

Spatio-temporal changes in the genetic structure of the *Passerina* bunting hybrid zone

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Abstract

Although theoretical models predict that the structure of a hybrid zone can change under a variety of scenarios, only a few empirical studies of hybrid zones have unequivocally demonstrated zone movement. These studies are rare because few data sets exist that include repeated, temporally spaced, samples of the same hybrid zone. We analysed mitochondrial DNA haplotype data from samples separated by 40–45 years from across the *Passerina amoena* (Lazuli Bunting) and *Passerina cyanea* (Indigo Bunting) hybrid zone to investigate whether the genetic structure of this zone has changed during that interval. Both cline and generalized linear mixed modelling analyses uncovered a significant narrowing and a substantial westward shift of the *Passerina* bunting hybrid zone, clearly illustrating hybrid zone movement. The cause of the change may be due to a combination of ecological, demographic and behavioural factors. Our results predict that the width of the hybrid zone will continue to narrow over time, a finding consistent with reinforcement theory.

Keywords: cline analysis, historical DNA, Indigo Bunting, Lazuli Bunting, mitochondrial DNA

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Introduction

Hybrid zones form when the geographic ranges of two closely related taxa overlap and those taxa interbreed (Harrison 1993). These ‘windows on the evolutionary process’ (Harrison 1990) have provided fruitful research avenues for a wide variety of studies, many of which have focused on trying to understand how patterns of gene flow and introgression between taxa influence the processes important in creating and maintaining biological diversity (e.g. Szymura & Barton 1986, 1991; Porter *et al.* 1997; Rieseberg *et al.* 1999; Payseur *et al.* 2004; Brumfield 2005; Carling & Brumfield 2008a; Nolte *et al.* 2009; Teeter *et al.* 2010). Accordingly, exploring the spa-

tio-temporal dynamics, that is movement or change in structure over time, of the hybrid zones themselves can provide important insights into how these evolutionary processes can change (Buggs 2007).

Although the potential causes of hybrid zone movement are varied (Buggs 2007), two general models of hybrid zone structure, the tension zone model (Key 1968) and the environmental gradient model (May *et al.* 1975), provide a framework for understanding the conditions under which hybrid zone movement may occur. In the tension zone model, endogenous selection against hybrid individuals is balanced by the continued dispersal of parentals into the zone from allopatric populations (Barton & Hewitt 1985, 1989). By contrast, the environmental gradient model maintains that exogenous selection creates fitness differences along the gradient, creating a steep cline despite dispersal along it (May *et al.* 1975). Both models assume ecological stability, which is important for inferences of

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hybrid zone stability because environmental changes would result in a shift in the geographic location of a hybrid zone under both types of selection pressures. Since selection is endogenous in the tension zone model, these zones are predicted to move according to population density gradients and should eventually settle in areas where the rate of gene flow into the zone is the same for both parental species. This typically happens at a population density trough and after landing in such a trough, which often occurs at ecotones (Endler 1977; Barton & Hewitt 1985, 1989), the hybrid zone becomes stable. Therefore, tension zones can move until reaching a population density trough or, even after a period of stability, if an ecotonal shift changes the population density gradient. Similarly, a shift in the location of an ecotone will produce a shift in the location of a hybrid zone maintained by an environmental gradient. Depending on the specifics of the fitness differences along the gradient, environmental changes can also produce changes in hybrid zone width. For example, if an intermediate environment, in which hybrids have higher fitness than either parental, expands the width of the hybrid zone should also expand (Moore 1977; Moore & Price 1993).

Despite the theoretical predictions of hybrid zone movement, empirical documentation of such events is difficult to obtain, due largely to the inherent difficulty of observing a transitory evolutionary phenomenon that requires the collection of geographically expansive, repeated, and comparable samples of organisms. Long-term studies of a small number of hybrid zones, in which the same populations were repeatedly sampled over a 15- to 20-year time period, have provided some evidence of hybrid zone movement (Hairston *et al.* 1992; Britch *et al.* 2001; Bull & Burzacott 2001). Movement has also been inferred indirectly based on the geographic distribution of molecular markers (e.g. alleles trailing in the wake of a moving hybrid zone; Moran 1981; Rowher *et al.* 2001; Gay *et al.* 2008), but others have argued that such patterns may be produced by differential introgression of loci rather than through actual hybrid zone movement (Barton & Hewitt 1985; Rieseberg & Soltis 1991; Goodman *et al.* 1999). Ideally, spatio-temporal changes in the genetic structure of hybrid zones should be investigated through the analysis of the same loci sampled from individuals collected through repeated sampling of geographic transects. To the best of our knowledge, only two studies have documented hybrid zone movement in such a manner (Britch *et al.* 2001; Dasmahapatra *et al.* 2002), both of which showed that the centre of the hybrid zone under study shifted over a period of 14–20 years.

Here, we investigate patterns of hybrid zone movement focusing on the hybrid zone formed between

Lazuli (*Passerina amoena*) and Indigo (*Passerina cyanea*) buntings (Aves: Cardinalidae), by comparing the historical (sampled from 1955 to 1969) genetic structure of the hybrid zone with its contemporary (sampled from 2004 to 2007) genetic structure.

Passerina hybrid zone

Passerina amoena and *Passerina cyanea* are sister species of songbirds (Carling & Brumfield 2008b) that hybridize where their breeding ranges overlap (Fig. 1). Sibley & Short (1959) used morphological and plumage data from 94 specimens they collected primarily in South Dakota and Nebraska (Table S1, Supporting information) and concluded that hybridization between *P. amoena* and *P. cyanea* was common, as they classified ~70% of their specimens (66/94) as either hybrid or backcross individuals. Approximately 15 years later, Emlen *et al.* (1975) collected 55 individuals along an east–west transect in northern Nebraska (Table S1, Supporting information). Emlen *et al.* (1975) also reexamined the specimens collected by Sibley and Short and, using all 149 individuals, concluded that the hybrid zone was narrower than had been estimated by Sibley & Short (1959) and further hypothesized that it may have moved westward in the c. 15 years between the two sampling periods. The hybrid index score developed by Sibley & Short (1959) to determine the ancestry of an individual was much more liberal than the one used by Emlen *et al.* (1975), potentially accounting for the differences in the estimated width of the hybrid zone between the two studies. Breeding Bird Survey data suggest that *P. cyanea* has been expanding its range westward over the past 50–60 years (Payne 2006), which may have contributed to the westward shift hypothesized by Emlen *et al.* (1975).

More recent work, using newly collected samples, has uncovered patterns of differential introgression among mitochondrial, autosomal and sex-linked loci across this hybrid zone (Carling & Brumfield 2008a, 2009) consistent with the predictions of Haldane's rule. Furthermore, coalescent based analyses have indicated a significant reduction in postdivergence gene flow of sex-linked genes, compared with autosomal or mitochondrial loci, that is likely not due to present-day hybridization (Carling *et al.* 2010). That said the long-term stability of the hybrid zone is unknown, which we address in this study.

Given the availability of temporally structured samples from across the hybrid zone between *P. amoena* and *P. cyanea* and the previous suggestion of zone movement (Emlen *et al.* 1975), this system is particularly well-suited for investigating the spatio-temporal changes in the genetic structure of hybrid zones.

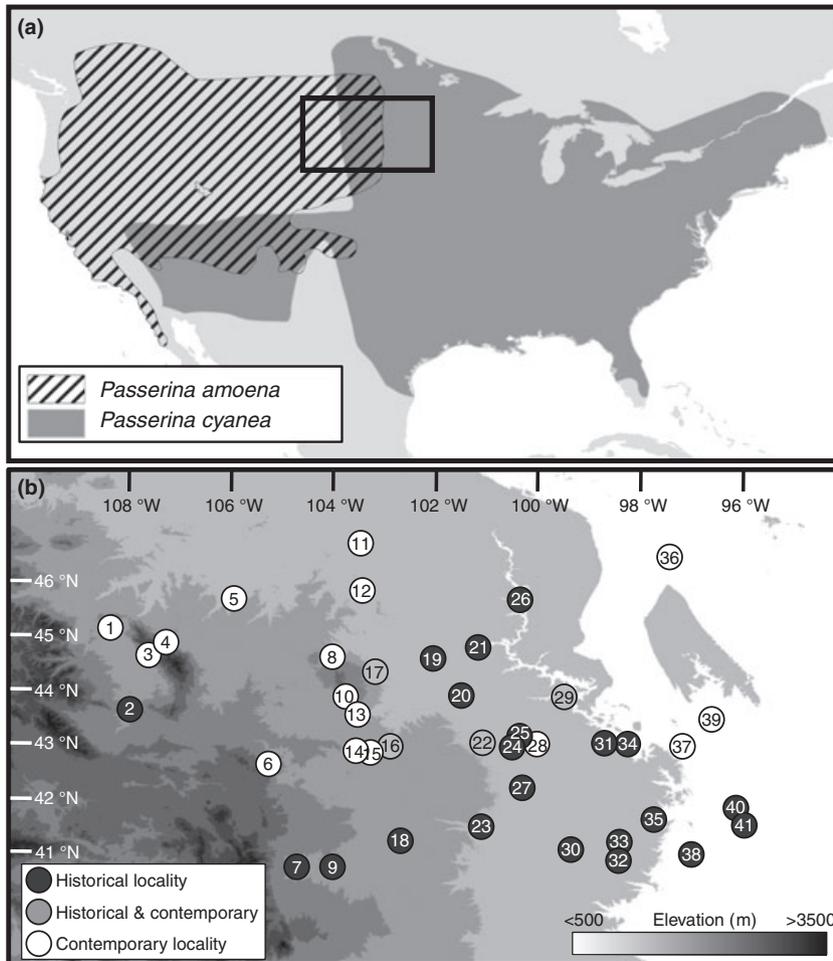


Fig. 1 *Passerina amoena* and *Passerina cyanea* breeding distributions and sampling localities. (a) Digital maps (Ridgely *et al.* 2003) were downloaded from NatureServe (2006) and modified. Area outlined by black box is magnified in B. (b) Schematic of sampling localities. Because of the close physical proximity of some sampling localities, not all localities are visible on this map. See Table S1 (Supporting information) for detailed locality information, including sample sizes.

Materials and methods

Sampling and molecular methods

We used single-use razor blades to sample toe pads from 149 museum specimens collected by Sibley & Short (1959) and Emlen *et al.* (1975) that are housed in the Ornithology Collection of the Cornell University Museum of Vertebrates (Fig. 1; Table S1, Supporting information). Hereafter, we refer to these samples as 'historical'. These historical samples consist of individuals collected in two different batches; 94 were collected from 1955 to 1957 (Sibley & Short 1959) and 55 were collected in 1969 (Emlen *et al.* 1975). The 163 individuals we refer to as the 'contemporary' samples were collected during the summers of 2004–2007 and skin and tissue specimens were deposited in the Louisiana State University Museum of Natural Science (Fig. 1; Table S1, Supporting information). We combined the two historical samples into a single sample because no analysis found any differences between them (1955–1957 and 1969, data not shown).

Mitochondrial DNA haplotypes for the contemporary samples were determined by polymerase chain reaction

amplification and cycle-sequencing a portion of the NADH dehydrogenase subunit 3 (ND3) gene using previously described methods (Carling & Brumfield 2008b) and were designated as either *Passerina amoena* or *Passerina cyanea* using haplotypes of individuals from allopatric populations as references.

We used a restriction fragment length polymorphism (RFLP) assay to determine the mitochondrial DNA ancestry of individuals in the historical sampling group. We designed the RFLP assay using methods more thoroughly described elsewhere (Carling *et al.* in press). Briefly, using ND3 sequences from individuals sampled from allopatric populations (Washington for *P. amoena*, Michigan and Illinois for *P. cyanea*) we designed fluorescently labelled primers that amplified a 133-bp portion of the ND3 gene that contained a diagnostic restriction site difference between the species. We extracted and amplified DNA from each toe pad sample in a dedicated ancient DNA facility and included negative controls at each step. Successful amplifications were digested with *Sfc 1* and the DNA fragment sizes were determined using an ABI 3100 Genetic Analyzer (Applied Biosystems) with a GeneScanTM-500 LizTM size

standard (Applied Biosystems), and then visualized using the program GeneMapper 3.7 (Applied Biosystems). Samples were then scored by visual examination as either *P. amoena* (one band, 133 bp in length) or *P. cyanea* (two bands, 48 and 85 bp in length). We validated the RFLP assay by employing it on contemporary samples, in all cases the mitochondrial DNA haplotype inferred was the same between the two methods.

Data analyses

We used a generalized linear mixed model (GLMM), using the logit link function (Pinheiro & Bates 2000; Faraway 2006), with a binomial error distribution to test for the effects of geography (latitude and longitude) and time period on the probability of an individual having a *P. cyanea* mitochondrial DNA haplotype. We treated the presence of a *P. cyanea* mitochondrial haplotype as a binary response that varies as a function of latitude (continuous), longitude (continuous), and time period modelled as a dummy variable (historical and contemporary). We included second-order interaction terms of longitude and latitude interacting with time period. Site location (SITE) was treated as a random effect to account for the nested nature of the data (multiple samples from sites), and all estimates were quantified using restricted maximum likelihood and Laplace approximation.

We constructed spatial interpolation maps based on model predictions to a grid of sampling points designed to cover the complete extent of the study region (bounded by the extreme north and south latitudes and east and west longitudes of the sampling localities). Using 800 unique combinations of latitude and longitude, we computed predictions based on the GLMM. For each of these locations, we had exact geographic coordinate information, but varied the time period covariate (historical and contemporary). For visualization purposes, we used kriging to create a continuous surface of predictions for each time period (Cressie 1990; Webster & Oliver 2001). The ordinary kriging procedure was based on a spherical semivariogram model with a fixed search radius of 9 of the nearest sample points for interpolation. Spatial interpolation techniques, such as kriging, are particularly relevant for estimating, predicting, and visualizing biological values at unsampled locations (Fortin & Dale 2005). In this case, our spatial method uses information from the models based on the sample data to effectively capture the main spatial signal and produce smoothed predictions of hybridization across the larger study region.

We performed a randomization procedure (Manly 1991) to ensure that the covariate relationships estimated in the GLMM were not a result of a geographic

shift in sampling (Fig. 1). The procedure consisted of first fitting a GLMM using only the historic data set and longitude and latitude as covariates (site was treated as a random effect). We then used this historic model to generate the expected probabilities of the *P. cyanea* mitochondrial DNA haplotype at each of the contemporary sampling locations. These predictions at contemporary sites are the quantification of the null hypothesis that the historical transition zone between bunting species has remained unchanged through time. Based on these null-hypothesis probabilities, we generated a set of Bernoulli (0/1) random response values at each of the contemporary sites and conducted a logistic regression (longitude and latitude as the two covariates) using these random data, saving the beta estimates. This randomization procedure was repeated 10 000 times. By comparing the actual beta estimates (i.e. those from the actual observed haplotypes at each contemporary location) with the distribution expected by chance, we were able to calculate probabilities that the contemporary transition zone differed from that expected by chance alone or whether there was a significant probability of a real change in location of the hybrid zone.

We also tested for temporal changes in the genetic structure of the hybrid zone using ClineFit (Porter *et al.* 1997), which uses methods developed by Szymura & Barton (1986, 1991) to estimate cline shape parameters (centre location and width). The sampling localities were transformed into a linear transect by first calculating the mean latitude of all localities (43.56°N). Then, assuming all localities were located on this 43.56°N latitudinal transect, we calculated the distance in kilometres between the longitude of each locality (Supporting Information). For both the historical and contemporary data sets, we estimated the location of the cline centre and the width, along with two likelihood support limits ($\ln L_{\max} - 2$) to assess support, using the following search parameters: burn-in: parameter tries per step—300; sampling for support: replicates saved—3000, 10 replicates per save. We determined differences in centre and width between the historical and contemporary clines using the two log-likelihood support limits, which are analogous to 95% confidence intervals; any differences in parameter estimates are significant if the support limits do not overlap (Porter *et al.* 1997).

Results

Mitochondrial DNA haplotypes were determined for 138 of the 149 individuals in the historical data set (92.6%) and for all 163 individuals in the contemporary data set (Table S1, Supporting information). As there were no differences between the two historical samples, we only present results for comparisons between a

single historical sample (all individuals collected prior to 1970) and the contemporary sample.

We found that the probability of a sampling a bird with a *Passerina cyanea* mitochondrial DNA haplotype was more likely in the contemporary time period ($\beta = 76.64$, SE = 36.62, $P = 0.04$) and in more southerly ($\beta = -0.52$, SE = 0.17, $P = 0.002$) and easterly ($\beta = 0.49$, SE = 0.14, $P < 0.001$) populations (Table 1). The increase in the probability over time, however, demonstrated a geographic trend. Although the predicted probability of an individual with a *P. cyanea* mitochondrial DNA haplotype increased in the contemporary time period, this increase was more prevalent in the easterly populations as evidenced by a significant interaction between longitude and time period ($\beta = 0.88$, SE = 0.34, $P = 0.001$). The interaction term between latitude and time was not significant ($\beta = 0.32$, SE = 0.27, $P = 0.22$). To adjust for what could be a bias in the distribution of sampling points between the two time periods (i.e. a scarcity of historical sampling localities in the west), we ran the same model including only those sites east of 106°W longitude (removing populations 1–4 in Fig. 1). The results remained the same (Table 1).

The mean β estimates from the randomization for longitude and latitude were -0.573 and 0.515 , respectively. The observed β estimates of the contemporary model associated with longitude ($\beta = 1.32$, SE = 0.28, $P < 0.001$) and latitude ($\beta = -0.21$, SE = 0.17, $P = 0.011$) were both significantly different than expected by chance (Fig. 2).

Spatial interpolation of the predicted probabilities of sampling a *P. cyanea* mitochondrial DNA haplotype clearly shows the differences between the two time

Table 1 Results from the GLMM model of the probability an individual will have a *Passerina cyanea* mitochondrial DNA haplotype. Predictor variables include longitude (continuous), latitude (continuous) and time period (historical and contemporary). The models are hierarchical where site is treated as a random effect

Fixed effects	β	SE	z-Value	P-value
All sites				
Time	76.64	36.62	2.09	0.04
Latitude	-0.52	0.17	-3.07	0.002
Longitude	0.49	0.13	3.58	<0.001
Time \times latitude	0.32	0.27	1.22	0.22
Time \times longitude	0.88	0.34	2.59	<0.001
Sites east of 106°W				
Time	77.31	37.31	3.94	<0.001
Latitude	-0.51	0.17	2.08	0.04
Longitude	0.47	0.14	3.29	<0.001
Time \times latitude	0.32	0.27	1.2	0.23
Time \times longitude	0.88	0.34	2.57	0.01

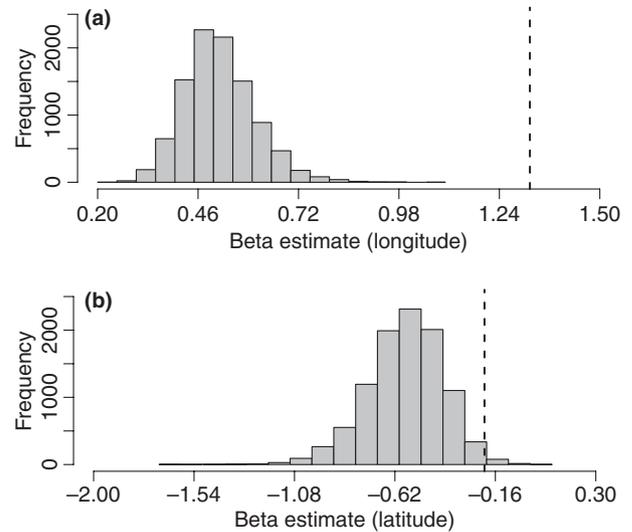


Fig. 2 The distributions of β estimates expected under a null hypothesis that the bunting hybrid zone has remained constant between historical and contemporary sampling periods. Distributions of coefficients are the result of 10 000 Monte Carlo simulations of the probability of sampling a bird with a *Passerina cyanea* mitochondrial DNA as a function of longitude (a) and latitude (b) of contemporary sites. The observed β estimates from the contemporary data are displayed as dashed vertical lines, and are larger than would be expected by chance.

periods (Fig. 3). The interquartile range (between the 0.25 and 0.75 probability isopleths) became substantially narrower between the historical and contemporary samples and the centre of the zone, as indicated by the 0.50 probability isopleth, shifted to a more north–south orientation.

Clinal analysis supported the spatial interpolation patterns (Fig. 4). The width of the mitochondrial DNA cline was significantly wider in the historical sample (695.7 km, 2 ln L support limits: 474.4–1212.9 km) than in the contemporary sample (234.9 km, 2 ln L support limits: 154.2–348.7 km). There was also a nearly significant 102.7 km westward shift in the centre of the clines, 571.6 km east of population 1 for the historical sample (2 ln L support limits: 503.3–642.8 km from population 1) and 468.9 km for the contemporary sample (2 ln L support limits: 436.9–510.7).

Discussion

Although hybrid zone movement is predicted under a variety of models, there are few conclusive empirical examples (Buggs 2007). Using historical and contemporary samples we document a significant temporal change in the genetic structure of the hybrid zone between *Passerina amoena* and *Passerina cyanea*. The GLMM (Table 1; Fig. 3), randomization procedure

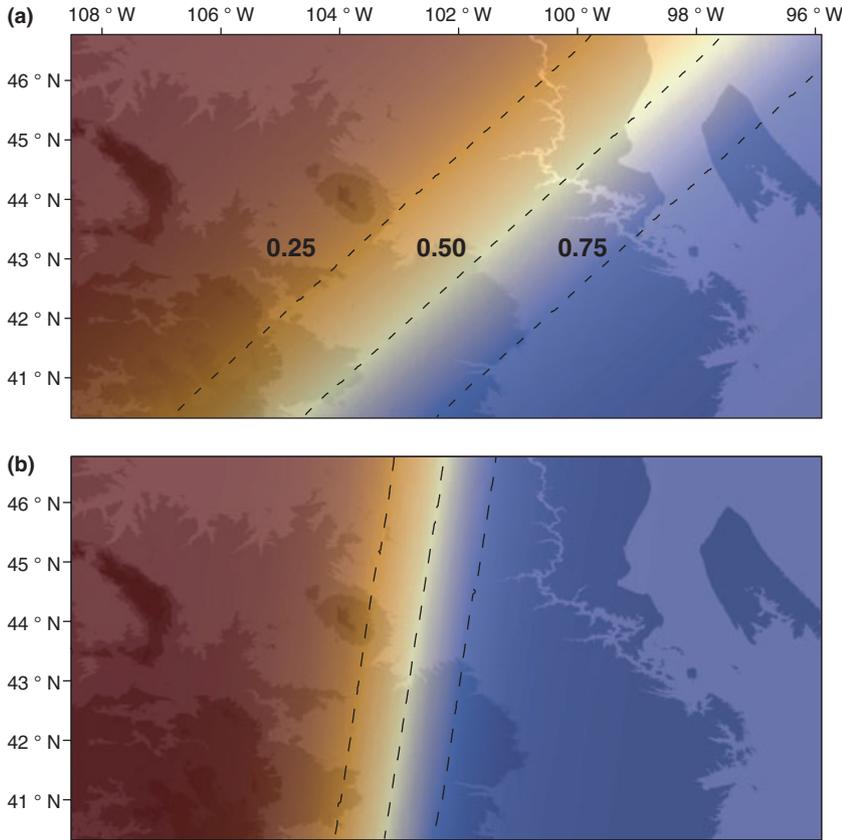


Fig. 3 Spatial interpolation of the predicted probability (red = low, blue = high) of sampling an individual with a *Passerina cyanea* mitochondrial DNA haplotype. The centre the hybrid zone is approximated by the 0.50 isopleth and the width is approximated by the 0.25–0.75 interquartile range. The predicted probability was estimated for the historical (a) and contemporary (b) samples. Underlying shading represents elevation as in Fig. 1.

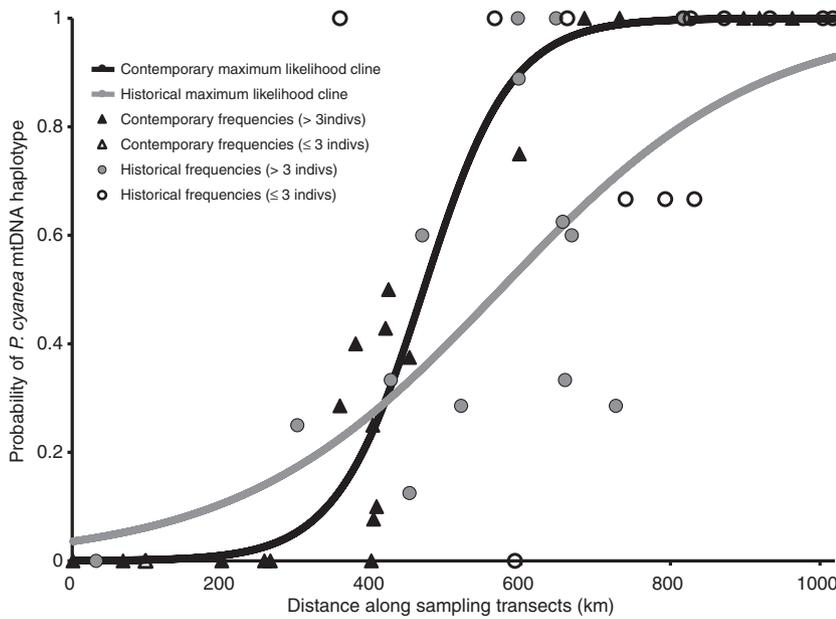


Fig. 4 Maximum-likelihood cline shapes (lines) and mitochondrial DNA haplotype frequencies (symbols), for historical and contemporary samples, plotted against geographic location of sampling locality, measured as the euclidian distance from population 1 (Fig. 1). Note that open symbols indicate those localities for which three or fewer individuals were sampled.

(Fig. 2) and cline analyses (Fig. 4) demonstrated substantial shifts in both the width and location of the centre of the hybrid zone, providing strong empirical support for theoretical predictions that hybrid zones can move under a variety of different scenarios.

The narrowing of the hybrid zone was particularly dramatic as there was a nearly threefold reduction in cline width between the two temporal samples (historical width = 695.7 km, contemporary = 234.9 km) according to the cline analysis. Although the difference

in the cline centres (102.7 km) only approached significance (see Results), the analyses suggest that the hybrid zone also shifted westward in the *c.* 40–45 years between the samples, with an annual movement rate of 2.28–2.57 km. Our spatial interpolations suggest that changes in the northern portion of the hybrid zone likely are responsible for the shift in cline centre (Fig. 3a, b).

Cause of change

There are many potential causes of spatio-temporal change in hybrid zones (Buggs 2007). Many studies that have inferred hybrid zone movement invoke ecological mechanisms for the change. For example, predator mediated selection may have played a role in the inferred movement of the *Heliconius erato hydara/H. e. petiverana* butterfly hybrid zone (Blum 2002) and Hairston *et al.* (1992) hypothesized that the movement of the *Plethodon jordani/Plethodon glutinosus* salamander hybrid zone may have resulted from selection for *P. glutinosus* traits along drier ridges. Using an ecological niche modelling approach, Swenson (2006) suggested that environmental variables, particularly temperature, do impact the geographic location of the *P. amoena/P. cyanea* hybrid zone. Therefore, it may be that *P. amoena* is better adapted to the western xeric environment and *P. cyanea* to the wetter mesic environment of the east, which has been suggested as a selective force maintaining the hybrid zone between *Icterus bullockii*, Bullock's Oriole, and *Icterus galbula*, Baltimore Oriole (Rising 1969). If *P. amoena* and *P. cyanea* are differentially adapted to environments on the west and east side of the ecotone between the Rocky Mountains and the Great Plains and if that ecotone has shifted and narrowed over the past 40–45 years, it is possible that the changes in the *Passerina* bunting hybrid zone found in this study are due to ecological mechanisms. In contrast, others have argued that *P. amoena* and *P. cyanea* do not differ in their ecological requirements (Emlen *et al.* 1975), suggesting that an environmental shift would do little to engender changes in the structure of the hybrid zone.

Under the tension zone model, stable hybrid zones should be located in population density troughs, so zones may move until a trough is reached (Barton & Hewitt 1985, 1989). It follows then that if one of the species in a hybrid zone is expanding its range, hybrid zone movement may occur as has been hypothesized for the hybrid zone between red deer, *Cervus elaphus*, and the expanding, introduced sika deer, *Cervus nippon* (Abernethy 1994; Goodman *et al.* 1999). Analyses of Breeding Bird Survey data show that *P. cyanea* has been expanding its range westward for at least the last

60 years (Payne 2006) and this demographic expansion could have played an important role in the conspicuous change in hybrid zone structure we found, particularly the westward shift in the centre of the zone. The increase in abundance of *P. cyanea* along the western edge of its range may have also contributed to the shift hypothesized by Emlen *et al.* (1975). Unfortunately, this increase is only evident at coarse geographic and time scales, the resolution of the Breeding Bird Survey data does not permit a robust analysis of how the range of *P. cyanea* may have expanded across our study area in the last 40–45 years. Previous work investigating patterns of differential introgression of 10 sex-linked loci across this hybrid zone found evidence of *P. amoena* alleles on the east side of the hybrid zone indicative of a westward moving hybrid zone (Carling & Brumfield 2009). It is certainly possible that greater numbers of *P. amoena* individuals existed on the east side of the hybrid zone in the past, but have been displaced by the expanding wave of *P. cyanea* individuals.

The theory of reinforcement has had a turbulent past (see Chapter 10 in Coyne & Orr 2004), and while we cannot conclusively demonstrate that reinforcement has occurred or is occurring in the *Passerina* bunting hybrid zone, multiple lines of available evidence are consistent with the theory. First, experiments with captive individuals have shown that although there is within population variation, both female *P. amoena* and *P. cyanea* are capable of distinguishing between conspecific and heterospecific mates (Baker 1996). Second, hybrids are at a selective disadvantage with respect to pure parentals (Baker & Boylan 1999). If this selection against hybrids serves to enhance the discriminatory power of females, one potential outcome is a reduction in cline width as hybridization rates decrease over time, which our results support. If the *Passerina* bunting hybrid zone conforms to the tension zone model, then the cline width is proportional to the ratio between dispersal and the square root of selection (σ/\sqrt{s} ; Barton and Gale 1993). Therefore, it is possible to calculate how either selection against hybrids (*s*) or natal dispersal (σ) has changed between the historical and contemporary samples to produce the observed change in cline width (contemporary width = 234.9 km, historical = 695.7 km). For a given dispersal distance, the strength of selection would have had to increase ~9-fold ($695.7^2/234.9^2$) to produce the observed reduction in cline width. Similarly, for a given strength of selection, mean natal dispersal distance would have had to decrease ~3-fold ($696.7/234.9$). As there is no evidence of temporal changes in avian natal dispersal distances, we suggest that under the tension zone model, the reduction in cline width is more likely a result of an increase in the strength of selection.

It is important to note that the tension zone model assumes random mating within the hybrid zone, which is violated in this system (Baker & Boylan 1999). Therefore, the apparent increase in the strength of selection against hybridization as calculated above, may more accurately indicate a reduction in the rate of hybridization, a consequence of reinforcement (Howard 1993), rather than an actual decrease in the fitness of hybrids. One possibility is that the mate discrimination abilities of females of both species have been enhanced over time, a situation known to result in reinforcement (Kelly & Noor 1996), which could result in a decrease in the width of the cline. However, further work is needed to determine whether female mate choice in *Passerina* buntings is hereditary and whether selection against hybridization is great enough to overcome continued gene flow from naive populations (Bigelow 1965).

Evolutionary implications

As *P. cyanea* continues to expand westward (Payne 2006), it is possible that a situation analogous to the *Vermivora pinus*/*Vermivora chrysoptera* hybrid zone will develop. As populations of *V. pinus* expand northward, they hybridize with the much rarer *V. chrysoptera*, contributing to the ongoing decline of *V. chrysoptera*, which has become a conservation concern (Dabrowski *et al.* 2005; Vallender *et al.* 2007). Under this scenario, *P. amoena* populations may become threatened by continued expansion of *P. cyanea* individuals and subsequent hybridization.

An alternative is that the hybrid zone may stabilize. As evidenced by the significant narrowing of the hybrid zone width, it may be that over time the rate of hybridization between these species diminishes and speciation would be completed as predicted by reinforcement theory (Dobzhansky 1937). Although only calibrated using data from two time points, our model predicts that the hybrid zone between *P. amoena* and *P. cyanea* will continue to narrow, consistent with a continuing reduction in hybridization rate.

Hybrid zones, particularly those that are moving, are excellent systems for viewing evolution in action, and as greater numbers of hybrid zones are repeatedly sampled it appears likely that greater numbers of moving hybrid zones will be identified (Buggs 2007).

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sampling localities and mitochondrial haplotypes of all individuals.

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