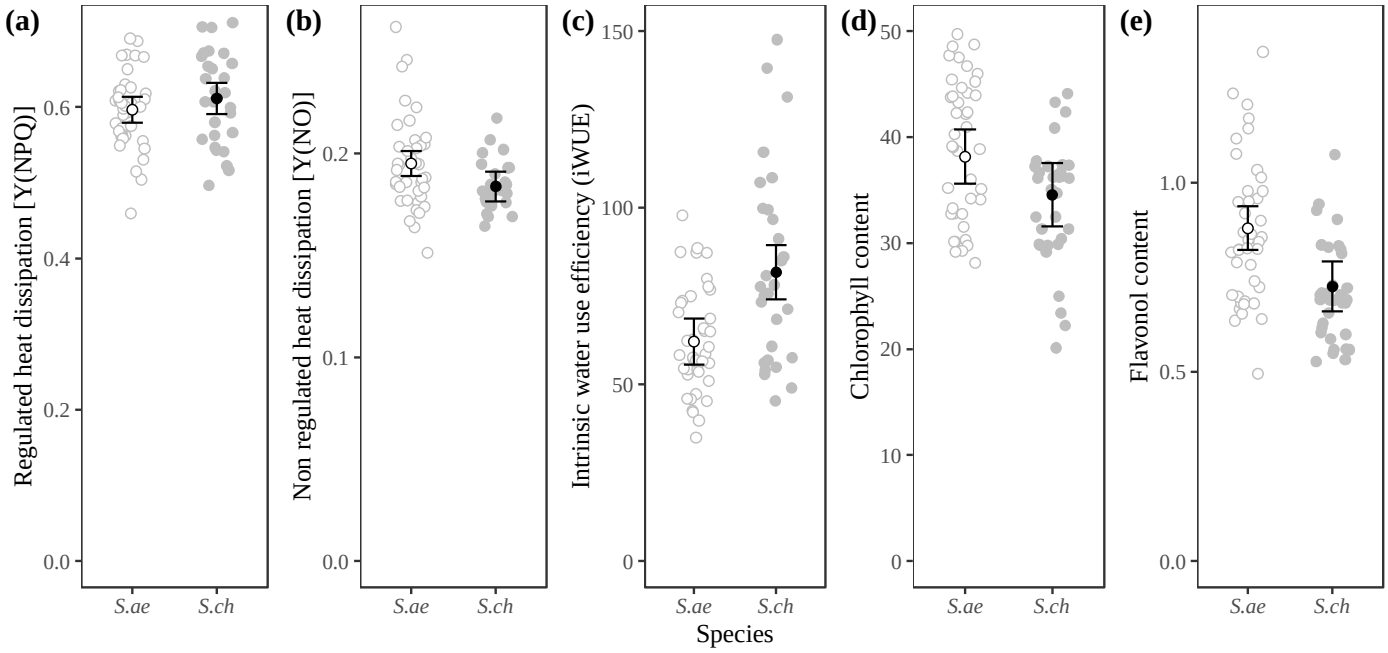

Fig. 2 Physiological differences between species grown from seeds under common garden conditions in the laboratory. Filled circles represent *S. chrysanthemifolius* (*S.ch*), while unfilled circles represent *S. aethnensis* (*S.ae*). Gray circles represent individual plants measured and credible intervals represent the 95% confidence intervals of the mean. **(a)** Both species showed similar values for regulated heat dissipation, Y(NPQ). **(b)** *Senecio aethnensis* showed greater values for the unregulated dissipation of heat, Y(NO). **(c)** *Senecio chrysanthemifolius* showed higher intrinsic water use efficiency, while *S. aethnensis* showed higher leaf chlorophyll content **(d)** and a higher flavonol content **(e)**.

Survival and growth of transplanted cuttings

The transplant sites experience contrasting climatic conditions associated with elevation, with extreme heat (regularly exceeding 40ºC) at 500m and 1,000m during summer, and extreme cold (regularly below 0ºC) at 1,500m and 2,000m during winter (**Fig. 3a**). Soil profiles separated the four transplant sites in a linear fashion along the first axis (MDS1), which represented a transition in soil type and reduction in nutrients (amount of organic material, total nitrogen, cation exchange capacity and exchangeable ions) at higher elevations (**Fig. 3b**). The second axis (MDS2) characterised differences between the 1,000m site and the other sites, associated with greater concentrations of various salts at 1,000m (soluble nitrates, calcium and magnesium).

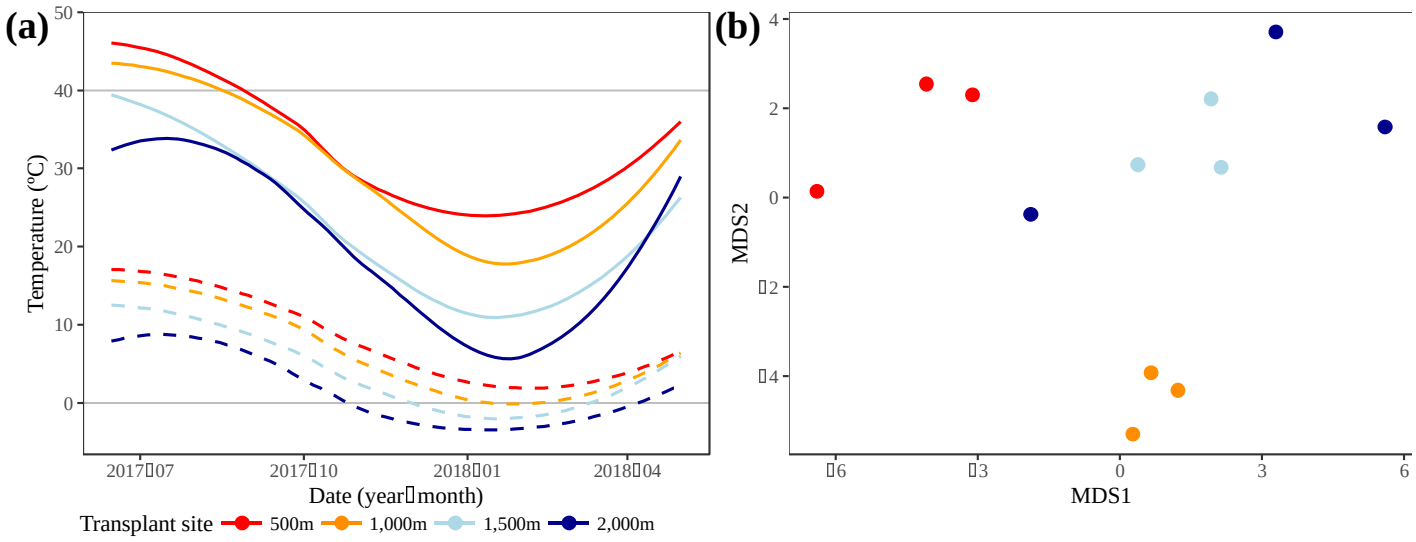

Fig. 3 Differences in environment for the four transplant sites at four elevations. **(a)** Average daily maximum (solid lines) and minimum (dashed lines) for three data loggers at each site, for the duration of the transplant. Gray shading represents the standard error for estimating the coefficients. Higher elevations remained below 40ºC in the summer and dropped well below zero in the winter. **(b)** Differences in soil composition for 35 soil variables captured by a multidimensional scaling analysis.

At the end of the experiment, *S. chrysanthemifolius* only showed high mortality at the highest elevation, whereas *S. aethnensis* showed higher mortality at the three lowest elevations compared to its native site (**Fig. 4a**; species×elevation $\chi^2(3)=37.46$, P<0.00001). At low elevations (500-1,500m), *S. aethnensis* showed greater mortality than *S. chrysanthemifolius* over summer (**Fig. 4b**; species×elevation $\chi^2(3)=20.94$, P=0.00011). By contrast, *S. chrysanthemifolius* showed greater mortality (>90%) over winter at 2,000m (**Fig. 4c**; species×elevation $\chi^2(3)=19.60$, P=0.00021). Both species showed similar reductions in height at higher elevations (**Fig. 4d**; species×elevation $\chi^2(3)=6.74$, P=0.0808). In the 2018 transplant experiment, where cuttings of both species were transplanted simultaneously in spring, and with less subsequent watering, we

found very similar patterns of survival to 2017. After summer, only 6% and 3% of *S. aethnensis* plants remained at 500m and 1,000m, respectively, as compared to 79% and 39% for *S. chrysanthemifolius* (**Fig. S1**). This consistency in patterns of mortality suggests that the 2017 experiment represented typical patterns of mortality.

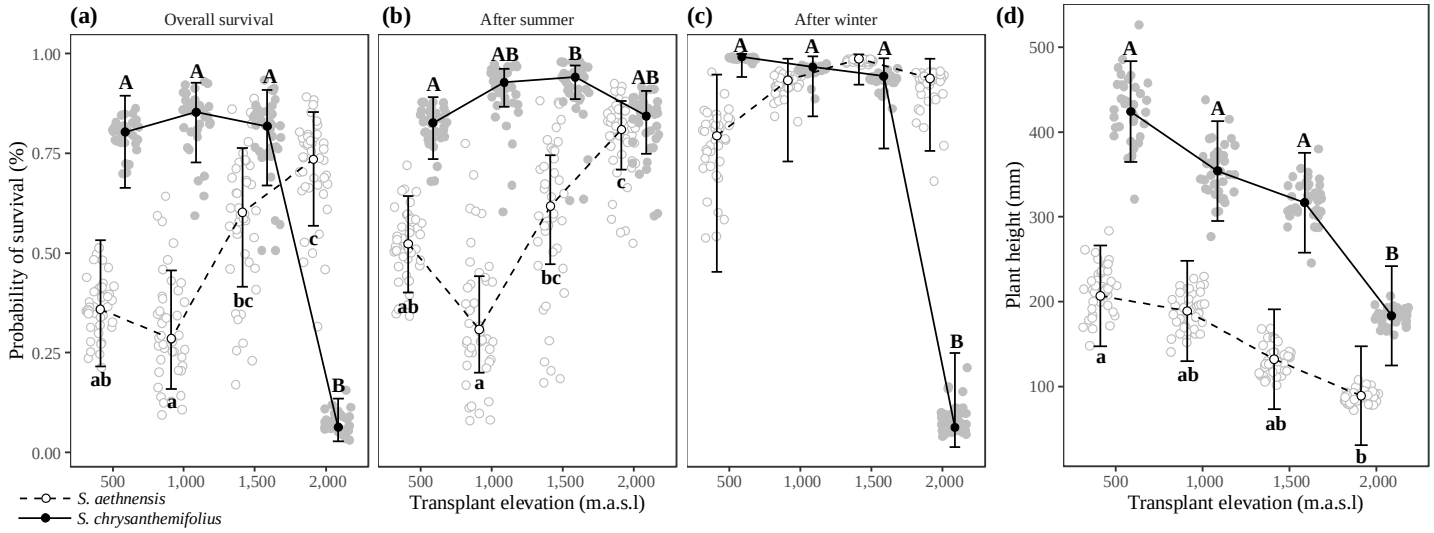

Fig. 4 Variation in survival and growth of both species across all transplant sites. Filled circles, solid lines and upper case letters represent *S. chrysanthemifolius*, while unfilled circles, dashed lines and lowercase letters represent *S. aethnensis*. Grey points represent the mean of all cuttings for each genotype sampled in the natural populations. Credible intervals represent 95% confidence intervals for the estimate of the mean and letters denote significant differences (full statistical summaries are located in **Table S2**). (**a**) At the end of the experiment, *S. chrysanthemifolius* showed low survival only at the highest elevation, while *S. aethnensis* showed lower survival at all three lower elevations. Each species suffered greater mortality during different seasons. (**b**) Survival after summer was high for *S. chrysanthemifolius*, but low for *S. aethnensis* away from its home site. (**c**) Both species survived well after winter, except for *S. chrysanthemifolius* at high elevation. (**d**) Plants grew larger at lower elevations, and *S. chrysanthemifolius* grew taller overall.

Morphological and physiological plasticity across species and elevation

Despite low survival of *S. aethnensis* at lower elevations, enough clones remained to measure almost all genotypes at each elevation (only 2-3 *S. aethnensis* genotypes were missing at lower elevations). Both species showed plasticity in leaf morphology traits across elevation **(Fig. 5)**. *Senecio aethnensis* showed an increase in leaf indentation at lower elevations, while *S. chrysanthemifolius* showed no significant change in leaf indentation across elevation (**Fig. 5a**; species×elevation $\chi^2(3)=30.97$, $P<0.0001$). However, *S. chrysanthemifolius* showed a reduction in leaf complexity at higher elevations, which was reflected by no change in *S. aethnensis* (**Fig. 5b**; species×elevation $\chi^2(3)=29.15$, $P<0.0001$). Both species showed similar increases in leaf area at lower elevations (**Fig. 5c**; species×elevation $\chi^2(3)=8.93$, $P=0.0302$). Both species also showed a similar increase in SLA (Specific Leaf Area) at lower elevations, but *S. aethnensis* showed a much greater increase at 500m (**Fig. 5d**; species×elevation $\chi^2(3)=22.51$, $P<0.0001$), suggesting that *S. aethnensis*

produced much thinner leaves than *S. chrysanthemifolius* at 500m.

We measured chlorophyll fluorescence to calculate the total performance index (PI_{total}), which reflects the capacity of the photosynthetic machinery. Although *S. chrysanthemifolius* showed no change in PI_{total} across elevation, *S. aethnensis* showed a steady decline, suggesting reduced photosynthetic activity of *S. aethnensis* at lower elevations (**Fig. 5e**; species×elevation $\chi^2(3)=24.59$, P<0.0001).

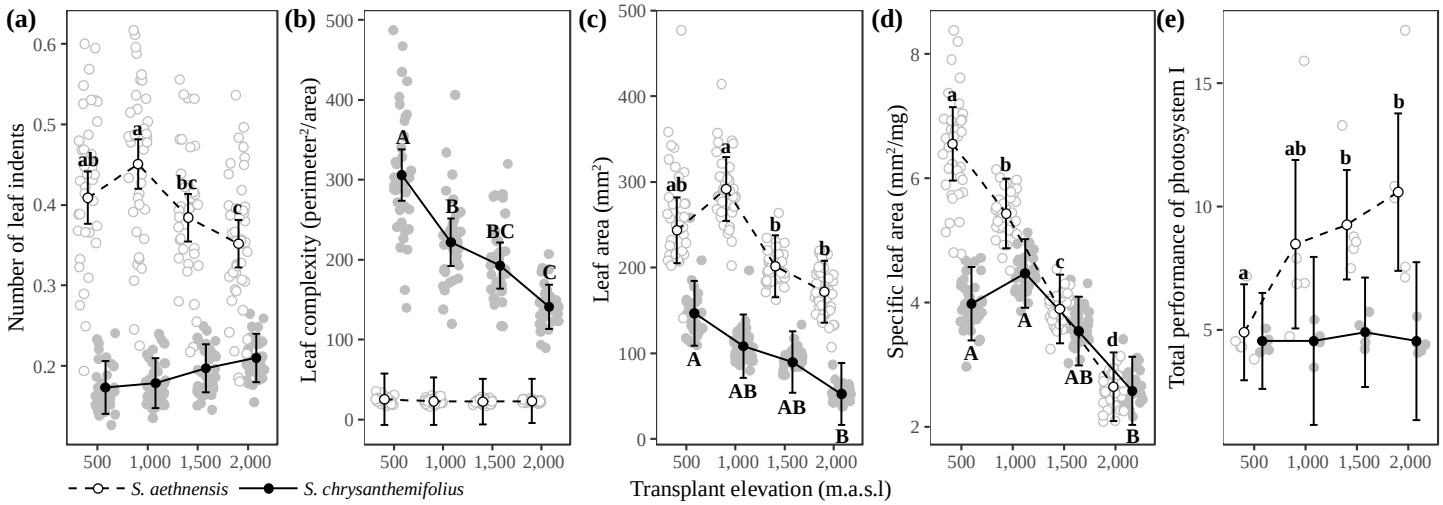

Fig. 5 Variation in univariate leaf traits across elevation for both species: **(a)** number of indents, **(b)** leaf complexity, **(c)** leaf area, **(d)** specific leaf area and **(e)** the performance of photosystem I. Filled circles, solid lines and upper case letters represent *S. chrysanthemifolius*, while unfilled circles, dashed lines and lowercase letters represent *S. aethnensis*. Credible intervals represent 95% confidence intervals for the estimate of the mean of each species at each elevation. Letters denote significant differences between transplant sites calculated using pairwise tests conducted within each species and adjusted for multiple comparisons (full statistical summaries are located in **Table S2**). Grey points represent the mean of all cuttings for each genotype, within species. *Senecio aethnensis* showed significant decreases in the number of indents with elevation, while *S. chrysanthemifolius* showed a decrease in leaf complexity at higher elevations. Leaf area changed similarly across elevation for both species, while specific leaf area was much greater for *S. aethnensis* at lower elevations. *Senecio aethnensis* also showed lower total photosynthetic performance (PI_{total}) at lower elevations, while *S. chrysanthemifolius* did not change.

To identify how multivariate phenotype changed across elevation, we quantified elevational changes in the first two principal components (**Fig. 6a**). Both species showed significant changes in principal component scores across the four transplant sites, but patterns differed between the two species. The first principal component described differences between species as well as phenotypic differences associated with elevation for *S. aethnensis* (**Fig. 6b**; PC1 species×elevation $\chi^2(3)=33.501$, P<0.0001). The second principal component described similar changes in multivariate morphology across the four transplant sites for both species (**Fig. 6b**; PC2 species×elevation $\chi^2(3)=3.526$, P=0.3174). Therefore, outside its native range, plasticity moved the phenotype of *S. aethnensis* further from the native phenotype of *S. chrysanthemifolius* (**Fig. 6a**). By contrast, *S. chrysanthemifolius* outside its range shifted its phenotype towards that expressed by *S. aethnensis* at high elevations.

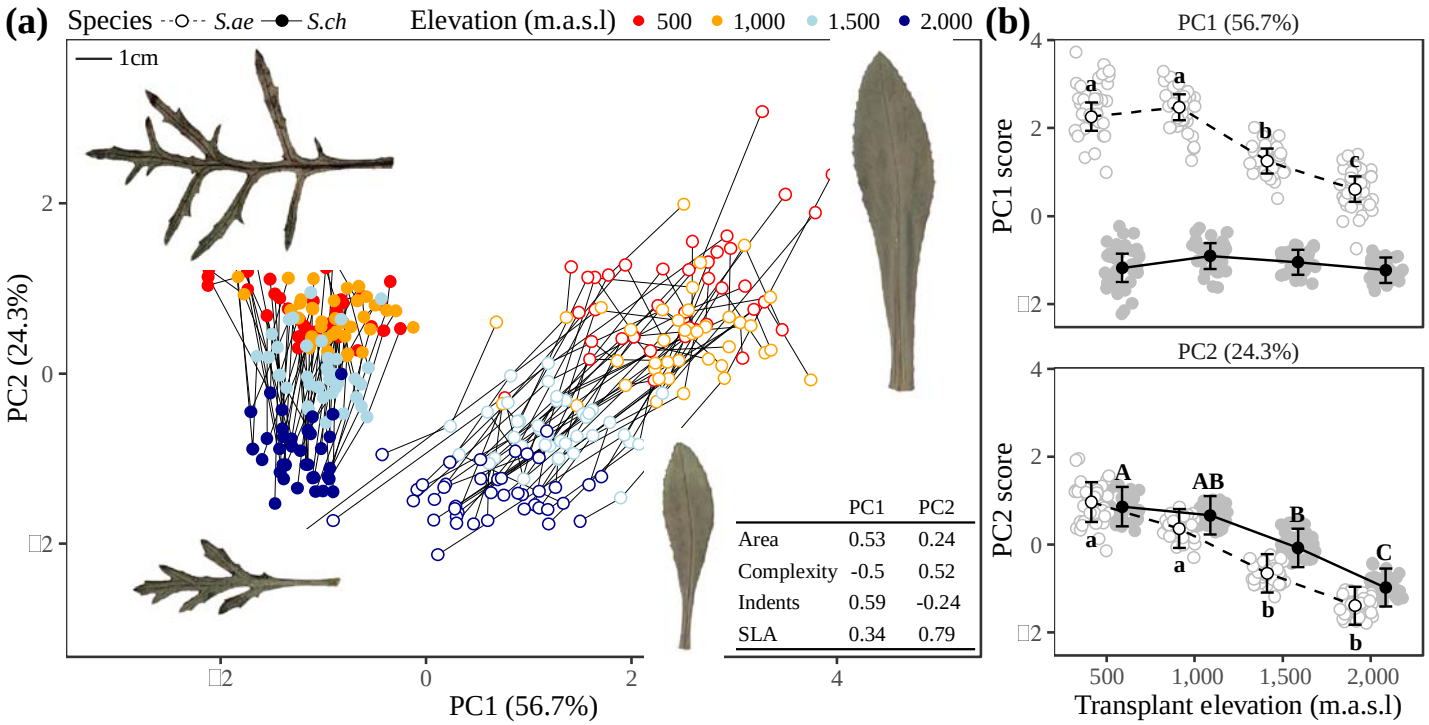

Fig. 6 Principal component analysis for leaf morphology of both species (*S.ae = S. aethnensis; S.ch = S. chrysanthemifolius*) measured at the four transplant elevations. **(a)** Filled circles and solid lines represent all genotypes of *S. chrysanthemifolius*, and unfilled circles and dashed lines represent the *S. aethnensis* genotypes. Table inset shows the trait loadings for both PC axes. *Senecio aethnensis* changed morphology across elevation for both PC1 (which also represents species differences) and PC2. *Senecio chrysanthemifolius* changed morphology across elevation only for PC2. Inset leaf images represent the extreme differences across elevation for one genotype of each species. **(b)** Analysing changes across elevation using linear mixed models for the first two principal components. Letters denote significant differences at P<0.05. *Senecio chrysanthemifolius* changed only in PC1, while *S. aethnensis* changes multivariate phenotype in both PC axes.

Genotypic variation in plasticity

G×E can be created either by a change in the magnitude of differences among genotypes across sites (i.e., a change in among-genotype variance), or by genotype-specific differences in their response to the environment, where genotypes change rank in different environments (i.e., where genotype reaction norms cross each other). We found that genotypes of both species varied significantly in their response to elevation (**Fig. 7a**). Generally, this meant that the magnitude of the variance among genotypes (within species) changed significantly between elevations, whereby >90% of G×E effects are characterised by changes in among-genotype variance between transplant sites (**Fig. 7a**), and visualised as a greater magnitude of differences among genotypes at 500m compared to 2,000m (**Fig. 7b-c** and **Fig S2**). However, genotypes did not change in rank between elevations. Furthermore, *S. chrysanthemifolius* showed no change in mean for PC1 across elevation, but a large change in PC2 **(Fig. 6)**, and this was reflected by the opposite pattern in G×E: strong G×E in PC1 and no G×E in PC2 (**Fig. 7**). By contrast, *S. aethnensis* showed changes in mean (**Fig. 6**) and significant G×E for both principal components (**Fig. 7**).

(a)

PC	Species	Genotype	Genotype×Elevation	Block	% of G×E change in: Reaction norm	Variance
PC1	*S.ae*	$\chi^2(1) = 73.15$ ***	$\chi^2(3) = 11.62$; P=0.0088	$\chi^2(1) = 59.51$ ***	8.09	91.9
	S.ch	$\chi^2(1) = 214.39$ ***	$\chi^2(3) = 102.05$ ***	$\chi^2(1) = 44.84$ ***	5.79	94.21
PC2	*S.ae*	$\chi^2(1) = 29.02$ ***	$\chi^2(3) = 35.75$ ***	$\chi^2(1) = 37.57$ ***	9.8	90.2
	S.ch	$\chi^2(1) = 103.56$ ***	$\chi^2(3) = 4.46$; P=0.2162	$\chi^2(1) = 178.43$ ***	0.78	99.22

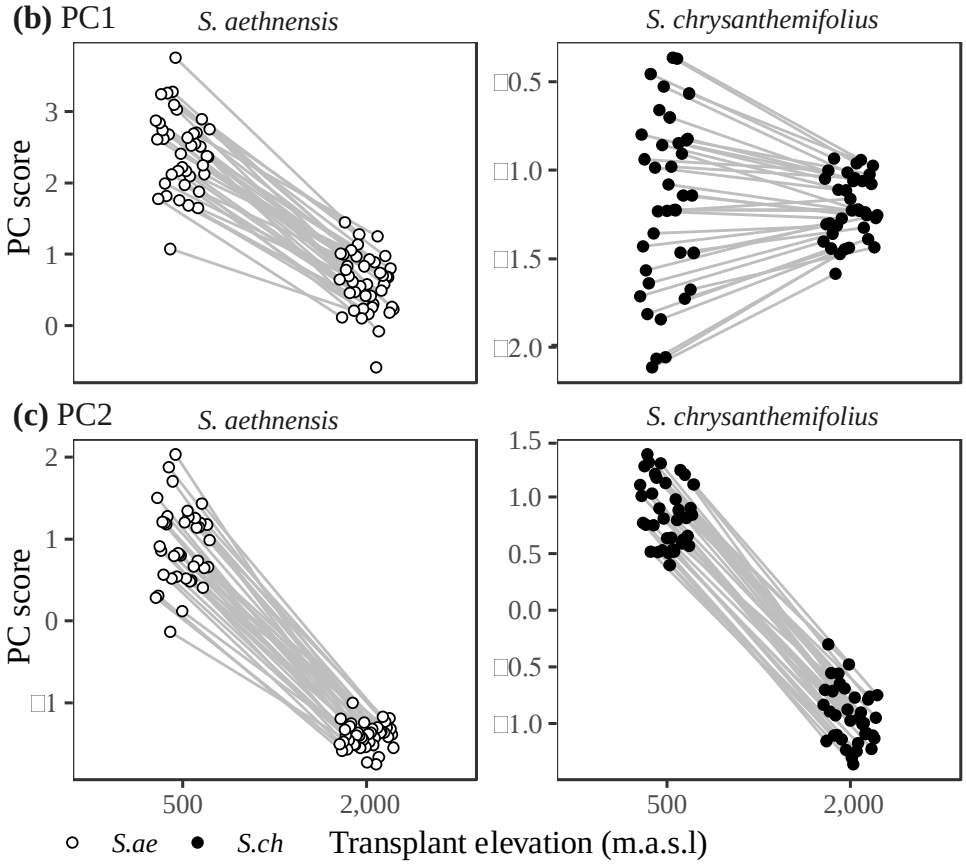

Fig. 7 Evidence for G×E in leaf morphology for both species (*S.ae = S. aethnensis; S.ch = S. chrysanthemifolius*). **(a)** χ^2 statistics presented from the likelihood ratio tests for genotype, genotype×elevation and block. The final two columns represent the calculation of the percentage contribution for G×E as either change in variance or a crossing of reaction norms. (**b-c**) Visualising G×E, where each line represents the change in morphology across elevation for a given genotype. Overall, G×E is evident as variation among the genotypes (of each species) in their response to elevation, largely due to changes in variance at a given site and not a crossing of reaction norm (see **a**). **(b)** For PC1, only *S. aethnensis* changed in mean across elevation, associated with significant, but small change in among-genotype variance between elevations. By contrast, *S. chrysanthemifolius* showed no change in mean, but a stronger pattern of G×E. **(c)** For PC2, both species changed similarly in mean, but only *S. aethnensis* showed significant G×E.

Differential gene expression between transplant sites and species

The two methods of estimating differential expression (*DESeq2* vs *limma/voom*) showed similar patterns of gene expression variation, although *limma/voom* detected fewer strongly differentially expressed genes in each species (**Fig. S3**). Patterns of gene expression between species revealed a high proportion of differentially expressed genes at each transplant site, with the most at 2,000m (1,677 genes) and decreasing at 1,500m (1,451 genes), 1,000m (1,079 genes) and 500m (1,051 genes) (**Fig. 8a**). In total 383 genes were

differentially expressed across all elevations. Functional enrichment of differentially expressed genes at each transplant site reflected differences in photoprotection, pigmentation and water use efficiency (**Fig. 8a**).

Within each species, more genes were differentially expressed as genotypes were moved further from their native elevations (**Fig. S3)**. Genes that were differentially expressed between the home site and the most novel environment (i.e. the elevational extremes) for each species indicated little overlap between the two species, with just 5.5% and 6.5% of overexpressed and underexpressed genes shared between species (**Fig. 8b**). For the ten genes of each species with the largest change in overexpression and underexpression between 2,000m and 500m, we observed contrasting patterns between the two species: strong overexpression or underexpression in one species but a relatively unchanged expression profile in the other species (**Fig. 9**).

Functional enrichment analyses of differentially expressed genes between the elevational extremes revealed 38 significant GO terms in *S. chrysanthemifolius* and 30 in *S. aethnensis*. Only four out of 68 functional categories of genes were shared between species (translation, response to blue light, photosynthesis and ribosomal small subunit assembly; **Tables S3-S4**). In *S. aethnensis*, GO terms indicated physiological changes to the leaf cuticle, including fatty acid, wax and cutin biosynthesis (**Table S3**). In *S. chrysanthemifolius*, GO terms involved responses to changing light conditions, including protein-chromophore linkage, light harvesting in Photosystem I and response to high light intensity (**Table S4**).

Network reconstruction resulted in 32 network modules ranging in size from 196 to 3,108, with 170 genes unassigned (**Table S5**). 17 modules showed a significant correlation with elevation (**Fig. 8c)**. 7 modules correlated with elevation uniquely in *S. aethnensis* and were annotated with terms including phyllome development, translational initiation, protein targeting to chloroplasts, response to heat and immune response. 6 modules correlated with elevation uniquely in *S. chrysanthemifolius* and were annotated with terms including cold-induced morphogenesis, response to nitrogen starvation, response to far red light, light harvesting and protein depolymerisation.

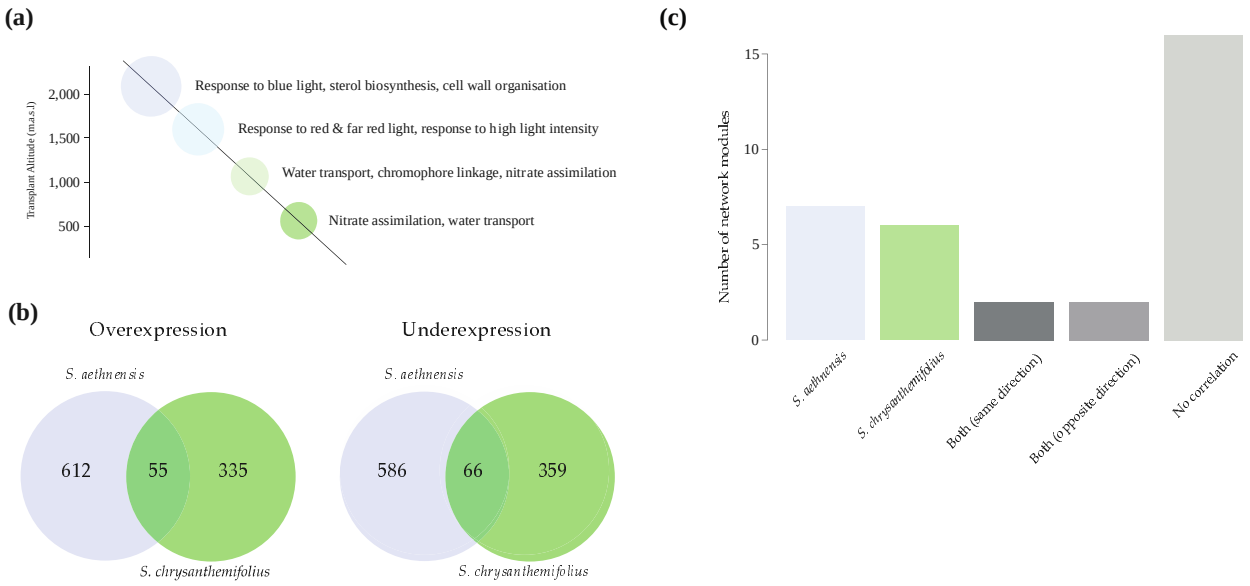

Fig. 8 Contrasting patterns of gene expression between species. (**a**) Total numbers of differentially expressed genes (-2 < lfc > 2) between species at each transplant site are represented by circles, with the diameter proportional to the number of genes. The most significantly enriched gene ontology categories are shown next to each circle (**b**) Overlapping overexpressed and underexpressed genes between the home and furthest transplant site in each species. **(c)** The number of significant correlations (adjusted $p < 0.05$) between network module eigengenes and elevation in each species.

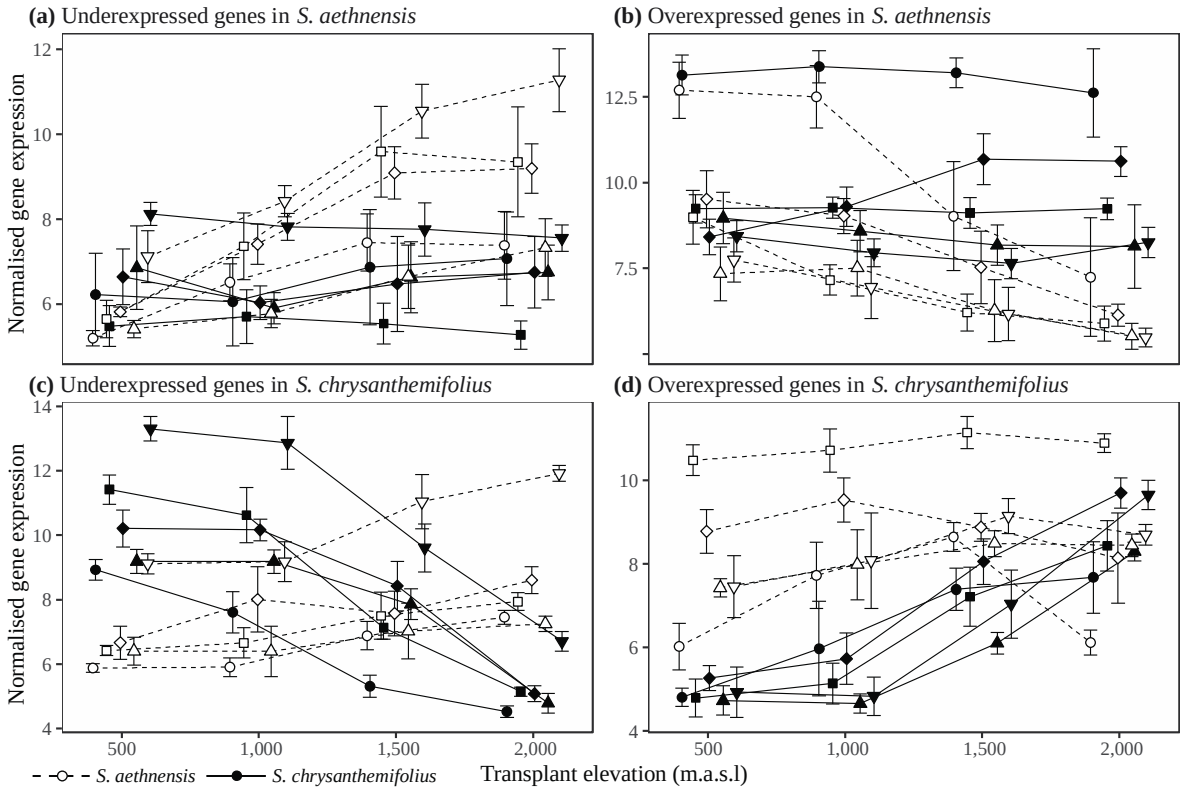

Fig. 9 Normalised expression profiles across all transplant sites for *S. chrysanthemifolius* (solid lines and circles) compared to *S. aethnensis* (dashed lines and unfilled circles). This includes the five genes (represented by different shapes) that are most strongly underexpressed in *S. aethnensis* **(a)**, overexpressed in *S. aethnensis* **(b)**, and the five genes most strongly underexpressed in *S. chrysanthemifolius* **(c)** and overexpressed in *S. chrysanthemifolius* **(d)**. Strong overexpression or underexpression in one species was typically reflected by little to no change in gene expression in the same gene for the other species.

Discussion

Given the two *Senecio* species on Mt. Etna are the result of recent ecological speciation, we predicted that (1) the two species would exhibit differences in physiology when grown in common garden conditions, and (2) that species would perform well in their native habitat, but poorly in the habitat of the other species. In addition, we predicted that, given *S. aethnensis* is endemic to high elevations on Mt. Etna, it would maintain high fitness across a narrower range of elevations than *S. chrysanthemifolius*. (3) We also predicted that due to their recent common ancestry, both species would display similar patterns of plasticity, and similar patterns of G×E underlying plasticity.

Consistent with our first prediction, genotypes of both species showed distinct behaviour in the common garden experiment (**Fig. 2**), indicating that adaptive divergence in high and low elevation habitats has generated substantial shifts in physiology. In support of our second prediction, *S. aethnensis* showed reduced survival at all elevations outside its native elevation, while *S. chrysanthemifolius* only showed reduced survival at the elevation furthest from its native elevation (**Fig. 4a**). Furthermore, *S. aethnensis* suffered greater mortality at the start of the transplant experiment (i.e. during summer), which occurred early in their flowering season and likely reduced flower and seed production (**Fig. 4b**). By contrast, high mortality for *S. chrysanthemifolius* was only recorded at the highest elevation after an extensive flowering season (i.e. during winter; **Fig. 4c**), suggesting that fecundity would likely be higher for *S. chrysanthemifolius* across the entire elevation gradient during the experiment. Reduced photosynthetic activity at lower elevations was also associated with lower survival for *S. aethnensis,* which contrasted with *S. chrysanthemifolius* that maintained a lower, but constant photosynthetic activity across elevation (**Fig. 5e**). These results support our prediction that *S. chrysanthemifolius* can tolerate a wider range of environmental variation compared to *S. aethnensis,* reflecting its broader distribution across a wider range of (lowland) habitats across Sicily.

However, contrary to our third prediction, we found different patterns of plasticity in phenotype (**Figs. 5-6)** and gene expression (**Figs. 8-9**) between the two species. *Senecio chrysanthemifolius* showed substantial reductions in leaf complexity at higher elevations but showed little change in the number of leaf indents. By contrast, *S. aethnensis* showed little change in leaf complexity, but a reduced number of leaf indentations when grown at higher elevations. These leaf morphology traits that showed divergence in plasticity between the two species were also associated with differences in trait mean, suggesting an important link between adaptive divergence and plasticity. Both species produced larger, thinner leaves at lower elevations, but *S. aethnensis* produced significantly thinner leaves than *S. chrysanthemifolius* at the lowest elevation. Taken together, phenotypic plasticity in *S. chrysanthemifolius* for these traits increased its resemblance to the

multivariate phenotype of *S. aethnensis* at high elevation. By contrast, phenotypic plasticity in *S. aethnensis* reduced its resemblance to *S. chrysanthemifolius* at lower elevations. We also detected significant G×E interactions underlying plasticity in leaf traits (**Fig. 7**), suggesting standing variation that could allow plasticity to evolve in response to novel environmental conditions. However, contrary to our prediction that both species would show similar levels of G×E interactions, we found evidence that patterns of G×E interactions were stronger for genotypes of *S. aethnensis* (**Fig. 7**).

Consistent with the distinct forms of phenotypic plasticity observed, but also contrary to our third prediction, gene expression changes across elevation involved surprisingly distinct gene networks for the two species. The number and nature of differentially expressed genes changed with elevation (**Fig 8a**), with only 1.6% of genes differentially expressed being shared between the two species within any given transplant site. This result also means that the vast majority of differentially expressed genes were induced by the elevational gradient. Genes showing large changes in expression in one species across elevations showed either small or non-existent changes in the other species (**Fig. 9**), while 13 gene network modules were strongly correlated with elevation in one species, but not in the other.

The genotypes taken and cultivated for transplant in the field were from large adult individuals in the wild, meaning that they represent a locally fit subset of genotypes taken from all the genotypes generated among the seeds of natural populations. The plastic responses of cuttings from these genotypes are also likely to have already been shaped by developmental decisions in earlier life, as well as individual differences among plants, for example in their health and age (Morey and Reznick 2000; Weinig and Delph 2001). Nevertheless, these data reflect clear differences in plasticity between species for genotypes growing in natural populations.

Putative function of differentially expressed genes

We identified c.600 loci per species that showed differential expression between the extreme elevations. *Senecio chrysanthemifolius* showed the greatest change in genes relating to photosynthesis, light response and circadian rhythm. These are traits that typically vary in response to the reduced temperature and increased light intensity associated with increasing elevation (Beis and Patakas 2012). By contrast, changes observed in *S. aethnensis* were in genes associated with the leaf cuticle, including the biosynthesis of cutin, waxes and fatty acids. Changes in the cuticle could reflect a response to biotic and abiotic stressors that are strongest at the lower elevation, such as pathogens or rates of water loss (Serrano et al. 2014).

At each transplant site, the genes differentially expressed between species were associated with functions that reflected the differences observed in physiological measures between species grown under common garden conditions. For example, genes relating to water use were differentially expressed between the species at lower elevations, which were reflected by greater water use efficiency in the leaves of *S.*

chrysanthemifolius in the common garden experiment. Likewise, genes differentially expressed at higher elevations were associated with light responses, photosynthesis and cuticle composition, which could reflect the differences in pigment composition observed between the leaves of the species grown in the common garden. This functional significance of the genes that vary most in expression, and differ between these species, provides further support that adaptive divergence has shaped the evolution of distinct forms of plasticity in these species.

Adaptive divergence between the two Sicilian Senecio *species*

Our data indicate that the plastic responses of either species are not sufficient for them to maintain fitness at the native elevations of the other species, and that this is especially the case for *S. aethnensis* (**Fig. 4a**). Adaptation of these species to the contrasting habitats on and around Mt. Etna is likely to result in selection against genotypes of either species outside their native habitat, which is likely to reduce any gene flow between the species (Ross 2010). Results from the common garden experiment suggest that such ecological divergence has resulted in differentiation in functional traits related to light defence and leaf pigments. Furthermore, the field transplants reveal that divergence of functional traits at the gene network and phenotypic level was associated with adaptation to the contrasting elevations. Therefore, reductions in survival outside each species native ranges seems likely to be due to divergence in traits that contribute to adaptation in their native habitat (Ross 2010).

This species pair forms a narrow hybrid zone (c.1.5 km wide) at approximately 1,500-1,700m elevation. Although the parental forms can form hybrids, there is evidence of intrinsic reproductive isolation that results in low fitness of the F2 and F3 generations (Hegarty et al. 2009). There is also evidence that parental individuals rarely meet on Mt. Etna, instead the hybrid zone is almost entirely populated by hybrid phenotypes that have only occasional contact with parental individuals due to the separation between the hybrid zone and parental populations (Brennan et al. 2009). However, it remains possible that some mutually beneficial alleles are able to spread into the parental genomes of either species, making it even more remarkable that we detected divergence in plasticity between the two species.

Adaptive divergence creates differences in plasticity

Our results suggest that adaptive divergence has a rapid effect on patterns of phenotypic plasticity and on the genetic basis of plasticity, even in two closely related species. It remains possible that genetic drift during the formation of these two species could cause the species-specific differences in plasticity that we observed. However, given substantial divergence in leaf form and physiology, the differences in plasticity of these same traits among species, and the higher fitness of each species at their native versus novel habitats, it seems far more likely that adaptive divergence between *S. aethnensis* and *S. chrysanthemifolius* is

responsible for their distinct plastic responses (Taylor and Aarssen 1988; Emery et al. 1994; Ho and Zhang 2018). In addition, genetic drift would also need to be exceptionally strong and persistent to create such distinct differences in plasticity.

Understanding how plasticity evolves is important for understanding how species can respond to novel environmental variation (Bradshaw 1965; Baythavong and Stanton 2010). Plants growing in more predictable environments should evolve reduced plasticity where stabilising selection is stronger (Emery et al. 1994; Alpert and Simms 2002; Baythavong 2011). In other studies, high-elevation populations showed reduced plasticity in flowering time (Schmid et al. 2017) and morphology (Emery et al. 1994) compared to low elevation populations. Similarly, we found that the high elevation species, *S. aethnensis*, showed a steeper decline in fitness than *S. chrysanthemifolius* across the elevational gradient (**Fig. 4a**), and this was associated with stronger reductions in leaf investment (**Fig. 5d**), lower photosynthetic activity (**Fig. 5e**) and reduced capacity to approach the native phenotype of *S. chrysanthemifolius* at lower elevations. Therefore, the distinct patterns of plasticity observed for the two species may be caused by *S. aethnensis* exhibiting maladaptive plasticity as a consequence of greater specialisation to the high elevation habitat.

We suggest two possible explanations for the distinct patterns of plasticity in these species. First, species may have reduced plasticity in traits that are not required for tracking environmental variation in their respective habitats. For example, as a perennial that grows from rootstock after winter each year, it is likely that the new leaves *S. aethnensis* produces each year possess different cuticles that are optimal for the specific environmental conditions, allowing *S. aethnensis* to track environmental variation across years. In this case, plasticity in leaf cuticle would be critical to buffer environmental variation across years at high elevations, and so would also show plasticity when transplanted to lower elevations, even if such plastic changes were maladaptive. By contrast, plasticity in traits that increase survival at low elevations may not be required to track environmental variation at high elevations, and are then lost. Therefore, as a consequence of adapting to the environmental variation specific to their native elevations, these species could show different patterns of plasticity because they have lost the plastic response that would increase survival in the other species' habitat.

The second explanation is that species will show reduced plasticity in the genes and traits that need to be continually expressed in their native habitat. This would occur if plastic responses required to initially colonise the high elevation habitat become genetically-based (via genetic assimilation) because consistently strong stabilising selection for a single phenotype is favoured (Waddington 1953). This would suggest that as divergent selection drives trait divergence between the two species, it changes the level of plasticity in the traits that are diverging. In this scenario, plasticity would only be retained in traits that maintain fitness in

response to the environmental variation experienced within their native habitat. For example, *S. aethnensis* shows a greater number of indents, as well as greater plasticity in this trait (**Fig. 5a**), while *S. chrysanthemifolius* shows both greater leaf complexity than *S. aethnensis*, as well as greater plasticity in this trait (**Fig. 5b**). At high elevations *Senecio aethnensis* is likely to experience consistently strong selection for less complex leaves, leading to a loss of plasticity in this trait, perhaps via genetic assimilation. By contrast, *S. chrysanthemifolius* likely experiences selection for more complex leaves, but the variety of habitats that this species occupies could maintain plasticity in this trait.

Traits, such as leaf complexity or leaf indentation, that show strong divergence between species as well as differences between species in plasticity, are likely to be under selection in at least one environment. During adaptive divergence, selection could either maintain or remove plasticity depending on which of the two explanations outlined above determine plasticity. Distinguishing between these explanations requires exploring how selection affects the evolution of plasticity in different traits and environmental regimes (Schmitt et al. 1999; Pratt and Mooney 2013; McLean et al. 2014). Such information could reveal how the fixation of alleles underlying adaptive trait divergence also affects trait plasticity.

Genotype-by-environment interactions and the evolutionary potential of plasticity

The lower tolerance of *S. aethnensis* to conditions beyond its native range suggests that adaptation to the high elevation environment has reduced its ability to respond to novel environmental variation at lower elevations. Such limits to plasticity threaten the persistence of high elevation species in response to climate change unless genotypic variation in plasticity (G×E) can promote rapid adaptation. In both species we found significant genotypic variation in plastic responses to the elevational gradient, suggesting substantial evolutionary potential. *Senecio aethnensis* showed substantial G×E in both the multivariate axis that separated the two species, and in the axis associated with consistent phenotypic change across elevation for both species. By contrast, *S. chrysanthemifolius* displayed substantial G×E in the multivariate axis that separated the two species, but not in the axis associated with consistent phenotypic change across elevation. This suggests little genetic variation for plasticity (and therefore adaptive potential) in response to elevational shifts in *S. chrysanthemifolius*, even though the plastic responses shown allow relatively good performance across a wide elevational range. Consistent with other studies (e.g., Friedman et al. 2019), G×E patterns in phenotype were largely created by changes in the amount of variance observed among genotypes across the elevational gradient (**Fig. 7**), rather than by genotype-specific (i.e., change in genotype rank between environments) responses to the environment. In other words, genotypes tended to vary in their magnitude of plasticity across elevation, which could promote adaptive responses across generations if the steepness of the reaction norm determines fitness in novel environments.

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