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Clines in traits compared over two decades in a plant hybrid zone

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• **Background and Aims** Clines in traits across hybrid zones reflect a balance between natural selection and gene flow. Changes over time in average values for traits, and especially the shapes of their clines, are rarely investigated in plants, but could result from evolution in an unstable hybrid zone. Differences in clines between floral and vegetative traits could indicate different strengths of divergent selection.
• **Methods** Five floral and two vegetative traits were measured in 12 populations along an elevational gradient spanning a natural hybrid zone between *Ipomopsis aggregata* and *Ipomopsis tenuituba*. We compared clines in the floral traits with those measured 25 years ago. Observed changes in mean trait values were compared with predictions based on prior estimates of natural selection. We also compared the steepness and position of clines between the floral and vegetative traits.
• **Key Results** Corolla length has increased over five generations to an extent that matches predictions from measurements of phenotypic selection and heritability. The shape of its cline, and that of other traits, has not changed detectably. Clines varied across traits, but not all floral traits showed steeper clines than did vegetative traits. Both suites of morphological traits had steeper clines than did neutral molecular markers.
• **Conclusions** The increase in corolla length provides a rare example of a match between predicted and observed evolution of a plant trait in natural populations. The clinal properties are consistent with the hypothesis that habitat-mediated divergent selection on vegetative traits and pollinator-mediated selection on floral traits both maintain species differences across the hybrid zone.

Key words: Cline, floral evolution, hybrid zone, *Ipomopsis*, leaf morphology, long-term study, predicted evolution.

INTRODUCTION

Hybridization is widespread in vascular plants, occurring in 40 % of families and 16 % of genera (Whitney *et al.*, 2010). Where hybrids form in the wild, the hybrid zone often shows a spatial pattern in which trait values change gradually over geographical location between values characteristic of the two species. These gradual changes in trait value, or clines, are generally thought to be maintained by a balance between natural selection and gene flow (Endler, 1977; Barton and Hewitt, 1985). The selection could take the form of selection against hybrids regardless of location (disruptive selection), or selection that depends upon the environment such that different trait values are favoured in the two species located at opposite ends of the cline (divergent selection). For flowering plants, two different agents of selection are often hypothesized to be important in this divergent or disruptive selection, which can also drive ecological speciation (Waser and Campbell, 2004). First, different pollinators could select for different traits, usually floral traits, in the two species (Grant, 1949; Campbell *et al.*, 1997; Sobel and Streisfeld, 2014), resulting in either disruptive or divergent selection (hereafter pollinator-mediated selection). Second, speciation could be driven by adaptation to different physical habitats such as soil types or water availability (habitat-mediated selection; McNeilly and Antonovics, 1968). Although less well studied, it is also possible that interactions with organisms other than pollinators, such as herbivores, could generate disruptive or divergent selection in hybrid zones (Marquis *et al.*, 2016).

Patterns in traits across hybrid zones could be stable over time, or could change for a variety of reasons. If traits characteristic of one parental species lead to higher fitness across the hybrid zone, those traits may introgress more rapidly and become more common. If hybridization is asymmetrical such that backcrossing occurs more often with one parental species, traits of that species may increase in frequency and the centre of the cline may shift (Buggs, 2007). If the hybrid zone reflects recent secondary contact of the two species, clines in traits may become less steep as gene flow between the two species comes into balance with selection (Barton and Hewitt, 1985). Selection against hybrids can also lead to reinforcement for traits, such as greater divergence in floral features, that reduce mating between species (Servedio and Noor, 2003; Hopkins *et al.*, 2014). A temporal change in the environment could lead to changes in spatial patterns of traits, either due to plastic responses or to evolutionary adaptation to the new conditions. Movement of clines across hybrid zones has been studied in a variety of animal systems (see review by Buggs, 2007) but very rarely in plants. In one unusual example from a sunflower hybrid zone, the phenotype shifted to more closely resemble that of *Helianthus annuus* over a period of 50 years (Carney *et al.*, 2000). Not only is little known about the stability of plant hybrid zones, we also know of no case in which investigators have compared an observed trait change across a hybrid zone with a change predicted by measurements of selection and heritability. Indeed, such comparisons of predicted and observed evolution are rare in general (Grant and Grant, 1995; Gordon *et al.*, 2015), particularly for plants (Galen, 1996; Gervasi and Schiestl, 2016).

Different traits can exhibit clines that vary in steepness, which generally reflects a balance between the strength of divergent or disruptive selection and the spatial extent of gene flow. Stronger selection on a trait produces a narrower cline (Endler, 1977), as does larger genetic variance in the case of a cline for a quantitative character rather than a single genetic locus (Barton and Gale, 1993), given a fixed amount of gene flow. Thus if two clines are different in width, that difference could reflect a difference in the strength of selection or in the extent of genetic variance. Clines can also differ in position in the hybrid zone (hereafter 'centre'), if selective pressures on different traits differ in spatial signature, for example because they reflect different features of the environment. If multiple traits are subject to selection with the same spatial signature, they should be coincident in centre. However, selection on a trait includes not only direct selection on that trait but also indirect selection on traits that are in linkage disequilibrium. Thus linkage disequilibrium can modify the spatial positioning of clines (Nurnberger *et al.*, 1995), such that if indirect selection of correlated traits outweighs direct selection, clines for different traits can become coupled with each other, showing similar widths and centres even if the spatial signature of selection differs across traits (Vines *et al.*, 2016).

Clines in floral traits could differ in width from those of vegetative traits if the intensity of selection differs on the two types of traits, subject to the assumptions that genetic variance and degree of plasticity are similar between the two types of traits. If divergent pollinator-mediated selection of floral traits is sufficiently stronger than divergent habitat-mediated selection of vegetative traits, then steeper clines across a hybrid zone are expected for floral traits than for vegetative traits. We are unaware of any such comparisons of floral and vegetative clines for a natural hybrid zone. There are a few examples in which clines in vegetative traits have been compared with clines in reproductive traits such as flowering time (Kooyers *et al.*, 2014; Wadgymar *et al.*, 2017), flower number (Hoffmann *et al.*, 2009) or fruiting success (Montesinos-Navarro *et al.*, 2011). But these rare examples did not include the floral traits that different pollinators are most likely to select for in divergent ways, such as floral shape, colour, rewards or scent (reviews in Raguso, 2008; Harder and Johnson, 2009).

We used long-term data to examine elevational clines across a natural hybrid zone between the herbs *Ipomopsis aggregata* and *Ipomopsis tenuituba* (Polemoniaceae). We asked two major questions:

(1) Have floral traits, or the shape of clines in those traits, changed over 25 years, a time period equivalent to approximately five generations? We expected some changes in this hybrid zone as a result of consistent selection on floral traits with high heritability (Campbell and Powers, 2015) and a prevalence of *I. tenuituba* cytoplasmic genes throughout most of the hybrid zone (populations D–J in Campbell *et al.*, 1997), suggesting the hybrid zone could be in historical transition with *I. aggregata* nuclear genes advancing into the contact zone (Wu and Campbell, 2005). Specific predictions for evolutionary change are detailed under the Statistical analysis section.

(2) How do clines in floral traits and vegetative traits compare in width and position?

We determined clines in corolla length, corolla width and anther position over a 25-year period and clines in flower colour, nectar production, specific leaf area (SLA) and leaf trichome density during two recent years. In addition, we compared the widths of clines for these morphological traits, many of which are known to be under selection (Campbell, 2004), with the width of clines for a set of previously studied neutral molecular markers in the same hybrid zone (Aldridge and Campbell, 2009).

MATERIALS AND METHODS

Study system

Ipomopsis aggregata ssp. *aggregata* and *Ipomopsis tenuituba* ssp. *tenuituba* are two closely related herbs (Porter *et al.*, 2010) that frequently hybridize in mountains of the western USA (Aldridge, 2005). Individuals of both species spend 2–13 years (median = 5 years at our study site) as a basal rosette of leaves before sending up a flowering stalk, setting seed almost always only in a single summer, and then dying (Campbell and Waser, 2007). The flowers are tubular, with numerous flowers per inflorescence.

We studied a natural hybrid zone at Poverty Gulch in Gunnison National Forest, Gunnison County, CO (Grant and Wilken, 1988; Campbell *et al.*, 1997). In this area, pure populations of *I. tenuituba* ssp. *tenuituba* (populations A–C at 3100–3250 m elevation) are separated from pure populations of *I. aggregata* ssp. *aggregata* (population L at 2900 m) by 1.63 km. In between, there are hybridizing populations. Common garden experiments have demonstrated local adaptation of these two species that is consistent with divergent selection; each species has high fitness in its home environment and performs poorly when planted in the locale of the other species (Campbell and Waser, 2007). Fitness of hybrids between these species depends upon the environment, and F_2 hybrids have average fitness as high as expected under an additive model of fitness, with no evidence for intrinsically low hybrid fitness (Campbell *et al.*, 2008).

For many of the traits, there is evidence for both genetic variation and selection. Plants of *I. tenuituba* have longer corolla tubes, narrower corolla tubes, more inserted anthers and paler flowers, and produce less nectar than do plants of *I. aggregata*. Species differences in these floral traits were retained when grown under common conditions (Campbell and Aldridge, 2006), indicating genetic differences. For corolla length, corolla width and anther position, genetic variance within *I. aggregata* has also been demonstrated (Campbell, 1996). In this system, hummingbird pollinators exert selection for wider corollas (Campbell *et al.*, 1996), exserted anthers (Campbell *et al.*, 1998), more intensely red flowers and more nectar (Meléndez-Ackerman and Campbell, 1998), all traits characteristic of *I. aggregata* ssp. *aggregata*. Hummingbirds also exert selection for longer corollas, at least in some years (Campbell *et al.*, 1991), even though *I. aggregata* has the shorter corollas of the two species. Selection by the hawkmoth pollinators has been less well studied, but they are known to exert selection for narrower corollas (Campbell *et al.*, 1997), paler flowers and emission of indole scent (Bischoff *et al.*, 2015), traits characteristic of *I. tenuituba*. Hummingbirds are more abundant at the *I. aggregata* end of the hybrid zone, while hawkmoths (in years when they are present) are less abundant there (Campbell *et al.*, 1997).

We also included two vegetative traits: SLA (leaf area divided by dry mass) and leaf trichome density. Plants of *I. aggregata* had higher SLA than those of *I. tenuituba* when grown in a common garden (Campbell and Wendlandt, 2013), indicating genetic differences between the species. Despite their lower elevation, the *I. aggregata* sites are cooler and more humid at the height of the vegetative rosettes (4 cm above the soil) than the *I. tenuituba* and hybrid sites (Wu and Campbell, 2006), likely due to a much shallower slope that prevents rapid water runoff. Low SLA (or high leaf mass area) often correlates with drier environments (Poorter *et al.*, 2009), suggesting it can be favoured by drought (Dudley, 1996; Agrawal *et al.*, 2008). Like low SLA, high trichome density can also improve drought avoidance (Ehleringer and Mooney, 1978). Although SLA and trichomes can also respond to selection via light intensity or herbivores (Roy *et al.*, 1999; Poorter *et al.*, 2009), no leaf-chewing herbivores have been observed on *Ipomopsis* rosettes anywhere in the hybrid zone. SLA and trichome density could potentially be under divergent selection along the hybrid zone associated with variation in water availability, but selection intensities on these traits have not been reported in this system.

Sampling of plants

Plants were measured in 12 populations (A–L in Aldridge and Campbell, 2009) along the Poverty Gulch hybrid zone in 1991, 1992, 2015 and 2016. In each year in each population (where possible) we sampled the nearest flowering individual to every 3-m mark along a 30-m transect. Two of the populations no longer spanned 30 m in 2015, and two more no longer spanned 30 m in 2016. In these cases, we sampled the nearest flowering individual to every 1-m mark along a 10-m transect. In 2015 and 2016 we also increased the sample size for the lowest elevation (pure *I. aggregata*) population by including a second 30-m transect. In total, traits were measured on 500 plants, with the occasional missing trait value accounting for a total number of degrees of freedom of <499 in some analyses. Populations were marked with permanent flags in 1991 and later characterized by GPS location. Distance along the hybrid zone was defined starting from the lowest point of the 30-m transect within the highest-elevation population A, which occurs in the range of *I. tenuituba* (see map in Aldridge and Campbell, 2009).

Trait measurements

Corolla length, corolla width and maximum stamen length were measured in all years as described in Campbell (1989). Anther insertion was then determined by subtracting the maximum stamen length from the corolla length. Those morphometric measurements were averaged over three or more flowers per plant in all years of the study. Several other traits were measured in 2015 and 2016 only. Nectar production rate over 48 h was measured for one or two flowers per plant as described in Meléndez-Ackerman (1997) and converted to µL/24 h. All nectar measurements for a given year were performed on the same day. Flower colour (measured in 2016 only) was averaged over two flowers per plants, measured as relative reflectance in the red compared with the green, using a reflectance spectrometer as described in Campbell and Powers (2015). Specific leaf area was measured according to Campbell and Wendlandt (2013) and averaged across two leaves per plant. Trichome density was determined for one leaf per plant by scanning the leaf with a flatbed scanner, counting the trichomes at 200 % using the program ImageJ (NIH freeware), and dividing by leaf area. Comparisons were also made with previously published information on 48 molecular markers [random amplification of polymorphic DNA (RAPD)] assessed for 214 individuals in the 12 populations across the hybrid zone during 1999 to 2001 (Aldridge and Campbell, 2009).

Statistical analysis

For question 1, we compared changes in traits across 25 years (approximately five generations) in two ways. First, we assessed changes in raw trait values. For this purpose, we used analysis of covariance to model a trait value as a function of the factor year, distance along the hybrid zone, and the year × distance interaction. If the interaction was not significant, we then removed it from the model to fit a standard analysis of covariance, supplemented by independent planned contrasts of 1991 versus 1992, 2015 versus 2016, and the average of 1991–92 versus the average of 2015–16. Changes between sequential years would most likely reflect phenotypic plasticity, as genetic changes could not be that rapid in this system, whereas changes over five generations could be due to evolution or plasticity. In previous studies of *Ipomopsis*, corolla length has increased as an environmental response to water, whereas other floral traits and SLA have not (leaf trichomes were not studied; Campbell and Wendlandt, 2013). To test for plasticity of corolla length in response to annual snowmelt date, a key indicator of water availability in this subalpine system, we examined corolla length as a function of snowmelt date and distance along the hybrid zone. The 1991 data were not included in the analyses of covariance for corolla width because only the population means and not the raw data were still available.

For corolla length and width, prior estimates of phenotypic selection differentials and narrow sense heritability allowed us to make predictions about changes in mean values between two generations. Selection was measured in one *I. aggregata* population (L) and one hybrid population (I), using total seed production by a plant as an estimate of fitness (Campbell and Powers, 2015). For purposes of prediction, we used only the estimates of selection differentials obtained in eight years between 2001 and 2011, because plants that were seeds in later years were unlikely to have reached reproduction by 2015, and selection on corolla length has become progressively weaker over time with drier conditions (Campbell and Powers, 2015). The selection differential (S) on corolla length averaged 0.72 mm for corolla length and 0.0086 mm for corolla width across eight years of measurement (Campbell and Powers, 2015). Multiplying those figures for *S* by the estimated narrow sense heritabilities of 0.74 for corolla length and 0.31 for corolla width in natural populations of *I. aggregata* (Campbell, 1996) predicts changes in mean between two generations of 0.53 and 0.0027 mm (Falconer and MacKay, 1996). Over five generations, the predicted shift is 2.66 mm for corolla length and 0.013 mm for corolla width. We compared these predictions with the observed changes in populations within 300 m of those where selection was measured. Excluded were the five uppermost populations (including all of the *I. tenuituba* populations), where selection could well be different because hummingbirds are much less common visitors, and hawkmoths more common (Campbell *et al.*, 1997).

If nuclear genes of *I. aggregata* are advancing quickly enough into the hybrid zone (Wu and Campbell, 2005), a shift towards a less steep cline centred closer to populations of *I. tenuituba* could be expected. Thus our second method of comparing patterns across the decades relied on cline analyses to evaluate whether the centre or width of the cline had changed. For this purpose, we fitted clines to standardized mean trait values for the 1991–92 data and the 2015–16 data separately. For each population, we determined the mean trait value and then standardized it between 0 and 1 by subtracting the minimum across the 12 populations and then dividing by the difference between the maximum and the minimum. Clines were fitted to a no-tails model:

$$Y = a + \frac{1}{1 + e^{-4\frac{(X-c)}{w}}}$$

where Y is the standardized trait value, X is distance, a is the intercept, c is the centre of the cline and w is the width of the cline (Derryberry *et al.*, 2014). The fit was obtained using Proc NLIN in SAS (ver. 9.3) using the Marquardt iterative method. Alternative cline functions with one or two tails either failed to converge or the Hessian was not positive definite. The fit of the cline was compared against a null model with trait values independent of distance, using Proc NLMIXED in SAS (ver. 9.3), which employs maximum likelihood estimation, assuming normally distributed errors. The cline and null models were compared using a likelihood ratio test based on the difference in reported values for −2 log likelihood. To compare clines across the two decades, we combined the data and then compared the fit for a reduced model with one parameter each for a, c and w with the fit for a full model in which a, c and w were allowed to differ for the two time periods. Models were compared using the reported differences in −2 log likelihood from Proc NLMIXED.

For question 2, to compare the width and centre of clines for floral and vegetative traits, we used the same methods of cline analysis as described above, for the 2015–16 period only, as that is when all traits were measured. Clines were compared between each pairs of traits using a likelihood ratio test comparing the reduced and full models. We also compared these morphological clines with clines in molecular markers. In a previous study, the Bayesian clustering method Structure 2.2 was used to estimate the assignment of individuals (Q scores) along the hybrid zone to *I. tenuituba* versus *I. aggregata* based on RAPD markers (Aldridge and Campbell, 2009). We fitted a

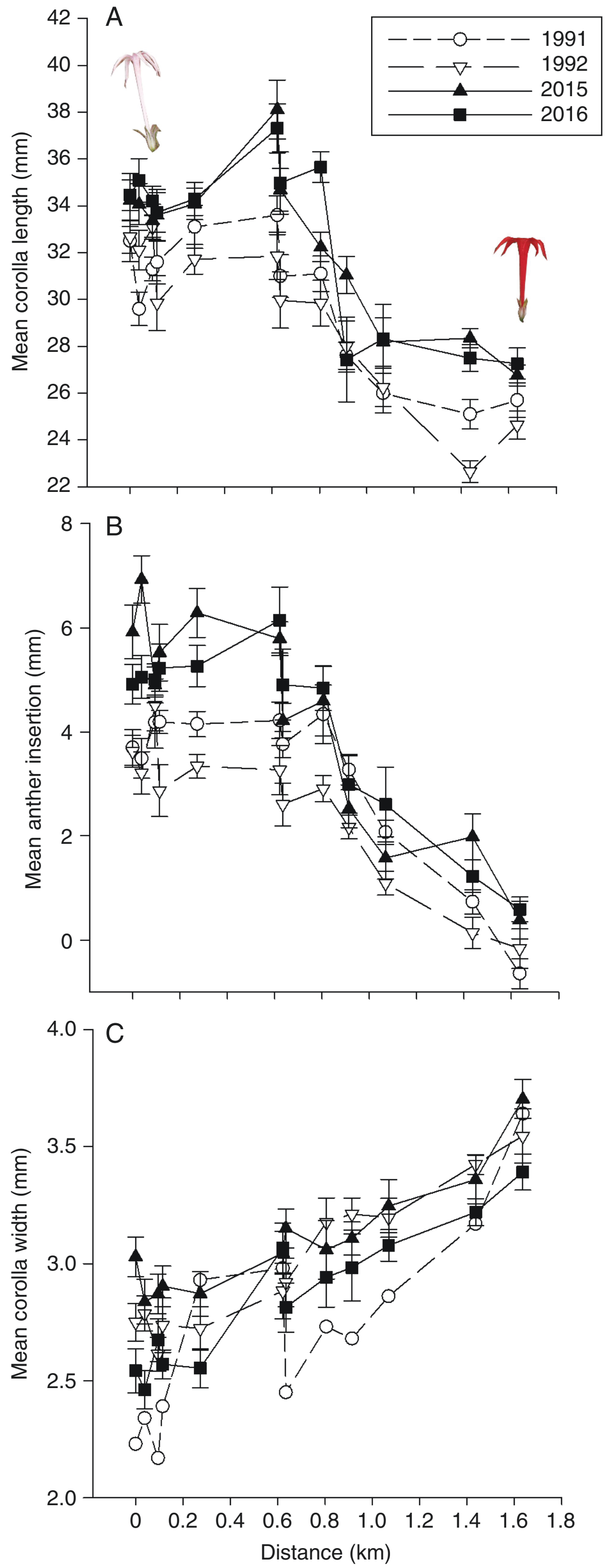

FIG. 1. Floral traits versus distance across the hybrid zone in four years. Means and standard errors across plant means are shown for 12 populations ranging from the highest-elevation *I. tenuituba* population A (elevation 3250 m at distance 0) to the lowest-elevation *I. aggregata* population (elevation 2900 m). (A) Mean corolla length. Insets show typical flowers of the two species. (B) Mean anther insertion. (C) Mean corolla width.

no-tails cline to these Q scores (Stankowski *et al.*, 2015) and compared its centre and width with those fitted to the morphological traits using log-likelihood ratio tests as described above. Because the genetic data came from a time point intermediate to our two sets of morphological data, we combined all years of morphological data for this analysis.

RESULTS

Changes in floral traits over two decades

Corolla length has generally increased over time throughout the hybrid zone (Fig. 1A). We detected no interaction between year of measurement and distance along the hybrid zone for corolla length ($F_{3,483}$ = 0.95, P = 0.42) or corolla width ($F_{2,365}$ = 1.92, P = 0.15). In a model without the interaction term, both traits changed significantly with distance (P < 0.0001). Corolla length did not differ significantly between sequential years (both contrasts P > 0.25), but was 2.7 mm greater on average in 2015–16 than in 1991–92 (contrast $F_{1,483}$ = 87.35, P < 0.0001). Variation in this trait was not explained by snowmelt date ($F_{1,488}$ = 0.99, P = 0.3211), which was latest in 1991 and earliest in 1992 for the four years included in the study. For the lowermost seven populations, near where phenotypic selection has previously been measured, the observed change in corolla length (mean ± s.e. = 2.43 ± 0.52 mm) was similar to the predicted value of 2.66 mm.

For corolla width, we detected no interaction between year of measurement and distance ($F_{2,365}$ = 1.92, P = 0.1474), and there was also no systematic change in mean corolla width over two decades (contrast $F_{1,367}$ = 0.20, P = 0.65 in model without interaction term; Fig. 1C). The non-significant trend towards wider corollas at the lower-elevation populations (0.02 ± 0.056 mm) could not be distinguished from the predicted value of 0.013 mm based on measurements of selection and heritability.

Anthers have become more inserted at the upper-elevation populations at the *I. tenuituba* end of the cline, while changing much less at the lower elevation populations (Fig. 1B), as shown in a significant year × distance interaction ($F_{3,482}$ = 5.29, P = 0.0013). Since anther position is a composite trait obtained as the difference between corolla and stamen length, this pattern means that stamen length changed more at the lower-elevation populations.

Shapes of floral clines over two decades

For every floral and vegetative trait, each cline model fitted significantly better than a null model in which trait values do not change with distance (likelihood ratio tests, P values in Table 1). Although flowers changed in morphological traits over two decades, the shape of the cline did not change significantly for any of the floral traits (Fig. 2; likelihood ratio test P = 0.13, 0.92, 0.36 for corolla length, width and anther insertion, respectively). For corolla length, the width of the cline was similar in 2015–16 to the value estimated in 1991–92 (0.45 and 0.56 km, respectively), and the centre of the cline remained near hybrid populations [populations I and J in Campbell *et al.* (1997), which are located at a distance of 0.91 and 1.07 km]. Both of the other floral traits also retained centres near these hybrid populations (Table 1).

Comparison of clines across floral and vegetative traits

In 2015–16, the steepest cline was in a floral trait, petal colour (Table 1). The cline for colour differed significantly from the much wider cline for corolla width (χ^2 = 17.8, P = 0.001; Table 2, Fig. 2). It also differed from the cline for nectar production (P < 0.05; Table 2), which was not only wider but also was centred at a lower elevation (Fig. 2). The cline for nectar production was also shifted significantly in comparison with the clines for corolla length and width (Fig. 2, Table 2). With the exception of nectar production, most floral traits were coincident in cline centre in 2015–16 (Table 1).

The cline for SLA did not differ significantly in shape from that of most of the floral traits, but was shallower than the very steep cline for petal colour (Table 2; compare Figs 2A and 3A). In contrast, leaf trichomes had a cline different in shape from that of any of the other traits (Fig. 3A, Table 2). Its cline was centred very near to the uppermost-elevation *I. tenuituba* population (at 0.08 km). The high-elevation populations had denser leaf trichomes, with comparatively little change from *I. aggregata* through hybrid populations (Fig. 3A).

The majority of morphological traits (all but corolla width) exhibited clines steeper than for molecular markers. A no-tails cline fitted to Q scores (assignment of individuals to *I. tenuituba* based on 48 markers) had a width equal to 1.47 km (s.e. = 0.23 km) and centre of 0.68 km (s.e. = 0.16 km; Fig. 3B). That cline

TABLE 1. *Width and centre of clines in floral traits and vegetative traits measured in 1991–92 and 2015–16*

Type of trait	Trait	Width of cline ± s.e. (km)		Center of cline ± s.e. (km)	
		1991–92	2015–16	1991–92	2015–16
Floral	Corolla length***	0.56 ± 0.18	0.45 ± 0.23	0.95 ± 0.06	0.95 ± 0.07
	Anther insertion****	0.85 ± 0.14	0.77 ± 0.21	1.14 ± 0.06	0.97 ± 0.07
	Corolla width****	1.55 ± 0.33	1.51 ± 0.26	1.02 ± 0.18	0.94 ± 0.15
	Petal colour****		0.33 ± 0.11		0.88 ± 0.03
	Nectar production****		0.78 ± 0.17		1.10 ± 0.07
Vegetative	SLA***		1.35 ± 0.43		1.22 ± 0.18
	Leaf trichomes**		0.56 ± 0.45		0.08 ± 0.08

s.e., standard error of the estimate.

Asterisks indicate significance levels for log-likelihood ratio tests against null models. ** P < 0.01; *** P < 0.001; **** P < 0.0001.

Approximate significance levels matched in the two time periods, except for corolla length (P < 0.0001 in 1992–92 and P < 0.001 in 2015–16).

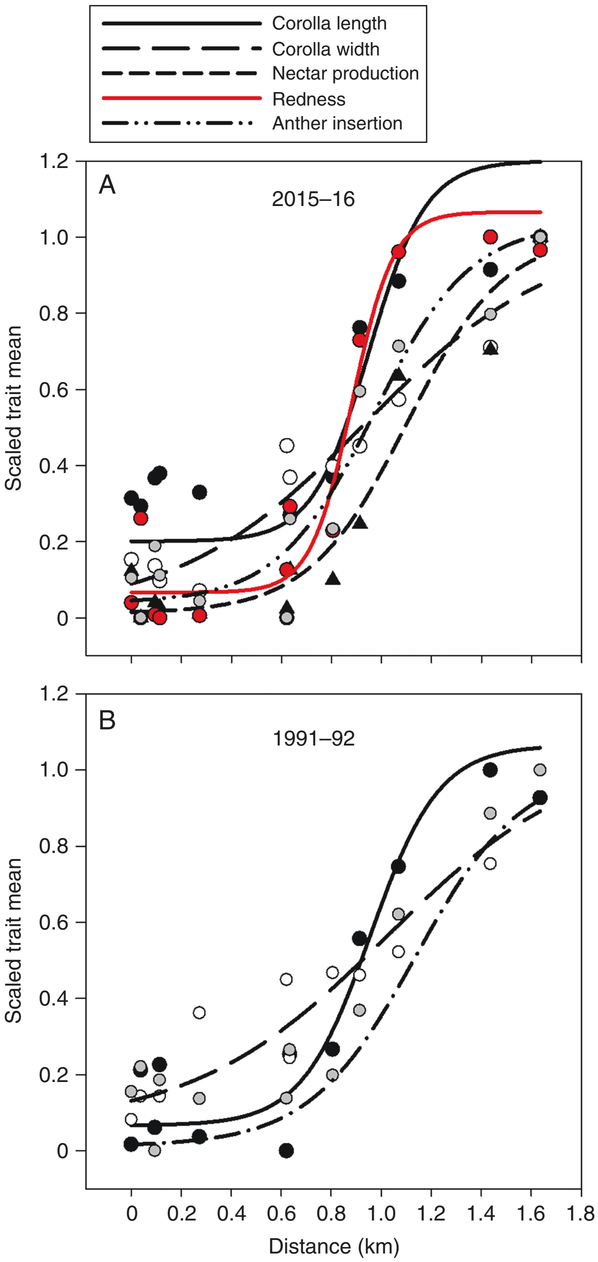

FIG. 2. Clines in floral traits. Symbols depict population means over two sequential years. Lines depict fits to the no-tail clines model. (A) Floral clines in 2015–16. (B) Floral clines in 1991–92. Red colour is used to depict the cline for flower colour.

differed significantly from clines in all morphological traits, including corolla width, the one trait that did not have a narrower cline (Table 2) largely because the centre of the corolla width cline was shifted downslope towards the *I. aggregata* sites.

DISCUSSION

Five generations of change

Two floral traits, corolla length and anther insertion, both showed some changes across 25 years in this hybrid zone. Corollas were longer in 2015 and 2016 than they were in 1991 and 1992, a time period of about five generations. In addition, anthers became more inserted at the upper-elevation portions of the hybrid zone. In principle, these morphological changes could be genetic, as the result of evolution, or plastic, as the result of environmental variation. Several pieces of evidence suggest that rapid evolutionary changes are more likely. First, we saw little change between sequential years even though these years were different in key environmental factors. For example, 1991 and 1992 were the most different years in snowmelt date, a variable with strong effects on soil moisture that can persist for up to 4 months (Blankinship *et al.*, 2014) and that influences natural selection in this plant species (Campbell and Powers, 2015). And yet, corolla length did not differ between these two years. Second, common garden studies have demonstrated genetic variation in corolla length, both among populations (Campbell and Aldridge, 2006) and within populations (Campbell, 1996), indicating potential for evolutionary change. Most importantly, the change in corolla length (and non-significant trend in corolla width) is consistent with predictions about evolution based on estimates of natural selection and heritability. Using total seed production as an estimate of fitness, the observed shift of 2.43 mm in mean corolla length at the lower end of the hybrid zone was similar to the predicted value of 2.66 mm. A caveat is that the rather crude prediction ignored some fitness components, such as male fitness (Campbell, 1989), relied on an estimate of heritability from populations ~10 km away, and did not incorporate selection on correlated traits (Campbell, 1996). The observed change in corolla length is equivalent to 0.15 haldanes or 0.15 standard deviations (within-population s.d. = 3.24 mm) per generation, which is considered rapid evolution that is difficult to sustain over long time periods (Kopp and Matuszewski, 2014) but within the range of commonly observed short-term evolution (Gingerich, 2009). The observed increase in corolla length per decade is equivalent to the starting difference between populations differing in elevation by 42 m. For context, this rate of change is far faster than the average elevational change in species distributions due to climate change (Parmesan and Yohe, 2003; Chen *et al.*, 2011). Other existing examples of comparing predicted and realized evolution in floral traits have generally relied on artificial selection (Galen, 1996; Gervasi and Schiestl, 2017) and thus are not directly comparable to the *Ipomopsis* results.

Despite some directional selection for longer corollas, the optimal corolla length would be shorter at the lower sites if hawkmoths, which prefer the high-elevation *I. tenuituba* and hybrids (Campbell *et al.*, 1997) favour even longer flowers than do hummingbirds. Such a divergence in selection could help maintain the observed cline in corolla length. Furthermore, if selection for long flowers is more extreme at high elevations, some of the increase in corolla length at the lower sites might also be due to spread of hybrids into those *I. aggregata* sites. First-generation hybrids with *I. aggregata* seed parents and *I. tenuituba* pollen parents have fitness as high as the home species, *I. aggregata*, at these sites (Campbell and Waser, 2007).

Stamen length, but not corolla width, also showed some detectable change over 25 years. This trait is more likely to be under selection through male function than female function (Campbell, 1989), as more exserted anthers increase

TABLE 2. *Significance values from likelihood ratio tests comparing cline shape between pairs of morphological traits or morphological traits and molecular marker assignment to* I. tenuituba *with Structure 2.2 (*Q*)*

	Corolla length	Corolla width	Anther insertion	Petal colour	Nectar production	SLA	Leaf trichomes
Corolla width	0.0937						
Anther insertion	0.0688	0.1386					
Petal colour	0.2407	**0.0010**	0.1951				
Nectar production	**0.0014**	**0.0005**	0.0979	**0.0004**			
SLA	0.1166	0.5724	0.2957	**0.0037**	0.0821		
Leaf trichomes	0.0085	**0.0011**	**0.0003**	**0.0006**	**<0.0001**	**0.0006**	
Molecular markers (*Q*)	**0.0300**	**0.0020**	**<0.0001**	**0.0005**	**<0.0001**	**0.0005**	0.0367

Morphological trait comparisons used data from 2015 to 2016 when all traits were measured. Morphological data from all years were combined for comparison with the molecular data from 1999 to 2001.

Bold type indicates $P < 0.05$ after correcting for multiple comparisons using a false discovery rate.

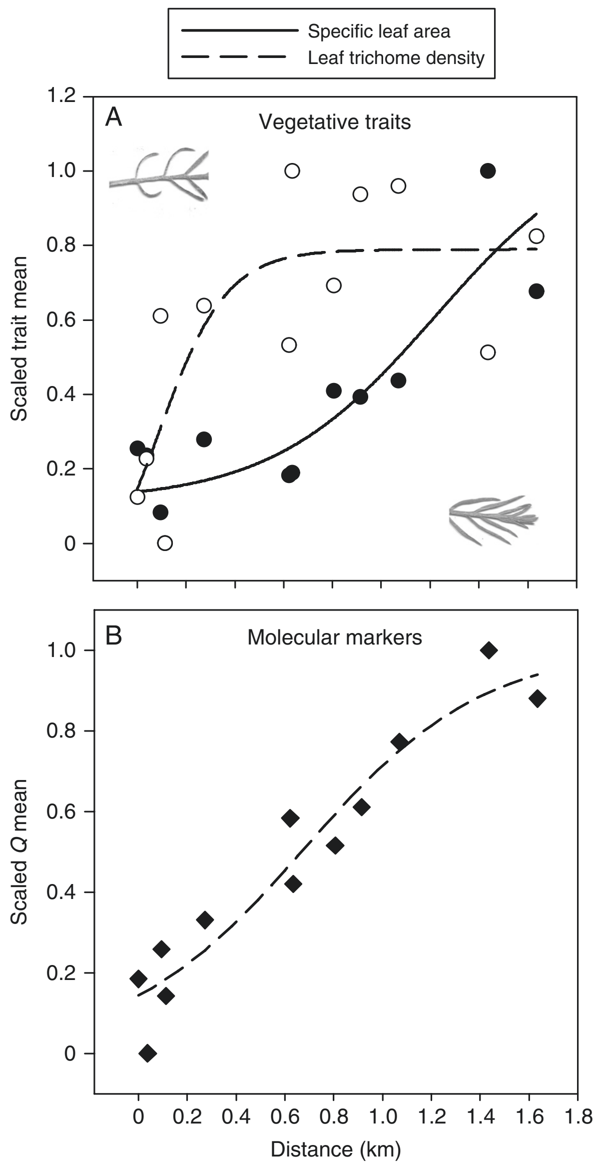

FIG. 3. Clines in vegetative traits and molecular markers. (A) For vegetative traits, symbols depict population means over 2015–16. Insets show typical leaves of the two species. (B) Mean values of *Q*, probability of assignment to *I. tenuituba* based on using the program Structure 2.2 for 48 RAPD markers (Aldridge and Campbell, 2009). Lines depict fits to the no-tail cline model.

pollen removal by hummingbirds (Campbell *et al.*, 1998). If hummingbirds drive the selection on stamen length, this could explain why stamens lengthened in lower-elevation but not higher-elevation populations, as hummingbird visits are more common at the lower sites (Campbell *et al.*, 1997).

Although corolla length and stamen length both showed some changes across 25 years, neither the centre nor the position of the cline changed detectably for either trait. Although the preponderance of *I. tenuituba* cytoplasmic genes in hybrid populations suggests that historically the centre of the hybrid zone might have been at lower elevation (Wu and Campbell, 2005), any such movement was not rapid enough to detect over 25 years, a period of just five generations. Spatial positions of clines, based on cline analysis as we used here, have not previously been followed over long periods of time in plants, but several cases of hybrid zone movement in animals have been attributed to asymmetrical hybridization, environmental change or recent invasion (Buggs, 2007; Glotzbecker *et al.*, 2016).

Comparisons of clines in floral versus vegetative traits

Clines across the hybrid zone varied in width from 0.33 to 1.51 km. Although the two steepest clines were for floral traits (petal colour and corolla length), so was the shallowest cline (for corolla width). Thus there was not a strong pattern of steeper clines for floral than vegetative traits. Such a pattern would be expected if divergent selection was stronger for floral traits or if genetic variance in those traits was larger, relative to the difference in trait values at the extremes of the cline (Barton and Gale, 1993). Measurements of divergent selection are not yet available, as most of the selection estimates are for the lower populations only, but there are already estimates of genetic variance for some of the floral traits in *I. aggregata* (Campbell, 1996). For these traits, the additive genetic variance expressed relative to the 2015–16 difference in trait values between the highest and lowest populations was 0.15 for corolla length, 0.18 for anther position, 0.04 for width and 0.05 for nectar. Thus, differences in genetic variance could be part of the explanation that corolla length shows a much steeper cline than width. The relatively steep cline for nectar (width 0.78 km) despite low genetic variance suggests strong divergent or disruptive selection on this trait.

The majority of morphological traits (all but corolla width) exhibited clines steeper than for assignment of individuals based on presumably neutral molecular markers. Most (all but leaf trichomes) also had clines with centres shifted downslope towards the *I. aggregata* sites in comparison with the molecular markers. One other molecular cline has been reported for this hybrid zone, for a single AFLP (amplified fragment length polymorphism) nuclear marker (Wu and Campbell, 2005). In that study, markers were assessed in pure populations of the parental species and only the one species-specific nuclear marker was then assessed in the other populations. Comparison with a cline in one marker has to be done cautiously, as cline widths can vary enormously among molecular markers, for example 40-fold in a genome-wide study of *Mimulus aurantiacus* (Stankowski *et al.*, 2017), and the one marker that stands out as species-specific is more likely than others to be under selection or linked to a gene under selection. Nevertheless, fitting the no-tails model to a cline for that marker yielded a width of 0.79 km, which is greater than for the majority of the floral traits. Overall, the steeper clines for phenotypic traits than presumed neutral molecular markers support the hypothesis that the phenotypic traits are under divergent or disruptive selection in this hybrid zone. Some of the floral traits may also be under selection in a region of contact between two other subspecies of *I. aggregata*, where populations differed more in corolla length, tube width, petal colour and nectar production than in microsatellite markers, as judged by F_{ST} (Milano *et al.*, 2016).

Coincidence between the centre positions of the clines for most floral traits is also consistent with a common pattern of selection on all of these traits, which could be driven by predominately hummingbird pollination at lower elevations and more common hawkmoth pollination at higher elevations (Campbell *et al.*, 1997), as these two sets of pollinators select for different trait values in some cases. For example, hummingbirds select for wider corollas (Campbell *et al.*, 1996) and more intensely red flowers (Meléndez-Ackerman and Campbell, 1998), whereas hawkmoths select for narrower corollas (Campbell *et al.*, 1997) and paler flowers in *Ipomopsis* (Bischoff *et al.*, 2015). The one trait with an offset cline is nectar production rate, for which the cline is shifted in centre towards lower elevations. A theoretically possible explanation is that nectar production is more sensitive to micro-environmental conditions. Nectar production increases linearly with soil moisture in *I. aggregata* (Waser and Price, 2016). The generally dry conditions in all of the hybrid sites (through a distance of 1.07 km) could be responsible for keeping nectar production low until approximately that point along the hybrid zone. It would be valuable to test the form of the relationship between soil moisture and nectar production along this hybrid zone.

The clines for the two vegetative traits are not coincident in centre position, nor are they coincident with clines for the floral traits. This difference suggests that they are under selection with a different spatial signature, or that selection is overwhelmed by phenotypic plasticity. Although selection intensities have not been reported on these specific traits, reciprocal transplants demonstrate divergent viability selection for *I. aggregata* at low-elevation sites and for *I. tenuituba* at the upper elevation (Campbell and Waser, 2007), suggesting that some traits expressed during the vegetative stage are also under selection. Like nectar production, SLA has a cline shifted towards lower-elevation populations, which is consistent with a hypothesis that both traits are influenced in part by soil moisture. In contrast, the centre of the cline for leaf trichomes is near the uppermost-elevation populations. Little is known about the selective advantage of trichomes in *Ipomopsis*, and it is unclear why its spatial pattern would differ so radically from another vegetative trait, low SLA, also thought to increase drought avoidance (Ehleringer and Mooney, 1978). Two potential hypotheses are: the increased trichome density offers protection against UV damage at high elevation (Karabourniotis *et al.*, 1995), or increased trichome density leads to increased emission of volatile compounds that influence attraction of pollinators, herbivores or pathogens (Maffei, 2010).

Conclusions

This hybrid zone shows a change in at least one floral trait (corolla length) over the past five generations. The shift to longer corollas is consistent with predicted evolution from past measurements of selection and heritability, and is inconsistent with phenotypic plasticity in which later snowmelt leads to larger flowers. The change in corolla length offers a rare example of a match between predicted and observed phenotypic evolution.

Although absolute values of corolla length changed, the shape of the cline was stable across 25 years, in the first such long-term comparison for a plant hybrid zone. Clines for both floral traits and vegetative traits were similar in steepness, but differed from those for presumed neutral markers. In combination with previous demonstrations of both fecundity and viability selection in this hybrid zone (Campbell *et al.*, 2007), these results are consistent with the hypothesis that both suites of morphological traits are under divergent or disruptive selection, due to pollinators and to other features of the habitat.

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