Evolutionary Biology
https://doi.org/10.1007/s11692-021-09541-1

RESEARCH ARTICLE

Evidence of Morphological Divergence and Reproductive Isolation in a Narrow Elevation Gradient

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Received: 17 January 2021 / Accepted: 19 May 2021

Abstract

Elevation gradients generate different environmental conditions. This environmental differentiation can influence morphological adaptation, habitat isolation, reproductive isolation, and pollinator limitation in plants. Habitat differentiation and isolation often act first on phenotypic traits and then on genotype variation, causing genetic divergences between populations. We evaluated the effect of elevation on morphological traits, reproductive isolation, and pollinator limitation in *Croton* aff. *wagneri* in dry shrublands of inter-Andean valleys in Ecuador. We measured morphological traits of *Croton* at three elevations and carried out experimental pollination crosses between and within each population at different elevations to assess the degree of reproductive isolation and pollinator limitation. Morphological traits such as leaf thickness, plant volume, inflorescence length and inflorescence number were dissimilar between plants in different elevations. There was evidence of incipient reproductive isolation between plants in populations at the highest and the lowest studied elevations. Pollination experiments within each elevation showed a limitation of pollinators in *Croton* in the highest elevation. Intrinsic barriers to pollen dispersal and ecological divergence can produce reproductive incompatibilities between individuals with different traits along the *Croton* elevation gradient.

Keywords *Croton* · Elevation gradient · Inter-andean shrubland · Morphological divergence · Pollen limitation · Reproductive isolation

Introduction

Speciation involves the evolution of reproductive isolation barriers. Reproductive isolation entails interruption of gene flow between populations of a species (Alix et al., 2017; White et al., 2020). Evolution of reproductive isolation barriers in plants originate by processes such as sexual and pollinator isolation (McKinnon et al., 2004; Rundle et al., 2005), gametic isolation (McCartney & Lessios, 2004; Ramsey et al., 2003) and habitat isolation (Matute et al., 2009; Rojo et al., 2020). Habitat isolation dissociates populations exposing them to novel environments in which they must adapt to persist (Noble et al., 2019). The direction of adaptive change depends on the phenotypic and genetic variation that is exposed to natural selection (Noble et al., 2019). Because selection acts on phenotypes, not directly on genotypes or genes, new traits can originate by environmental induction, as well as mutation, and then undergo selection and genetic accommodation (Levis & Pfennig, 2020). These processes enhance morphological variation and genetic structuring between populations because of new genetic rearrangements that can cause reproductive incompatibility (Draghi & Whitlock, 2012; Radersma et al., 2020). As an example, in the Galapagos the cyclical phenomenon of "El Niño" causes intense rains followed by periods of drought (Snell & Rea, 1999). These cyclical phenomena cause a prolonged change in habitat and in seed size (Grant & Grant, 1996). Variation in seed size is associated with the bimodal size of beaks of "El Garrapatero" (*Geospiza fortis*; Huber

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et al., 2007). The *G. fortis* population has large-beaked individuals that prefer large seeds and small-beaked individuals that prefer small seeds (Grant & Grant, 1996). This morphological divergence of beak size in *G. fortis* reveals both restrictions in gene flow between two morphs as well as two distinct gene pools showing a widely accepted speciation pattern in many taxa (Grant, 1999; Huber et al., 2007; Schluter, 2000). Such phenotypic plasticity could facilitate speciation through morphological divergence between populations (Gomez-Mestre & Buchholz, 2006; Jiang et al., 2019; Rundle & Nosil, 2005).

Environmental changes often trigger gene flow restriction, phenotypic and population divergence (Minelli, 2016; Pfennig & McGee, 2010; Shaw & Mullen, 2011). Phenotypic divergence produced through selection and reproductive isolation can generate ecologically differentiated adaptive populations that could result in the generation of new species (Adams & Huntingford, 2004; Mallet, 2008). Once a phenotype is expressed in a population, to support local environmental changes through physiological tolerance (phenotypic plasticity), selection can favor the expression of these traits through genetic adaptation (Chevin et al., 2010; Pfennig et al., 2010; Sun et al., 2020). Several studies of adaptive traits in plants suggest that genetic adaptation is omnipresent due to clinal variation in phenotypes and genotypes between populations (Depardieu et al 2021; Kremer et al., 2014; Pais et al., 2017). In *Vitis vinifera*, the variation of adaptive traits of their leaves (plants with large leaves inhabit hot and humid climates, and plants with small leaves inhabit cold and dry climates) showed high heritability (Chitwood et al., 2014). Divergent populations of *Cornus florida* showed evidence of local genetic adaptation at various loci under selection that express traits of foliar osmotic potential for adaptation to drought (Pais et al., 2017). These studies suggest that plasticity is an evolutionary source for plant populations to adapt to environmental change (Radersma et al., 2020). Adaptations of populations to new environmental conditions can be become genetically fixed (Corl et al., 2010; Levis & Pfennig, 2020). This process can accumulate genetic differences between populations and contribute to reproductive isolation (Alix et al., 2017; Cardona et al., 2020; Pfennig et al., 2010). It is necessary to understand how processes that generate phenotypic variation interact with natural selection to explain and predict evolutionary paths (Uller et al., 2020).

Variation in reproductive morphology and pollinators plays an important role in plant species isolation with elevation. Two Andean species of *Polylepis* decreased their number of inflorescences in an elevation gradient between 3500 to 4100 m above sea level (m a.s.l.; Cierjacks et al., 2008). In the Helan Mountain Range, China, inflorescence size increased with elevation in insect-pollinated plants on a gradient between 1300 to 3100 m a.s.l. (Zhu et al., 2009). Inflorescence size is key to pollination of flowers that exhibit differences in their sexual expression (Harder & Prusinkiewicz, 2013). Increased number of open flowers in an inflorescence can promote more pollinator visits and greater reproductive success (Gurung et al., 2019; Harder & Prusinkiewicz, 2013).

Several studies have demonstrated the effect of elevation over pollination interactions (Gugerli, 1998; Ramos-Jiliberto et al., 2010; Zhao & Wang, 2015). Often, pollinator availability is low at higher altitudes, so populations at these elevations tend to develop self-compatibility (Alonso, 2005; Arroyo et al., 2017; Gugerli, 1998). An understanding of the influence of environmental heterogeneity on phenotypic and genotypic adaptation, pollination systems and sexual expression is essential to comprehend isolation and speciation processes (Matesanz et al., 2020; Minelli, 2016; Olito et al., 2018; Pélabon et al., 2011).

In tropical mountainous regions temperature and humidity vary according to elevation gradients (Apaza-Quevedo et al., 2015). Elevation generates morphological variations in plants allowing them to adapt locally (Scheepens et al., 2010). Some of the adaptations that plants develop in response to changes in temperature and moisture in elevation gradients are specific leaf area (SLA; Cruz-Nicolás et al., 2020; Jian et al., 2009), leaf thickness (LT; Scheepens et al., 2010), plant size (Badr et al., 2017), inflorescence length (Wang et al., 2019; Wu et al., 2016) and number of inflorescences (Quilot-Turion et al., 2013). In the Jura region and the Alps, populations of *Campanula thyrsoides* showed phenotypic plasticity in both SLA and LT, and large genetic variation across an elevation gradient between 1600 to 2200 ma.s.l. (Scheepens et al., 2010). In Egypt, populations of *Achillea fragrantissima* growing at high altitudes had larger plant size and greater number of total and polymorphic ISSR markers compared to populations growing at low elevations in more arid sites (Badr et al., 2017). In *Glycine max* (soybean) and *Penstemon centranthifolius* inflorescence length was determined as a hereditary trait (Mitchell & Shaw, 1993; Wang et al., 2019). These environment-induced phenotypic variations could also be influenced by genetic effects due to local adaptation or genetic drift (Scheepens et al., 2010).

In mountainous regions in the Andes, the Alps and Tibet, there has been evidence of pollinator limitation at high elevations (e.g., Alonso, 2005; Arroyo et al., 2017; Ramos-Jiliberto et al., 2010; Zhao & Wang, 2015). However, there is a paucity of studies evaluating the effect of elevation gradients on pollen limitation (Levis & Pfennig, 2020). These processes are essential to understand selection forces favoring plant speciation along elevation gradients (Cardona et al., 2020; Matesanz et al., 2020; Sun et al., 2020).

We evaluated morphological divergence and reproductive isolation on *Croton* aff. *wagneri* populations at an elevation gradient in the Andes of Loja, Ecuador. *Croton* aff. *wagneri* is a dominant member of plant communities occurring in inter-Andean regions of Ecuador (León-Yánez et al., 2011). Our study occurred in the lowest zone of the Andes called the Andean Depression (Richter et al., 2009). In this zone, elevation gradients cause abrupt changes in moisture and temperature, generating different microenvironments (Quintana et al., 2017; Richter & Moreira-Muñoz, 2005). Our study comprised a narrow elevation range of 300 m (Vélez-Mora et al., 2020). We hypothesize that the variation of moisture and temperature throughout this elevation gradient influence plant morphological traits (specific leaf area and leaf thickness) and reproductive isolation between *Croton* aff. *wagneri* populations located at different elevations. Our research objectives were: (1) Determine morphological divergence of *Croton* aff. *wagneri* at different elevations; (2) Evaluate reproductive isolation of *Croton* aff. *wagneri* at different elevations, and (3) Assess pollen limitation at each elevation. We refer to pollen limitation as limited pollen receipt resulting from insufficient visits by pollen-bearing animals (Willmer, 2011). We measured vegetative and reproductive trait variation among populations at three different elevations and performed experimental pollination crosses among and within elevations. We measured fitness components such as fruit set, and seed set associated with the different crosses as indicators of reproductive isolation and pollinator limitation.

Methods

Study Species

Croton aff. *wagneri* (Euphorbiaceae, hereafter *Croton*) is a 0.5—1.5 m tall monoecious shrub (Ulloa & Jørgensen, 1995; Fig. 1B). *Croton* has axillary or terminal inflorescences with small unisexual flowers (Ulloa & Jørgensen, 1995). It has pubescent female flowers located at the base of the inflorescence and male flowers at the top (Ulloa & Jørgensen, 1995; Fig. 1C and D). Male *Croton* flowers alternate occasionally in the middle with a few female flowers (Ulloa & Jørgensen, 1995; Webster, 1993). Number of flowers per inflorescence can vary between 10 and 60 male flowers and between 4 and 16 female flowers (Vélez-Mora unpublished data). Female *Croton* flowers are sessile or united with short pedicels (Ulloa & Jørgensen, 1995). The female flower calyx has five segments attached at the base with petals and glands generally absent (Ulloa & Jørgensen, 1995). The gynoecium consists of a compound pistil (van Ee et al., 2011). The

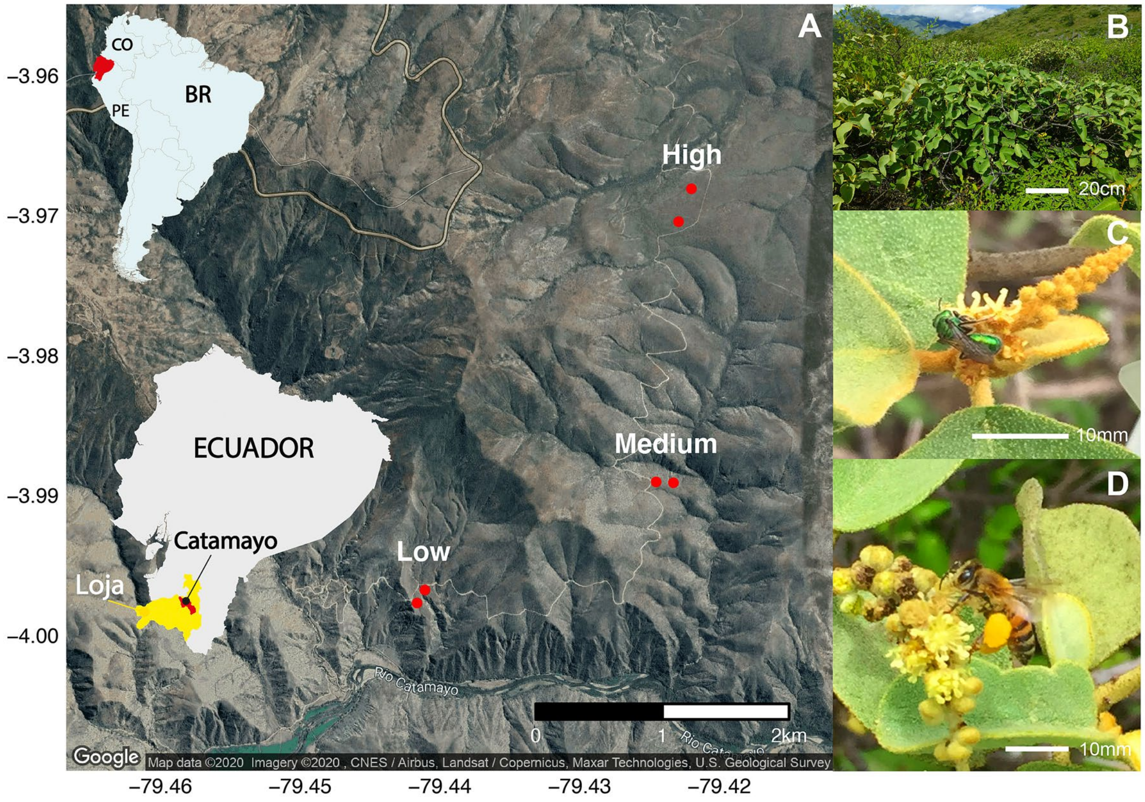

Fig. 1 A: Map of Ecuador showing the locations of studied sites colored red at each elevation. B: Study species. C: A species of wasp visiting female *Croton* flowers. D: A bee visiting male *Croton* flowers

ovary has three locules with one seminal primordium per locule (Ulloa & Jørgensen, 1995; van Ee et al., 2011). Styles are bifid and are 3 to 6 mm long (Ulloa & Jørgensen, 1995; Webster, 1993; personal observation). There may be two to three male flowers that are supported by a bract with five sepals attached at the base and five reduced petals (Ulloa & Jørgensen, 1995). Number of stamens varies from 10 to 20 and the filaments are free (Ulloa & Jørgensen, 1995; Webster, 1993). Stamens are 3 to 6 mm long (personal observation). *Croton* is likely pollinated by a variety of nectar-seeking pollinator species, particularly those belonging to the Hymenoptera and Diptera insect orders (Webster, 2014). Average number of fruits per inflorescence is 4.85 + SE 0.04 (Vélez-Mora unpublished data). Most seed dispersal occurs within a few meters (Espinosa et al., 2019; Jara-Guerrero et al., 2015). The female flowers bloom before the male flowers, possessing a well-established dichogamy avoiding geitonogamy in the same inflorescence (Domínguez & Bullock, 1989; Webster, 2014). However, pollination between male and female flowers from different inflorescences in the same plant can occur (c.f. Domínguez & Bullock, 1989).

Study Site

Our study was performed at an inter-Andean valley at Catamayo, Loja Province, Ecuador. This valley extends into the basin of the Río Catamayo from the riverbed at 1100 m a.s.l. to approximately 2700 m a.s.l. at the local highest point. In this valley *Croton* is distributed continuously between 1100 and 2080 m of elevation (Leal, 2015; personal observation). *Croton* shrubs are conspicuous elements in a sparse xerophytic and spiny vegetation (Fig. 1B). Plant species surrounding *Croton* are perennials such as *Lantana canescens* Kunth, *Vachellia macracantha* (Humb. & Bonpl. ex. Willd.) Seigler & Ebinger, *Bursera graveolens* (Kunth) Triana y Planch., *Colicodendron scabridum* (Kunth) Seem. and *Opuntia quitensis* F.A.C. Weber (Espinosa et al., 2013, 2019; Sierra, 1999; Ulloa & Jørgensen, 1995). The topography is rugged with moderately steep slopes (Fig. 1A). Ungulates (donkeys, cows, goats) wander on the property (Espinosa et al., 2013). We have not seen these animals browsing on *Croton,* and local herders confirmed that these animals seldom feed on it, but we have observed livestock trample seedlings and small plants and damaging plant branches. This ecosystem experiences hot (24.8 °C mean annual temperature) and dry weather, with an extended dry season (317 mm mean annual precipitation, 57% of it occurring between February and May) (Espinosa et al., 2019; Figure S1A). Water deficit is prevalent 10 months a year (Espinosa et al., 2019; Figure S1A).

We selected six sites, two per elevation, at three elevations within the Hacienda Alamala: 1700 m a.s.l. identified as High Elevation (3° 58′ 07.90″ S, 79° 25′ 19.71″ W), 1550 m a.s.l. identified as Medium Elevation (3° 59′ 20.09″ S, 79° 25′ 28.64″ W) and 1400 m a.s.l. identified as Low Elevation (3° 59′ 40.15″ S, 79° 26′ 31.29″ W; Fig. 1A). Distances among sites in different elevations were greater than 2.5 km (but less than 6 km) while sites within the same elevation were at least 100 m apart (but less than 300 m apart).

Morphological Traits of Croton Plants Between Elevations

To evaluate morphological traits of *Croton* we selected haphazardly breeding plants at two sites at each elevation. We selected eight plants per site for a total of 48 plants. We randomly collected four leaves per plant (n = 192 leaves). To measure leaf area, we used a CI-202 Portable Laser Area Meter (CID Bio-Science Inc, Camas, Washington, USA). Fresh weight of these leaves was obtained 1 h after collection. Leaves were dried for 42 h in a forced air oven (DFO-36, MRC ltd., United Kingdom). Fresh weight and dry weight of leaves were measured on an analytical scale (OHAUS PA84C, Analytical Pioneer, China). Specific leaf area (*SLA*; cm^2 g^{-1}), leaf dry matter content (*LDMC*; mg g^{-1}) and leaf thickness (*LT*; µm) were calculated with the following equations (Vile et al., 2005):

$$SLA = \frac{LA}{LD}$$

$$LDMC = \frac{LD}{1-(LW)}$$

$$LT = \frac{1}{(SLA \times LDMC)}$$

where *LA* is leaf area (cm^2), *LD* is leaf dry mass (g) and *LW* is leaf water content (g). We characterized each plant by their volume (see Vélez-Mora et al., 2020), number of inflorescences and average inflorescence length. We evaluated plant volume as a measure of resource availability (Shivanna & Tandon, 2014) and number of inflorescences and average inflorescence length as a measure of reproductive effort (Harper & Ogden, 1970). When plants had ~ 100 inflorescences, we counted all inflorescences on the plant. In some individuals the number of inflorescences was estimated because they were too numerous (from January to March; Vélez-Mora 2019, unpublished data). We divided plant canopy into four quadrants, selected one at random, counted the number of inflorescences and multiplied it by four. For inflorescence length, we measured five inflorescences randomly per plant.

We used generalized linear mixed effect models (using R package *nlme*; Pinheiro et al., 2020) to evaluate leaf thickness variation as a function of specific leaf area

(Supplementary Information Table S1) and elevation (elevation and specific leaf area as fixed factors and sites and plants as random factors). We evaluated variation on number of inflorescences (transformed with logarithms) and inflorescence length as a function of plant volume (transformed with logarithms) and elevation (plant volume and elevation as fixed factors and sites and plants as a random factor). Hierarchically, we evaluated the random effects of plants within sites and sites within elevations. The inclusion of plant volume and specific leaf area as state variables allowed us to account for the effect of variation in plant and leaf size among individuals. We identified the most likely model for each set using small sample Akaike criteria (AICc; Burnham & Anderson, 2002; see Supplementary Information Tables S1-S9). For inflorescence length, there were no clear differences between the two most likely models. We choose the model with the effects of elevation and volume because it had the highest r-squared among the set and it was consistent with previous findings (Supplementary Information Tables S7). All analyses were performed in version 3.6.2 of R software (R Core Team, 2019).

Study Site Micro-Climate

During our field season between 1 and 28 Feb. 2019, we monitored at each elevation air temperature and humidity, soil temperature and moisture at a depth of 10 cm, and photosynthetic light, all with a sampling interval of five minutes, using HOBO data loggers S-TMB-M006, S-SMC-M005, and S-LIA-M003 (Onset, Bourne, Massachusetts, USA). We built an ordination regularized discriminant analysis (RDA; *rda* function in package vegan of R Core Team, 2019; Oksanen et al., 2019) to describe the combined association of these variables with elevation in our sites. Regularized discriminant analysis ordination included the mean and the standard deviation of daily air temperature, daily soil temperature, relative air humidity, photosynthetic active radiation, soil moisture and dew point (Supplementary Information Fig. S1 and Fig. S2). We measured with Spearman correlations (*cor* function; stats package of R Core Team, 2019) the association of these scores with the studied leaf attributes to identify possible different selection pressures across elevations.

Reciprocal Pollinations Between Elevations to Assess Reproductive Isolation

We performed reciprocal hand pollinations between the High, Medium, and Low elevations. Pollinations began on 7 Feb. 2019, during the species' peak bloom. Two sites were selected per elevation and eight breeding plants were chosen at each site (total 48 plants). One day before the first pollination, inflorescences with pre-anthesis female buds were bagged to ensure that they were not pollinated. Reciprocal pollinations consisted of hand pollinations between elevations and bagging of the inflorescences after being pollinated. Reciprocal pollinations were contrasted with cross pollination within each elevation. This treatment consisted of bagging and hand pollination with pollen from other plants of the same population. In the first attempt, one day after bagging, two trials of hand pollination were carried out per plant and inflorescence: one in the morning (between 700 and 1100 h) and another in the afternoon (between 1400 and 1800 h) in the same inflorescence. Additional pollinations were attempted on the fourth day for those flowers that were not in anthesis before. A third and last pollination was repeated after 15 days for remaining flowers that did not open before. No female flowers remained available in the chosen inflorescences after this final attempt. A total of 144 pollination trials were carried out (eight plants × two sites × three elevations × two pollen provenances from respective elevation + crosses within elevation). The experimental unit was the inflorescence for each treatment.

For hand pollination, fresh inflorescences with male flowers at each site were collected one hour before each pollination attempt. Inflorescences were kept in a cooler in plastic bags. Three male flowers with pollen available from different donor plants were used. To check availability of pollen, anthers were rubbed against a clean piece of black cloth. Anthers of each male flower were gently rubbed one by one over stigmas of all open female flowers (Supplementary Information Fig. S3). After 36 days we collected the fruits of the reciprocal pollinations. Not all the fruits reached full maturity because we had to collect them before livestock could damage the bags from the treatments. We counted number of aborted fruits, number of fruits developed and number of seeds for each fruit. We consider as aborted fruits scars left on the inflorescences by female reproductive structures that fell before developing (see Domínguez & Bullock, 1989). Total fruits were the sum of aborted fruits and developed fruits in the inflorescence. Fruit set of each treatment was calculated as proportion of fruits developed throughout the inflorescence. Seed set for each treatment was calculated as proportion of seeds developed in all fruits of the entire inflorescence. We calculated the reference potential number of seeds assuming three seeds per fruit. Reproductive isolation (RI) was calculated following Sobel and Chen (2014). The reproductive isolation for fruit set and seed set was calculated as:

$$RI = 1 - 2 \times \left(\frac{X_{CBE}}{X_{CWE} + X_{CBE}} \right)$$

where X_{CBE} is the value of fruit and seed set of the reciprocal crosses between elevations and X_{CWE} is the value of fruit and seed set of the crosses within each elevation. Reproductive isolation values range from 1 (complete isolation) to -1 (complete disassortative mating). RI = 0 indicates random mating (Sobel & Chen, 2014; Ramírez-Aguirre et al., 2019). We considered for the analysis of reproductive isolation only plants greater than 400,000 cm^3 (41 large plants of a total of 48) because we observed that smaller plants have reduced fertility (mean of large plant fruits 5.1 ± 0.66 SE and small plants 2.9 ± 0.98 SE; Espinosa et al., 2019; see Supplementary Information Fig. S4). We calculated the mean RI (and its 95% confidence interval) for each of the pairs of crosses per elevation. We considered clear evidence of reproductive isolation when crosses with the reproductive isolation index (RI) had a positive value and its confidence interval bar did not overlap zero.

Pollen Limitation Within Each Elevation

We carried out four pollination treatments to estimate pollen limitation: (1) Crosses within elevation (also incorporated in reproductive isolation analysis); (2) Hand-geitonogamous pollination: bagging and manual pollination with pollen of the same plant; (3) Open pollination: not bagging and no manual pollination, and (4) Control: bagged flowers to eliminate access of animal pollinators. These treatments were carried out in parallel with reciprocal pollination between elevations for reproductive isolation, so they were carried out under the same protocol. Inflorescences of open pollination treatment were bagged six days after last pollination to protect developing fruits. A total of 144 trials were conducted excluding treatment of crosses within elevation (eight plants × two sites × three elevations × three treatments). The experimental unit was the inflorescence for each treatment. Plants for the pollination treatments were different from the plants used in the measurement of leaves so as not to influence their reproductive investment. For the treatments that involved bagging, 4 × 6" pollination bags of non-woven transparent polypropylene fabric were used (Carolina Biological Supply Company, Burlington, North Carolina). We also only considered plants larger than 400,000 cm^3 to analyze pollen limitation. We compared the four pollination treatments using a non-parametric paired Wilcoxon test.

Results

Morphological Traits of Croton Plants Between Elevations

Leaf thickness decreased with specific leaf area (Fig. 2A). Leaf thickness was higher in the High Elevation compared to the other two elevations (Fig. 2A, Supplementary Information Table S3). Number of inflorescences and inflorescence length increased with plant volume (Fig. 2B and C). Number of inflorescences was different between elevations and was higher in High Elevation (Fig. 2B). Inflorescence length was greater in the Low Elevation compared to the High Elevation

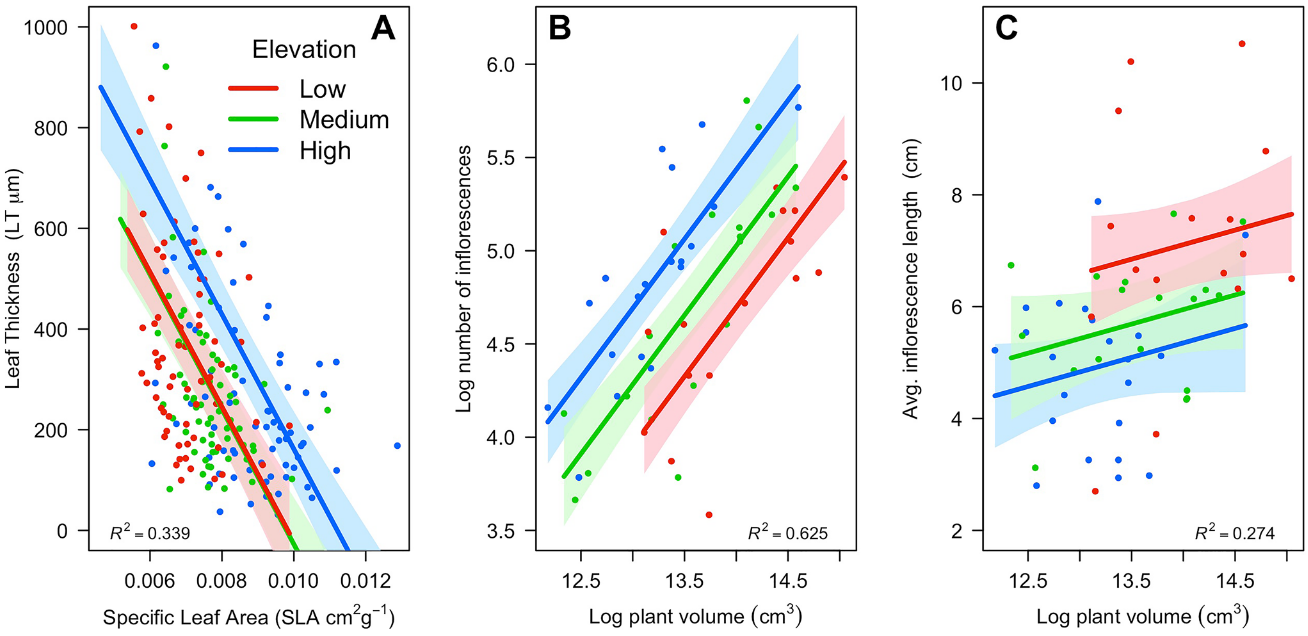

Fig. 2 Variation of morphological traits of *Croton* according to elevation. A: Leaf thickness increased according to specific leaf area and was higher in the High Elevation. B: Number of inflorescences increased according to plant volume and was higher in the High Elevation. C: Average inflorescence length increased according to plant volume and was higher in the Low Elevation

(Fig. 2C). We did not observe differences in inflorescence length between Medium Elevation and High Elevation or between Medium Elevation and Low Elevation.

Climate

The regularized discriminant analysis differentiated the elevations with the climate variables studied. The percentage of the variance explained by the first axis (PC1) was 93% and the percentage explained by the second axis (PC2) was 7%. The first axis (PC1—Fig. 3) clearly separated the three elevations while the second axis (PC2) clearly separated the Low Elevation from the Medium Elevation and the High Elevation that were like each other in this axis. In the first axis, the High Elevation had the lowest scores associated with high average soil and air humidity and high soil water content variation but low values for all the other environmental variables. In the first axis, the Low Elevation had intermediate scores and the Medium Elevation the high scores. In the second axis, the Low Elevation had the highest scores, while the High Elevation and Medium Elevation had lower scores. High scores in the second axis were associated with more variable air and soil humidity, higher temperatures, and higher dew points. Change in leaf thickness with elevation, adjusted by leaf area (coefficients for elevation in Supplementary Information Table S1) was associated with the first axis of the environmental regularized discriminant analysis (Spearman correlation = -1).

Reciprocal Pollinations Between Elevations to Assess Reproductive Isolation

We used fruit and seed set variation of reciprocal pollinations between elevations contrasted with cross pollinations within each elevation to calculate reproductive isolation (RI). Using fruit set we recognized reproductive isolation between Low Elevation (as pollen donors) and High Elevation plants (as pollen receptors) and between Medium Elevation (as pollen donors) and High Elevation plants (as pollen receptors; Fig. 4). Using seed set, the results were consistent with those of fruit set but less clear (Fig. 4). We did not find clear evidence of reproductive isolation or disassortative mating of plants in any other cross between populations.

Pollen Limitation Within Each Elevation

Fruit and seed set with open pollination were clearly higher at Low Elevation compared to the other two elevations and treatments (Fig. 5). Fruit and seed set with open pollination were higher compared to control treatment

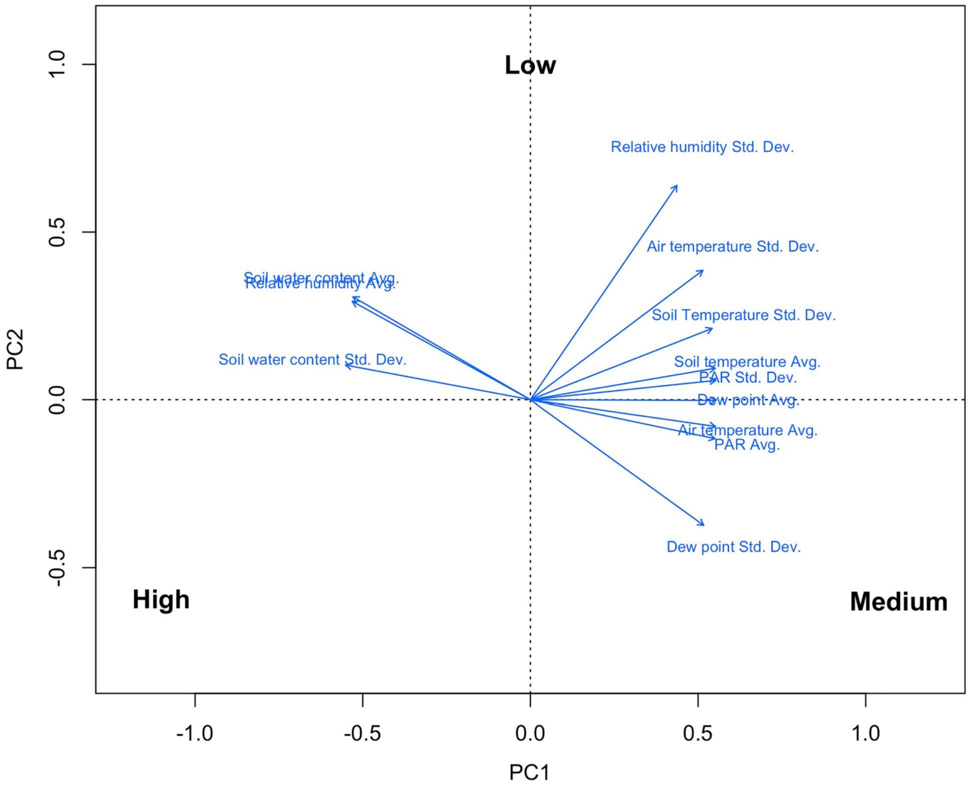

Fig. 3 The regularized discriminant analysis differentiated the elevations with the climate variables studied. The first axis (PC1 explained 93% of the variance) clearly separated the three elevations while the second axis (PC2 explained 7% of the variance) clearly separated the Low Elevation from the Medium Elevation and the High Elevation that were like each other in this axis

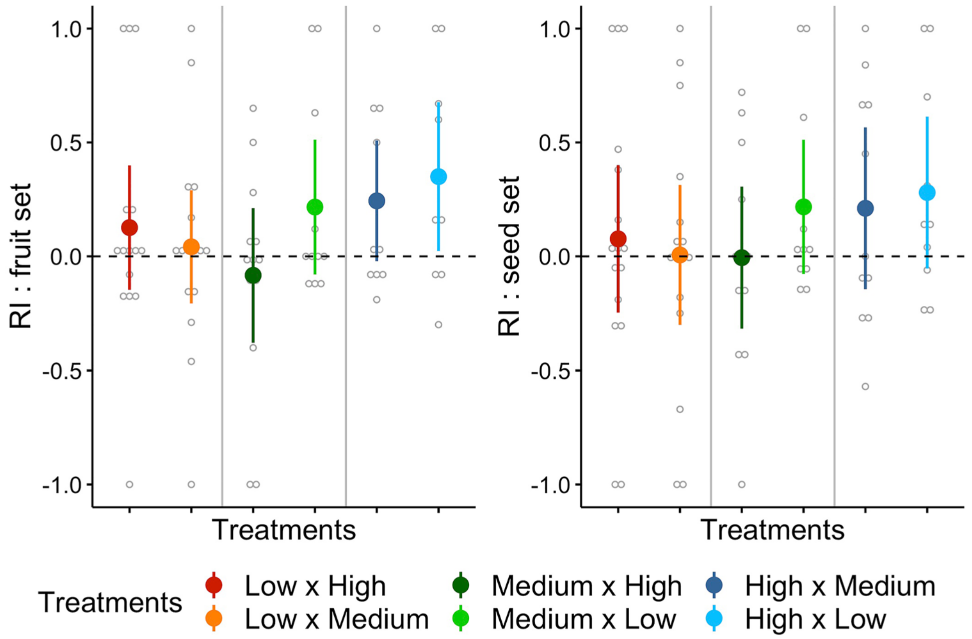

Fig. 4 Reciprocal crosses between elevations to determine reproductive isolation (RI) by fruit and seed set of Croton. The mean and the confidence intervals indicated reproductive isolation between Low Elevation plants and High Elevation plants according to fruit set

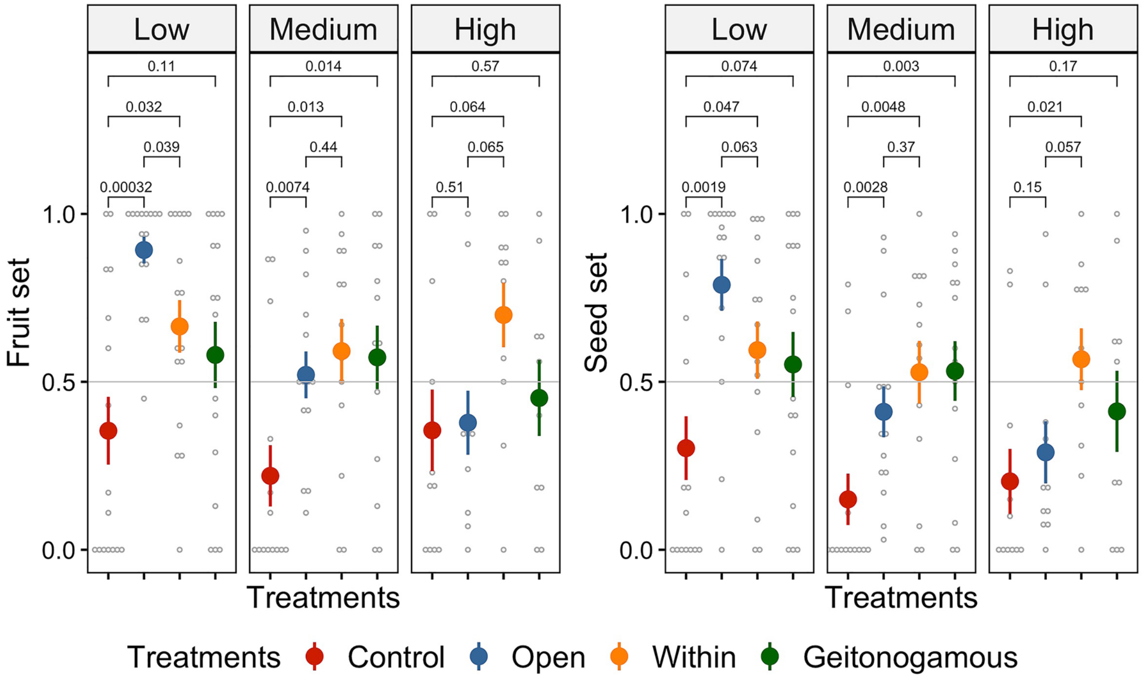

Fig. 5 Fruit and seed set by treatment within each elevation. We compared differences among the four pollination treatments using a non-parametric paired Wilcoxon test after an analysis of variance. Fruit and seed set with open pollination were higher at Low Elevation compared to the other two elevations and treatments. Fruit and seed set was marginally different between within crosses and open bag treatment at High Elevation

at Low and Medium elevations (Fig. 5). Fruit and seed set were clearly higher with crosses within and hand-geitonogamy compared to control treatment at Medium Elevation (Fig. 5). Fruit and seed set did not differ between the treatments at High Elevation, except between the treatments within and control by seed set (Fig. 5).

Discussion

Our data contribute to a better understanding of the interaction between elevation, morphological trait variation, reproductive isolation, and pollinator limitation among *Croton* in the southern Ecuadorian Andes. Individual morphological trait variation of *Croton* plants was concordant with reproductive isolation between individuals of populations at different elevations.

Morphological Traits of *Croton* Plants Between Elevations

Our results evidenced morphological divergences between *Croton* populations at different elevations. The plants at the High Elevation produced a greater number of inflorescences. Their leaves were thicker and with a greater specific leaf area. However, plants at the High Elevation had less volume, and the inflorescence length was shorter. Higher inflorescence production suggests that plants possess sufficient resources to invest in flower and seed production and prioritize reproduction instead of growth (Fabbro & Körner, 2004). Allocation of resources to reproductive structures in alpine plant species was three times greater at high elevations than at low elevations (Fabbro & Körner, 2004). High number of *Croton* flowers at the High Elevation could be associated with a higher availability of nitrogen and organic matter existing at this elevation (Wright et al., 2004; Supplementary Information Fig. S5). In addition, in the High Elevation resources for the reproductive function are in greater proportion for production of male *Croton* flowers, the most economical reproductive function (Velez-Mora et al., 2020). In the high tropical mountains, climate is cold and dry, and air and soil temperatures decrease with increasing elevation (Domic & Capriles, 2009). Under these conditions, plants produce thick leaves to counteract drought and mechanical stress caused by the wind (Pérez et al., 2020; Vogel, 2009). Several studies indicate reduction of specific leaf area with increasing elevation to withstand unfavorable environmental conditions (Apaza-Quevedo et al., 2015; Jian et al., 2009; Scheepens et al., 2010). Our results showed an opposite relationship in *Croton*, with a lower specific leaf area at Low Elevation probably due to higher soil water stress. In *Viola maculata* in the Central Andes of Chile, for example, the specific leaf area and stomatal conductance increased with elevation, reducing water stress (Seguí et al., 2018). This relationship is consistent with specific leaf area and soil moisture content data in *Croton* (Supplementary Information Fig. S1). This relationship suggests that *Croton* can develop differential responses to the environment at relatively short distances, either by phenotypic plasticity or by genetic adaptations.

Leaf thickness and specific leaf area generally tend to evolve separately from plant size (Pérez et al., 2020). Reduction in plant size at high elevations serves as self-shading where plants adhere to the ground to decouple their climate from the surrounding environment, prevent desiccation, and accumulate heat in the plant canopy (Hallik et al., 2009). Inflorescence length also shortens with increasing elevation in alpine environments (Fabbro & Körner, 2004). Shortening of inflorescences at high elevations creates a warm environment that could substitute for the advantage of large plants to attract the most pollinators (Donnelly et al., 1998). These strategies can increase seed set and attractiveness to pollinators (Fabbro & Körner, 2004).

Reciprocal Pollinations Between Elevations to Assess Reproductive Isolation

Our results indicated an incipient reproductive isolation between *Croton* populations at the most extreme studied elevations in this relatively short gradient. Environmental heterogeneity generated by elevation can lead to local adaptation in plants (Chapman et al., 2016; Cordell et al., 1998). Recent studies have suggested that phenotypic plasticity may signal genetic divergence between populations (Caetano et al., 2020; Walter et al., 2020). The *Achillea fragrantissima* populations that grew at high altitudes differed from the rest by being larger and presenting a greater number of ISSR markers in Egypt (elevation range between 132 and 1154 m; Badr et al., 2017). A genetic study on *Croton* populations in the same geographic area as ours but at different elevations and using AFLP markers provided evidence of clear genetic differentiation between high and low elevation populations (Leal, 2015). *Croton* populations of each elevation still maintained a partial compatibility with each other in agreement with our results.

Pollen Limitation Within Each Elevation

Open pollination produced higher fruit and seed set at Low Elevation and similar values as other treatments (except bagged flowers) in Medium Elevation. This did not occur at High Elevation where fruit and seed set were higher with hand pollination with pollen from neighboring plants compared to the other treatments, including open pollination. The lower fruit and seed set at High Elevation in open pollination offers us indirect evidence of limitation of pollinators in *Croton*. Our study did not evaluate diversity of pollinators associated with *Croton* pollination. However, several studies have shown decreases in abundance and diversity of pollinators as elevation increases consistent with our preliminary observations in this region (Arroyo et al., 2006;

Ramos-Jiliberto et al., 2010; Torres-Díaz et al., 2011). In two subspecies of *Campanula spatulata*, insect visitation rate decreased with elevation on Mount Olympos (elevation range 400—2200 m.a.s.l; Blionis & Vokou, 2002). In the same study, plant size, flower size and number of flowers differed between elevations indicating reproductive isolation and morphological divergence in these subspecies (Blionis & Vokou, 2002). Low visit rates of pollinators at high elevation were consistent with low output of fruit and seed of *Croton* at this elevation. Scarcity or ineffectiveness of pollinators to ensure pollen dispersal at high elevations and limited dispersal of *Croton* seeds (Espinosa et al., 2019) could restrict gene exchange between populations at different elevations (Cardona et al., 2020; van der Niet et al., 2014).

Intrinsic barriers to gene flow and ecological divergence produce reproductive incompatibilities between ecotypes (Schliewen et al., 2001; Walter et al., 2018, 2020). There were clear differences in temperature and moisture between high and low elevation in our study site. These climatic conditions could generate a strong selection for both plants and pollinators (Cardona et al., 2020; Halbritter et al., 2018). Several studies show how insect composition and environmental conditions change along elevation in narrow geographic spaces in the Andes (Arroyo et al., 2017; Hall, 2005; Medina et al., 2002; Pyrcz, 2004; Ramos-Jiliberto et al., 2010). Change in composition of insects could generate a restriction of gene flow between elevations and strengthen local adaptation of *Croton* at each elevation (Peakall & Whitehead, 2014; van der Niet et al., 2014). Local adaptation could help *Croton* plants to adjust in response to their habitat and pollination environments (Dai et al., 2017). Our findings are consistent with other studies where ecological adaptations (SLA for example according to Scheepens et al., 2010) in combination with reproductive isolation could lead to a divergence of *Croton* populations in parapatry (Itino & Hirao, 2016; Nosil, 2012; Walter et al., 2020). Low gene flow through reduced dispersal of pollen and seeds (Espinosa et al., 2019) could be causing reproductive isolation between populations of *Croton* at high and low elevations. Abrupt changes in temperature and humidity along elevation gradient could be selective forces that disrupt pollinator movement between elevations (Bridle & Vines, 2007; Halbritter et al., 2018; Lenormand, 2002). This could reinforce genetic isolation barriers for *Croton* in High Elevation populations probably leading to divergence and local adaptation at each elevation (White et al., 2020). Elevation gradients offer an interesting opportunity to study adaptive traits under strong selection pressure and homogenizing effect of gene flow (Gonzalo-Turpin & Hazard, 2009; Halbritter et al., 2018; Sexton et al., 2011). In these regions of the Andes there is a great diversity of species, many of them endemic with restricted elevation which will benefit from a better understanding of their population ecology and evolution (c.f. Herzog et al., 2011; Homeier et al., 2010; Josse et al., 2009; Quintana et al., 2017).

Limitations

In this study, it was not possible to determine differences in other fitness components between pollination treatments and elevations since fruits were collected before their complete development to prevent cows and goats in the study site from damaging the experimental bags. However, independent information indicated that Low Elevation seeds (0.0090 ± SE = 0.00013 g; n = 716) were larger and heavier compared to Medium Elevation seeds (0.0066 ± SE = 0.00032 g; n = 111) and High Elevation (0.0064 ± SE = 0.00019 g; n = 330; unpublished data from Vélez-Mora). Future work should directly evaluate composition and visitation rates of pollinators in this elevation gradient. Likewise, experiments based on reciprocal transplants of plants or experiments in common garden conditions should be carried out to evaluate genotypic and phenotypic differences between populations to reaffirm our interpretations.

Conclusion

Our study provides observational and experimental evidence for simultaneous variation of plant morphological traits and incipient reproductive isolation of *Croton* in a narrow elevation gradient with contrasting climatic conditions. Lower number of fruits and seeds of the High Elevation population compared to the Low Elevation population and pollination experiments within each elevation evidenced a limitation of pollinators in *Croton* in the High elevation population. Intrinsic barriers to pollen and seed dispersal, and ecological divergence may produce reproductive incompatibilities between individuals with different traits along the *Croton* elevation gradient.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11692-021-09541-1.

Acknowledgements We thank to Inés Vélez, Ismael Vélez and many *Universidad Técnica Particular de Loja* students for their help in the field. Thanks to Monterrey Azucarera Lojana C.A. and the Jaramillo family for access to their beautiful property. We also thank Chris Brinegar and Javier Morente-López for valuable comments on this document.

Author's contributions DPVM and PFQA conceived the study and design. DPVM, KTA and PFQA collected the samples and measurements. DPVM carried out the pollinations. PFQA conducted the analyses. DPVM and PFQA wrote the draft of the manuscript. All authors edited and reviewed the final version of the manuscript.

Funding This work was supported by the *Secretaría de Educación Superior, Ciencia, Tecnología e Innovación, Ecuador*

PIC-13-ETAPA-005 to DPVM, and The Winter Park Garden Club through a University of Central Florida endowment to PFQA.

Availability of data and material If this paper is accepted our data will be deposited in Dryad Digital Repository. However, they will be available for Evolutionary Biology and for peer reviewers if required.

Code availability Code will be sent to the reviewers when they require it.

Compliance with ethical standards

Conflict of interest: The authors have no conflict of interest to declare.

References

Adams, C. E., & Huntingford, F. A. (2004). Incipient speciation driven by phenotypic plasticity? Evidence from sympatric populations of Arctic charr. *Biological Journal of the Linnean Society, 81*(4), 611–618.

Alix, K., Gérard, P. R., Schwarzacher, T., & Heslop-Harrison, J. S. P. (2017). Polyploidy and interspecific hybridization: Partners for adaptation, speciation and evolution in plants. *Annals of Botany, 120*(2), 183–194.

Alonso, C. (2005). Pollination success across an elevation and sex ratio gradient in gynodioecious *Daphne laureola*. *American Journal of Botany, 92*(8), 1264–1269.

Apaza-Quevedo, A., Lippok, D., Hensen, I., Schleuning, M., & Both, S. (2015). Elevation, topography, and edge effects drive functional composition of woody plant species in tropical Montane forests. *Biotropica, 47*(4), 449–458.

Arroyo, M. T. K., Muñoz, M. S., Henríquez, C., Till-Bottraud, I., & Pérez, F. (2006). Erratic pollination, high selfing levels and their correlates and consequences in an altitudinally widespread above-tree-line species in the high Andes of Chile. *Acta Oecologica, 30*(2), 248–257.

Arroyo, M. T. K., Pacheco, D. A., & Dudley, L. S. (2017). Functional role of long-lived flowers in preventing pollen limitation in a high elevation outcrossing species. *AoB Plants, 9*(6), 195–212.

Badr, A., El-Shazly, H. H., Ahmed, H. I. S., Hamouda, M., El-Khateeb, E., & Sakr, M. (2017). Genetic diversity of *Achillea fragrantissima* in Egypt inferred from phenotypic variations and ISSR markers associated with traits of plant size and seed yield. *Plant Genetic Resources, 15*(3), 239–247.

Blionis, G. J., & Vokou, D. (2002). Structural and functional divergence of *Campanula spatulata* subspecies on Mt Olympos (Greece). *Plant Systematics and Evolution, 232*(1), 89–105.

Bridle, J. R., & Vines, T. H. (2007). Limits to evolution at range margins: When and why does adaptation fail? *Trends in Ecology & Evolution, 22*(3), 140–147.

Burnham, K. P., & Anderson, D. R. (2002)**.** *Model selection and multimodel inference*: *a practical information-theoretic approach* (2nd Edn.). Springer.

Caetano, R. A., Sanchéz, S., Costa, C. L. N., & de Aguiar, M. A. M. (2020). Sympatric speciation based on pure assortative mating. *Journal of Physics A: Mathematical and Theoretical*, *53*(15), 155601.

Cardona, J., Lara, C., & Ornelas, J. F. (2020). Pollinator divergence and pollination isolation between hybrids with different floral color and morphology in two sympatric *Penstemon* species. *Scientific Reports, 10*(1), 8126.

Chapman, M. A., Hiscock, S. J., & Filatov, D. A. (2016). The genomic bases of morphological divergence and reproductive isolation driven by ecological speciation in *Senecio* (Asteraceae). *Journal of Evolutionary Biology, 29*(1), 98–113.

Chevin, L.-M., Lande, R., & Mace, G. M. (2010). Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLoS Biology, 8*(4), e1000357.

Chitwood, D. H., Ranjan, A., Martinez, C. C., Headland, L. R., Thiem, T., Kumar, R., Covington, M. F., Hatcher, T., Naylor, D. T., Zimmerman, S., Downs, N., Raymundo, N., Buckler, E. S., Maloof, J. N., Aradhya, M., Prins, B., Li, L., Myles, S., & Sinha, N. R. (2014). A modern ampelography: a genetic basis for leaf shape and venation patterning in grape. *Plant Physiology, 164*(1), 259–272.

Cierjacks, A., Rühr, N. K., Wesche, K., & Hensen, I. (2008). Effects of altitude and livestock on the regeneration of two tree line forming *Polylepis* species in Ecuador. *Plant Ecology, 194*(2), 207–221.

Cordell, S., Goldstein, G., Mueller-Dombois, D., Webb, D., & Vitousek, P. M. (1998). Physiological and morphological variation in *Metrosideros polymorpha*, a dominant Hawaiian tree species, along an altitudinal gradient: the role of phenotypic plasticity. *Oecologia, 113*(2), 188–196.

Corl, A., Davis, A. R., Kuchta, S. R., & Sinervo, B. (2010). Selective loss of polymorphic mating types is associated with rapid phenotypic evolution during morphic speciation. *Proceedings of the National Academy of Sciences, 107*(9), 4254–4259.

Cruz-Nicolás, J., Giles-Pérez, G., González-Linares, E., Múgica-Gallart, J., Lira-Noriega, A., Gernandt, D. S., Eguiarte, L. E., & Jaramillo-Correa, J. P. (2020). Contrasting evolutionary processes drive morphological and genetic differentiation in a subtropical fir (*Abies*, Pinaceae) species complex. *Botanical Journal of the Linnean Society, 192*(2), 401–420.

Dai, W., Kadiori, E. L., Wang, Q., & Yang, C. (2017). Pollen limitation, plasticity in floral traits, and mixed mating system in an alpine plant *Pedicularis siphonantha* (Orobanchaceae) from different altitudes. *Journal of Systematics and Evolution, 55*(3), 192–199.

Depardieu, C., Gérardi, S., Nadeau, S., Parent, G. J., Mackay, J., Lenz, P., Lamothe, M., Girardin, M. P., Bousquet, J., & Isabel, N. (2021). Connecting tree-ring phenotypes, genetic associations and transcriptomics to decipher the genomic architecture of drought adaptation in a widespread conifer. *Molecular Ecology*. https://doi.org/10.1111/mec.15846

Domic, A. I., & Capriles, J. M. (2009). Allometry and effects of extreme elevation on growth velocity of the Andean tree *Polylepis tarapacana* Philippi (Rosaceae). *Plant Ecology, 205*(2), 223–234.

Domínguez, C. A., & Bullock, S. H. (1989). La reproducción de *Croton suberosus* (Euphorbiaceae) en luz y sombra. *Revista De Biología Tropical, 37*(1), 1–9.

Donnelly, S. E., Lortie, C. J., & Aarssen, L. W. (1998). Pollination in *Verbascum thapsus* (Scrophulariaceae): The advantage of being tall. *American Journal of Botany, 85*(11), 1618–1625.

Draghi, J. A., & Whitlock, M. C. (2012). Phenotypic plasticity facilitates mutational variance, genetic variance, and evolvability along the major axis of environmental variation. *Evolution, 66*(9), 2891–2902.

Espinosa, C. I., Luzuriaga, A. L., de la Cruz, M., Montero, M., & Escudero, A. (2013). Co-occurring grazing and climate stressors have different effects on the total seed bank when compared to the persistent seed bank. *Journal of Vegetation Science, 24*(6), 1098–1107.

Espinosa, C. I., Vélez-Mora, D. P., Ramón, P., Gusmán-Montalván, E., Duncan, D. H., & Quintana-Ascencio, P. F. (2019). Intraspecific interactions affect the spatial pattern of a dominant shrub in a semiarid shrubland: A prospective approach. *Population Ecology, 61*(2), 217–226.

Fabbro, T., & Körner, C. (2004). Altitudinal differences in flower traits and reproductive allocation. *Flora—Morphology, Distribution, Functional Ecology of Plants, 199*(1), 70–81.

Fenster, C. B. (1995). Mirror image flowers and their effect on outcrossing rate in *Chamaecrista fasciculata* (Leguminosae). *American Journal of Botany, 82*(1), 46–50.

Gomez-Mestre, I., & Buchholz, D. R. (2006). Developmental plasticity mirrors differences among taxa in spadefoot toads linking plasticity and diversity. *Proceedings of the National Academy of Sciences, 103*(50), 19021–19026.

Gonzalo-Turpin, H., & Hazard, L. (2009). Local adaptation occurs along altitudinal gradient despite the existence of gene flow in the alpine plant species Festuca *eskia*. *Journal of Ecology, 97*(4), 742–751.

Grant, B. R., & Grant, P. R. (1996). High survival of Darwin's finch hybrids: Effects of beak morphology and diets. *Ecology, 77*(2), 500–509.

Grant, P. R. (1999). *Ecology and evolution of Darwin's finches*. Princeton University Press.

Gugerli, F. (1998). Effect of elevation on sexual reproduction in alpine populations of *Saxifraga oppositifolia* (Saxifragaceae). *Oecologia, 114*(1), 60–66.

Gurung, S., Pradhan, A., & Chettri, A. (2019). Pollination in an endemic and threatened monoecious herb *Begonia satrapis* CB Clarke (Begoniaceae) in the eastern Himalaya, India. *Journal of Threatened Taxa, 11*(10), 14328–14333.

Halbritter, A. H., Fior, S., Keller, I., Billeter, R., Edwards, P. J., Holderegger, R., Karrenberg, S., Pluess, A. R., Widmer, A., & Alexander, J. M. (2018). Trait differentiation and adaptation of plants along elevation gradients. *Journal of Evolutionary Biology, 31*(6), 784–800.

Hall, J. P. (2005). Montane speciation patterns in *Ithomiola* butterflies (Lepidoptera: Riodinidae): are they consistently moving up in the world? *Proceedings of the Royal Society b: Biological Sciences, 272*(1580), 2457–2466.

Hallik, L., Niinemets, Ü., & Wright, I. J. (2009). Are species shade and drought tolerance reflected in leaf-level structural and functional differentiation in Northern Hemisphere temperate woody flora? *New Phytologist, 184*(1), 257–274.

Harder, L. D., & Prusinkiewicz, P. (2013). The interplay between inflorescence development and function as the crucible of architectural diversity. *Annals of Botany, 112*(8), 1477–1493.

Harper, J. L., & Ogden, J. (1970). The reproductive strategy of higher plants: I. The concept of strategy with special reference to *Senecio vulgaris* L. *The Journal of Ecology, 58*(3), 681–698.

Herzog, S. K., Martínez, R., Jørgensen, P. M., & Tiessen, H. (2011). *Climate change and biodiversity in the tropical Andes*. Inter-American Institute for Global Change Research (IAI) and Scientific Committee on Problems of the Environment (SCOPE).

Homeier, J., Breckle, S., Günter, S., Rollenbeck, R. T., & Leuschner, C. (2010). Tree diversity, forest structure and productivity along altitudinal and topographical gradients in a species-rich Ecuadorian montane rain forest. *Biotropica, 42*(2), 140–148.

Huber, S. K., León, L. F. D., Hendry, A. P., Bermingham, E., & Podos, J. (2007). Reproductive isolation of sympatric morphs in a population of Darwin's finches. *Proceedings of the Royal Society B: Biological Sciences, 274*(1619), 1709–1714.

Itino, T., & Hirao, A. S. (2016). Plant genetic diversity and plant–pollinator interactions along altitudinal gradients. In *Structure and function of mountain ecosystems in Japan* (pp. 63–88). Springer.

Jara-Guerrero, A., De la Cruz, M., Espinosa, C. I., Méndez, M., & Escudero, A. (2015). Does spatial heterogeneity blur the signature of dispersal syndromes on spatial patterns of woody species? A test in a tropical dry forest. *Oikos, 124*(10), 1360–1366.

Jian, Q., Keming, M., & Yuxin, Z. (2009). Leaf-trait relationships of *Quercus liaotungensis* along an altitudinal gradient in Dongling Mountain, Beijing. *Ecological Research, 24*(6), 1243–1250.

Jiang, S., Luo, M.-X., Gao, R.-H., Zhang, W., Yang, Y.-Z., Li, Y.-J., & Liao, P.-C. (2019). Isolation-by-environment as a driver of genetic differentiation among populations of the only broad-leaved evergreen shrub *Ammopiptanthus mongolicus* in Asian temperate deserts. *Scientific Reports, 9*, 12008.

Josse, C., Cuesta, F., Navarro, G., Barrena, V., Cabrera, E., Moreno, E. C., Ferreira, W., Peralvo, M., Saito, J., & Tovar, A. (2009). *Ecosistemas de los Andes del Norte y Centro*. Universidad de los Andes.

Kremer, A., Potts, B. M., & Delzon, S. (2014). Genetic divergence in forest trees: understanding the consequences of climate change. *Functional Ecology, 28*(1), 22–36.

Leal, M.C. (2015). *Cambios en las características morfológicas y genéticas de Croton sp. en un gradiente altitudinal en matorral seco*. Undergraduate Thesis, Universidad Técnica Particular de Loja, Ecuador.

Lenormand, T. (2002). Gene flow and the limits to natural selection. *Trends in Ecology & Evolution, 17*(4), 183–189.

León-Yánez, S., Valencia, R., Pitmam, N., Endara, L., Ulloa, C., & Navarrete, H. (2011). *Libro rojo de plantas endémicas del Ecuador: Croton wagneri*. Pontificia Universidad Católica del Ecuador.

Levis, N. A., & Pfennig, D.W. (2020). Phenotypic plasticity and the origins of novelty. In Levine, H., Jolly, M. K., Kulkarni, P., & Nanjundiah, V. (Eds.), *Phenotypic switching: Implications in biology and medicine* (pp. 443–458). Academic Press.

Mallet, J. (2008). Hybridization, ecological races and the nature of species: Empirical evidence for the ease of speciation. *Philosophical Transactions of the Royal Society b: Biological Sciences, 363*(1506), 2971–2986.

Matesanz, S., Ramos-Muñoz, M., Blanco-Sánchez, M., & Escudero, A. (2020). High differentiation in functional traits but similar phenotypic plasticity in populations of a soil specialist along a climatic gradient. *Annals of Botany, 125*(6), 969–980.

Matute, D. R., Novak, C. J., & Coyne, J. A. (2009). Temperature-based extrinsic reproductive isolation in two species of *Drosophila*. *Evolution, 63*(3), 595–612.

McCartney, M. A., & Lessios, H. A. (2004). Adaptive evolution of sperm bindin tracks egg incompatibility in neotropical sea urchins of the genus *Echinometra*. *Molecular Biology and Evolution, 21*(4), 732–745.

McKinnon, J. S., Mori, S., Blackman, B. K., David, L., Kingsley, D. M., Jamieson, L., Chou, J., & Schluter, D. (2004). Evidence for ecology's role in speciation. *Nature, 429*(6989), 294–298.

Medina, C. A., Escobar, F., & Kattan, G. H. (2002). Diversity and habitat use of dung beetles in a restored Andean landscape. *Biotropica, 34*(1), 181–187.

Minelli, A. (2016). Species diversity vs. morphological disparity in the light of evolutionary developmental biology. *Annals of Botany, 117*(5), 781–794.

Mitchell, R. J., & Shaw, R. G. (1993). Heritability of floral traits for the perennial wild flower *Penstemon centranthifolius* (Scrophulariaceae): Clones and crosses. *Heredity, 71*(2), 185–192.

Noble, D. W. A., Radersma, R., & Uller, T. (2019). Plastic responses to novel environments are biased towards phenotype dimensions with high additive genetic variation. *Proceedings of the National Academy of Sciences, 116*(27), 13452–13461.

Nosil, P. (2012). *Ecological speciation*. Oxford University Press.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O´Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2019). vegan: Community ecology package. R package version 2.5-7 https://cran.r-project.org/web/packages/vegan/index.html

Olito, C., Abbott, J. K., & Jordan, C. Y. (2018). The interaction between sex-specific selection and local adaptation in species without separate sexes. *Philosophical Transactions of the Royal Society B, 373*(1757), 20170426.

Pais, A. L., Whetten, R. W., & Xiang, Q. Y. (2017). Ecological genomics of local adaptation in *Cornus florida* L. by genotyping by sequencing. *Ecology and Evolution, 7*(1), 441–465.

Peakall, R., & Whitehead, M. R. (2014). Floral odour chemistry defines species boundaries and underpins strong reproductive isolation in sexually deceptive orchids. *Annals of Botany, 113*(2), 341–355.

Pélabon, C., Armbruster, W. S., & Hansen, T. F. (2011). Experimental evidence for the Berg hypothesis: Vegetative traits are more sensitive than pollination traits to environmental variation. *Functional Ecology, 25*(1), 247–257.

Pérez, F., Lavandero, N., Ossa, C. G., Hinojosa, L. F., Jara-Arancio, P., & Arroyo, M. T. K. (2020). Divergence in plant traits and increased modularity underlie repeated transitions between low and high elevations in the Andean genus *Leucheria*. *Frontiers in Plant Science, 11*, 714.

Pfennig, D. W., & McGee, M. (2010). Resource polyphenism increases species richness: A test of the hypothesis. *Philosophical Transactions of the Royal Society B: Biological Sciences, 365*(1540), 577–591.

Pfennig, D. W., Wund, M. A., Snell-Rood, E. C., Cruickshank, T., Schlichting, C. D., & Moczek, A. P. (2010). Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology & Evolution, 25*(8), 459–467.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team (2020). nlme: Linear and nonlinear mixed effects models. R package version 3.1 https://cran.r-project.org/web/packages/nlme/index.html

Pyrcz, T. W. (2004). Pronophiline butterflies of the highlands of Chachapoyas in northern Peru: faunal survey, diversity and distribution patterns (Lepidoptera, Nymphalidae, Satyrinae). *Genus, 15*(4), 455–622.

Quilot-Turion, B., Leppälä, J., Leinonen, P. H., Waldmann, P., Savolainen, O., & Kuittinen, H. (2013). Genetic changes in flowering and morphology in response to adaptation to a high-latitude environment in *Arabidopsis lyrata*. *Annals of Botany, 111*(5), 957–968.

Quintana, C., Girardello, M., Barfod, A. S., & Balslev, H. (2017). Diversity patterns, environmental drivers and changes in vegetation composition in dry inter-Andean valleys. *Journal of Plant Ecology, 10*(3), 461–475.

R Core Team. (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Radersma, R., Noble, D. W. A., & Uller, T. (2020). Plasticity leaves a phenotypic signature during local adaptation. *Evolution Letters, 4*(4), 360–370.

Ramírez-Aguirre, E., Martén-Rodríguez, S., Quesada-Avila, G., Quesada, M., Martínez-Díaz, Y., Oyama, K., & Espinosa-García, F. J. (2019). Reproductive isolation among three sympatric *Achimenes* species: pre- and post-pollination components. *American Journal of Botany, 106*(7), 1021–1031.

Ramos-Jiliberto, R., Domínguez, D., Espinoza, C., López, G., Valdovinos, F. S., Bustamante, R. O., & Medel, R. (2010). Topological change of Andean plant–pollinator networks along an altitudinal gradient. *Ecological Complexity, 7*(1), 86–90.

Ramsey, J., Bradshaw, H. D., & Schemske, D. W. (2003). Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution, 57*(7), 1520–1534.

Richter, M., Diertl, K.-H., Emck, P., Peters, T., & Beck, E. (2009). Reasons for an outstanding plant diversity in the tropical Andes of Southern Ecuador. *Landscape Online, 12*(1), 1–35.

Richter, M., & Moreira-Muñoz, A. (2005). Heterogeneidad climática y diversidad de la vegetación en el sur de Ecuador: un método de fitoindicación. *Revista Peruana De Biología, 12*(2), 217–238.

Rojo, J. H., Fernández, D. A., Figueroa, D. E., & Boy, C. C. (2020). Phenotypic and genetic differentiation between diadromous and landlocked puyen *Galaxias maculatus*. *Journal of Fish Biology, 96*(4), 956–967.

Rundle, H. D., Chenoweth, S. F., Doughty, P., & Blows, M. W. (2005). Divergent selection and the evolution of signal traits and mating preferences. *PLoS Biology, 3*(11), e368.

Rundle, H. D., & Nosil, P. (2005). Ecological speciation. *Ecology Letters, 8*(3), 336–352.

Scheepens, J. F., Frei, E. S., & Stöcklin, J. (2010). Genotypic and environmental variation in specific leaf area in a widespread Alpine plant after transplantation to different altitudes. *Oecologia, 164*(1), 141–150.

Schliewen, U., Rassmann, K., Markmann, M., Markert, J., Kocher, T., & Tautz, D. (2001). Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. *Molecular Ecology, 10*(6), 1471–1488.

Schluter, D. (2000). *The ecology of adaptive radiation*. Oxford University Press.

Seguí, J., Lázaro, A., Traveset, A., Salgado-Luarte, C., & Gianoli, E. (2018). Phenotypic and reproductive responses of an Andean violet to environmental variation across an elevational gradient. *Alpine Botany, 128*(1), 59–69.

Sexton, J. P., Strauss, S. Y., & Rice, K. J. (2011). Gene flow increases fitness at the warm edge of a species' range. *Proceedings of the National Academy of Sciences, 108*(28), 11704–11709.

Shaw, K. L., & Mullen, S. P. (2011). Genes versus phenotypes in the study of speciation. *Genetica, 139*(5), 649–661.

Shivanna, K. R., & Tandon, R. (2014). *Reproductive ecology of flowering plants: A manual*. Springer.

Sierra, R. (1999). *Propuesta preliminar de un sistema de clasificación de vegetación para el Ecuador continental*. Proyecto INEFAN/GEF-BIRF y EcoCiencia.

Snell, H., & Rea, S. (1999). The 1997–98 El Niño in Galápagos: Can 34 years of data estimate 120 years of pattern? *Noticias De Galápagos, 60*, 111–120.

Sobel, J. M., & Chen, G. F. (2014). Unification of methods for estimating the strength of reproductive isolation. *Evolution, 68*(5), 1511–1522.

Sun, S. J., Catherall, A. M., Pascoal, S., Jarrett, B. J. M., Miller, S. E., Sheehan, M. J., & Kilner, R. M. (2020). Rapid local adaptation linked with phenotypic plasticity. *Evolution Letters, 4*(4), 345–359.

Torres-Díaz, C., Gómez-González, S., Stotz, G. C., Torres-Morales, P., Paredes, B., Pérez-Millaqueo, M., & Gianoli, E. (2011). Extremely long-lived stigmas allow extended cross-pollination opportunities in a high Andean plant. *PLoS ONE, 6*(5), e19497.

Uller, T., Feiner, N., Radersma, R., Jackson, I. S., & Rago, A. (2020). Developmental plasticity and evolutionary explanations. *Evolution & Development, 22*(1–2), 47–55.

Ulloa, C., & Jørgensen, P. (1995). *Árboles y arbustos de los Andes del Ecuador* (2nd Edn). Abya-Yala.

Van der Niet, T., Peakall, R., & Johnson, S. D. (2014). Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Annals of Botany, 113*(2), 199–212.

van Ee, B. W., Riina, R., & Berry, P. E. (2011). A revised infrageneric classification and molecular phylogeny of new world *Croton* (Euphorbiaceae). *Taxon, 60*(3), 791–823.

Vélez-Mora, D., Ramón, P., Vallejo, C., Romero, A., Duncan, D., & Quintana-Ascencio, P. F. (2020). Environmental drivers of femaleness of an inter-Andean monoecious shrub. *Biotropica, 53*(1), 17–27.

Vile, D., Garnier, E., Shipley, B., Laurent, G., Navas, M. L., Roumet, C., Lavorel, S., Díaz, S., Hodgson, J. G., Lloret, F., Midgley, G. F., Poorter, H., Rutherford, M. C., Wilson, P. J., & Wright, I. J. (2005). Specific leaf area and dry matter content estimate thickness in laminar leaves. *Annals of Botany, 96*(6), 1129–1136.

Vogel, S. (2009). Leaves in the lowest and highest winds: temperature, force and shape. *New Phytologist, 183*(1), 13–26.

Walter, G. M., Aguirre, J. D., Blows, M. W., & Ortiz-Barrientos, D. (2018). Evolution of genetic variance during adaptive radiation. *The American Naturalist, 191*(4), E108–E128.

Walter, G. M., Abbott, R. J., Brennan, A. C., Bridle, J. R., Chapman, M., Clark, J., Filatov, D., Nevado, B., Ortiz-Barrientos, D., & Hiscock, S. J. (2020). *Senecio* as a model system for integrating studies of genotype, phenotype and fitness. *New Phytologist, 226*(2), 326–344.

Wang, J., Zhao, X., Wang, W., Qu, Y., Teng, W., Qiu, L., Zheng, H., Han, Y., & Li, W. (2019). Genome-wide association study of inflorescence length of cultivated soybean based on the high-throughout single-nucleotide markers. *Molecular Genetics and Genomics, 294*(3), 607–620.

Webster, G. L. (1993). A provisional synopsis of the sections of the genus *Croton* (*Euphorbiaceae*). *Taxon, 42*(4), 793–823.

Webster, G. L. (2014). Euphorbiaceae. In Kubitzki K. (Ed), *Flowering plants. Eudicots* (pp. 51–216). Springer.

White, N. J., Snook, R. R., & Eyres, I. (2020). The past and future of experimental speciation. *Trends in Ecology & Evolution, 35*(1), 10–21.

Willmer, P. (2011). *Pollination and floral ecology*. Princeton University Press.

Wright, I. J., Reich, P. B., Westoby, M., Ackerly, D. D., Baruch, Z., Bongers, F., Cavender-Bares, J., Chapin, T., Cornelissen, J. H. C., Diemer, M., Flexas, J., Garnier, E., Groom, P. K., Gulias, J., Hikosaka, K., Lamont, B. B., Lee, T., Lee, W., Lusk, C., & Villar, R. (2004). The worldwide leaf economics spectrum. *Nature, 428*(6985), 821–827.

Wu, X., Li, Y., Shi, Y., Song, Y., Zhang, D., Li, C., Buckler, E. S., Li, Y., Zhang, Z., & Wang, T. (2016). Joint-linkage mapping and GWAS reveal extensive genetic loci that regulate male inflorescence size in maize. *Plant Biotechnology Journal, 14*(7), 1551–1562.

Zhao, Z. G., & Wang, Y. K. (2015). Selection by pollinators on floral traits in generalized Trollius ranunculoides (Ranunculaceae) along altitudinal gradients. *PLOS ONE*, *10*(2), e0118299.

Zhu, Y., Jiang, Y., Liu, Q., Kang, M., Spehn, E. M., & Körner, C. (2009). Elevational trends of biodiversity and plant traits do not converge—A test in the Helan Range, NW China. *Plant Ecology, 205*(2), 273–283.