


Geographically consistent hybridization dynamics between the Black-crested and Tufted titmouse with evidence of hybrid zone expansion

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ABSTRACT

We studied hybridization between the Black-crested and Tufted titmouse across two geographically distinct transects that differ in the timing of secondary contact by hundreds to thousands of years. We found that hybridization patterns correspond to localized hybrid swarms and that the titmouse hybrid zone is likely slowly expanding over time, a product of short post-natal dispersal distances coupled with weak or absent selection against admixture. We show the southern part of the hybrid zone located in Texas is four times wider than the northern region of hybridization in Oklahoma, which is likely due to geographic differences in hybrid zone age. Despite differences in width, most individuals in both transects are advanced-generation hybrids and backcrosses, suggesting geographically consistent hybridization dynamics. We documented a strong correlation between genotypes and plumage index, suggesting that hybridization has not yet resulted in the decoupling of plumage and genome-wide ancestry as observed in some other avian hybrid zones. Although our results suggest the ongoing expansion of the hybrid zone, the rate of expansion appears to be slow, on the scale of tens of meters a year, and it will likely take hundreds of thousands to millions of years before homogenization of the parental populations. While we did not find support for partial reproductive isolation in the hybrid zone itself, there is the possibility that ecological or sexual selection limits introgression into allopatric regions. Broadly, the results of our study highlight the value of multiple, geographically distant, transects across a hybrid zone for assessing the evolutionary dynamics of hybridizing lineages.

Keywords: Black-crested titmouse, hybridization, hybrid swarm, introgression, reproductive isolation, Tufted titmouse

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LAY SUMMARY

- We studied hybridization patterns between Black-crested and Tufted titmice in two areas of their contact zone that differ in the age of onset of hybridization by hundreds or thousands of years.
- We show that northern (younger) parts of the hybrid zone have a genetic transition four times narrower than the southern (older) region, which plausibly reflects the differences in the hybrid zone age or is a result of differences in the breadth of the ecotone between titmouse habitats in the north and south.
- Our results suggest that the Black-crested and Tufted titmouse hybrid zone might be in the process of slowly expanding following selectively neutral hybridization dynamics, although we cannot completely rule out a possible role of ecological or sexual selection in limiting gene flow outside of the hybrid zone.

Dinámicas de hibridación geográficamente consistentes entre *Baeolophus atricristatus* y *B. bicolor* con evidencia de expansión de la zona híbrida

RESUMEN

Estudiamos la hibridación entre *Baeolophus atricristatus* y *B. bicolor* a través de dos transectos geográficamente distintos que difieren en el momento del contacto secundario por cientos a miles de años. Encontramos que los patrones de hibridación corresponden a enjambres híbridos localizados y que la zona híbrida de *Baeolophus* probablemente se está expandiendo lentamente con el tiempo, producto de distancias cortas de dispersión postnatal, junto con una selección débil o ausente en contra de la mezcla genética. Mostramos que la parte sur de la zona híbrida ubicada en Texas es cuatro veces más ancha que la región norte de hibridación en Oklahoma, lo cual probablemente se debe a diferencias geográficas en la edad de la zona híbrida. A pesar de las diferencias en el ancho, la mayoría de los individuos en ambos transectos son híbridos de generaciones avanzadas y retrocruzas, lo que sugiere dinámicas de hibridación geográficamente consistentes. Documentamos una fuerte correlación entre los genotipos y el índice de plumaje, lo que sugiere que la hibridación aún no se ha traducido en el desacople del plumaje y la ancestralidad a nivel de todo el genoma, como se observa en algunas otras zonas híbridas de aves. Aunque nuestros resultados sugieren la expansión en curso de la zona híbrida, la tasa de expansión parece ser muy lenta, en la escala de decenas de metros por año, y probablemente tomará cientos de miles a millones de años antes de la homogeneización de las poblaciones parentales. Aunque no encontramos apoyo para el aislamiento reproductivo parcial en la zona híbrida en sí misma, existe la posibilidad de que la selección ecológica o sexual limite la introgresión en regiones alopátricas. En términos generales, los resultados de nuestro estudio destacan el valor de múltiples transectos geográficamente distantes a través de una zona híbrida para evaluar las dinámicas evolutivas de linajes hibridizantes.

Palabras clave: aislamiento reproductivo, *Baeolophus atricristatus*, *Baeolophus bicolor*, enjambre híbrido, hibridación, introgresión

INTRODUCTION

Hybrid zones continue to advance our understanding of the mechanisms promoting or reversing reproductive isolation between nascent species and, on a broader scale, the origin of biological diversification (Hewitt 1988, Payseur and Rieseberg 2016, Taylor and Larson 2019). In particular, the dynamic nature of hybridization provides an opportunity to use phenotypic and genetic patterns to assess the strength of reproductive barriers and to evaluate the evolutionary trajectory of hybridizing taxa. Moreover, a combination of selection on certain genomic regions coupled with introgression throughout the rest of the genome—which sometimes results in the strong decoupling of phenotypic variation from genome-wide genetic ancestry—has enabled a growing collection of studies to successfully dissect the genetic basis of traits involved in reproductive isolation and speciation (e.g., Poelstra *et al.* 2014, Toews *et al.* 2016, Semenov *et al.* 2021).

Many hybrid zones follow the tension hybrid zone model (Slatkin 1973, Barton and Hewitt 1985), where zone width is determined by the interplay between the dispersal of parental genotypes into the region of hybridization and selection against admixed genotypes that are maladaptive. Under the tension zone model, the hybrid zone width will remain narrower than expected under a scenario of unrestricted hybridization and neutral diffusion, and a comparison of observed and expected hybrid zone widths can provide insight into the action of selection. Further, the interaction between dispersal and selection can produce an array of patterns informative about hybrid zone dynamics. For example, strong selection against hybridization (e.g., due to positive assortative mating) or hybrids (e.g., due to postzygotic selection) coupled with ongoing dispersal of parentals will result in a bimodal distribution (i.e., two modes corresponding to each parental genotype) of genetic ancestries in the hybrid zone center (Gay *et al.* 2008). With some level of hybridization and introgression, as well as selection against hybrids and backcrosses, the distribution of genetic ancestries will appear trimodal (two modes for parental genotypes and one for the hybrids; Gay *et al.* 2008). In such scenarios, sample variance in genotypes across the hybrid zone will increase towards the hybrid zone center (Gay *et al.* 2008, Semenov *et al.* 2017). Conversely, small dispersal distances coupled with weak (or no) selection will produce a hybrid

swarm that is characterized by unimodal genetic ancestry distributions and no change in the variance associated with hybrid indexes across a hybrid zone (Gay *et al.* 2008). In addition, a comparison of the frequencies of individual hybrid classes (F1s, F2s, backcrosses and advanced-generation hybrids) can provide insights into mechanisms of reproductive isolation. For example, the presence of F1s but a lack of other hybrid classes can be indicative of strong selection due to reduced fecundity of F1s or selection against recombined genomes (F2s and backcrosses) due to the buildup of Bateson–Dobzhansky–Muller genetic incompatibilities (Bateson 1909, Dobzhansky 1937, Muller 1940, 1942). Alternatively, a high frequency of advanced-generation hybrids and backcrosses may suggest weak reproductive isolation and extensive introgression.

Many studies of hybrid zones sample a single transect and make inferences about reproductive isolation or introgression from one location of contact. In cases where multiple transects across a hybrid zone are sampled, they are commonly temporal replicates, rather than geographic replicates, and are used to assess hybrid zone spatiotemporal stability or quantify changes in hybridization rates over time (e.g., Mettler and Spellman 2009, Taylor *et al.* 2014, Wang *et al.* 2019, Walsh *et al.* 2020). Less commonly, multiple transects are sampled across geographically distinct parts of a hybrid zone (Rohwer and Wood 1998, Brelsford and Irwin 2009, Scordato *et al.* 2020). Importantly, ecological context matters and species interactions can vary with geography (Harrison 1993, Moor and Price 1993, Harrison and Larson 2016). As such, comparisons of patterns of hybridization across multiple transects in geographically distinct parts of a hybrid zone have the potential to provide valuable insights into the mechanisms maintaining or eroding species boundaries. For example, variable degrees of hybridization between different regions of a hybrid zone may be due to the context-specific breakdown of reproductive isolation (e.g., due to anthropogenic habitat transformation; Grabenstein and Taylor 2018, Grabenstein *et al.* 2023). In a hybrid zone with differently aged regions of secondary contact, comparisons between geographically distant transects can provide insight into the robustness of reproductive isolation over larger historical scales (compared to temporal transects) and can help with interpreting the evolutionary fate of hybridizing lineages.

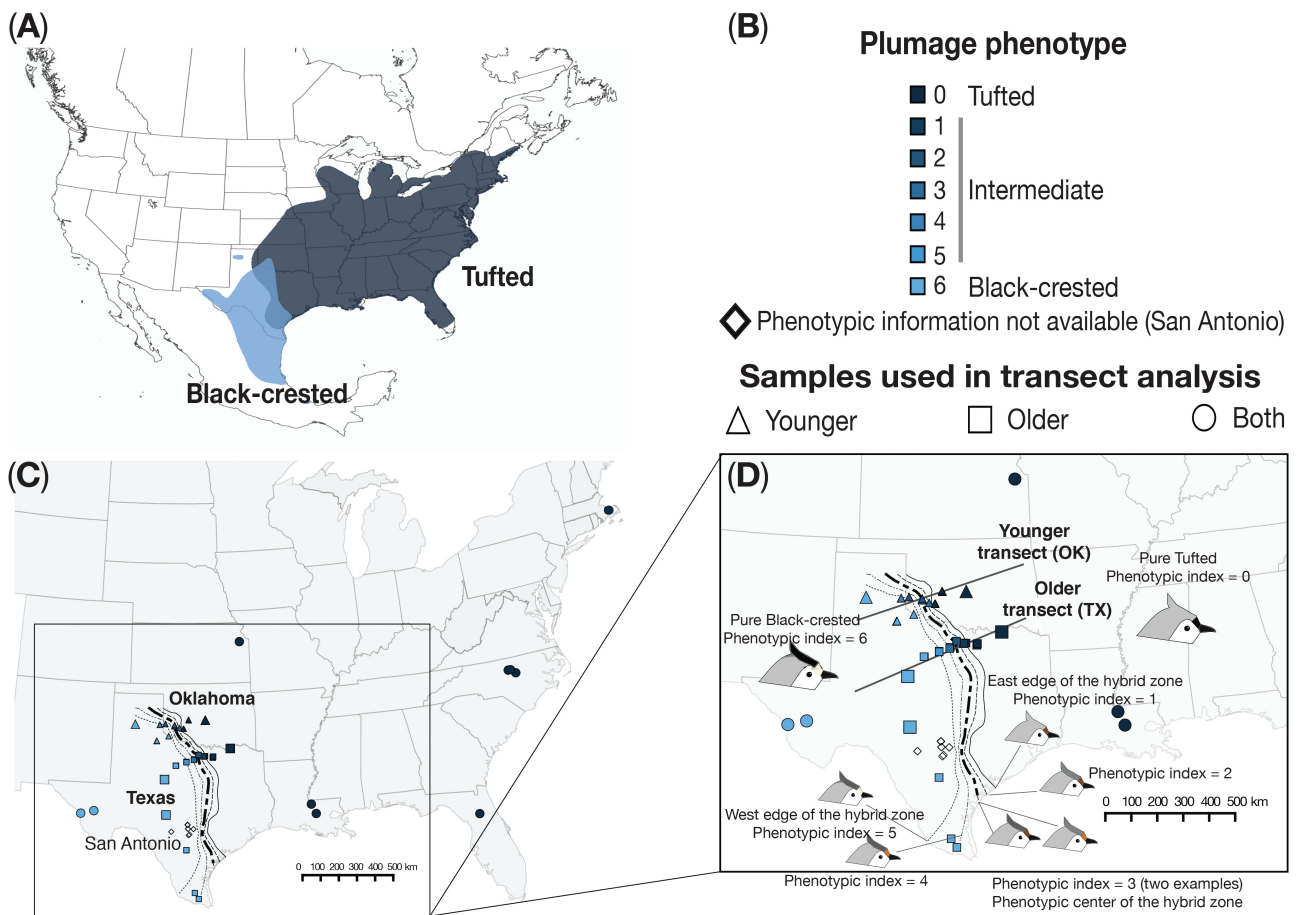


FIGURE 1. Distribution, population sampling, transects across hybrid zones, and variation in crest color in the Black-crested and Tufted titmouse. (A) Approximate distributions of Black-crested and Tufted titmouse in North America (redrawn from <https://www.allaboutbirds.org>). (B) Plumage index color scheme used throughout this study. (C and D) Population sampling and examples of plumage phenotypes and corresponding phenotypic indices. In panel (D), predominant phenotypes are shown for some key areas. Larger and smaller symbols (squares for older transect, triangles for younger transect) represent parental samples from remote allopatric populations and parental samples adjacent to the hybrid zone respectively. Circles indicate samples of both taxa used as allopatric in both older and younger transect analyses. Solid lines indicate transect directions.

The Black-crested Titmouse (*Baeolophus atricristatus*) and Tufted Titmouse (*Baeolophus bicolor*) are sister lineages (Johansson *et al.* 2013) distributed in the southern and eastern parts of North America (Figure 1). They are non-migratory and differ in several aspects of plumage, most markedly in the amount of melanin in the crest (Dixon 1955; Figure 1), exhibit song differences (Dixon 1955, Coldren 1992, Curry and Patten 2019), and have differential habitat preferences but similar foraging microhabitats (Dixon 1955). The two taxa have a spatially extensive area of secondary contact, where the presence of intermediate phenotypes (Figure 1) suggests ongoing or historical hybridization. Southern parts of the contact zone in central and north-central Texas are presumably older and may have formed a few thousand years ago. This estimate is conjectural, however, given that it mainly relies on data that suggest a relatively stable climate in the region for the last four millennia (Dixon 1955, 1978, 1990). The two taxa also co-occur farther north in a southwestern Oklahoma contact zone (Figure 1), where evidence suggests they came into secondary contact as a result of shrub invasion within the past century (Nice 1931, 1943, Sutton 1967, Arnold 1972, Rising 1983, Van Auken 2000, 2009, Seyffert 2001, Callahan 2002, Patten and Smith-Patten 2020). Morphological evidence shows the younger (Oklahoma, OK) contact zone is

geographically narrower than the older (Texas, TX) contact zone, which was hypothesized to be due to its more recent origin (Curry and Patten 2014). Sixty years of phenotypic data from the older contact zone indicate its width and position are relatively stable (Dixon 1990, Curry and Patten 2014), suggesting its boundaries may be maintained by natural selection.

Despite long-term studies of the titmouse hybrid zone, the genetics of hybridization between the Black-crested and Tufted titmouse have not yet been characterized in detail (for characterizations of species divergence and phylogenetic placement as sister taxa, see Braun *et al.* 1984, Avise and Zink 1988, Gill and Slikas 1992, Sheldon *et al.* 1992, Gill *et al.* 2005, Johansson *et al.* 2013). In the current study, we sampled two geographically distant transects across both the southern (older) and northern (younger) parts of the hybrid zone (Figure 1) and used a genotyping-by-sequencing approach to develop genetic markers and characterize patterns of hybridization. We first explored whether parental populations demonstrate a signal of population differentiation and structure and quantified admixture in our two sampled areas of range overlap. Next, we assessed whether the southern region of the hybrid zone is broader than the younger northern region (as suggested by previous plumage studies) by comparing the

width of ancestry-informative geographic clines, and used dispersal estimates to determine if the titmouse hybrid zone is a tension zone. We also compared the observed patterns of variance in hybrid index across both transects to evaluate their correspondence to different hybrid zone models and examined hybrid index and inter-specific heterozygosity to identify distinct hybrid classes (F1s, F2s, backcrosses, and parental genotypes). Finally, we compared plumage, as represented by crest and forehead color, and ancestry to evaluate the strength of the relationship between genotype and phenotype, and to test whether admixture results in substantial decoupling between phenotypic and genetic markers as in some other avian systems (e.g., White Wagtail [*Motacilla alba*], Semenov *et al.* 2021; Blue-winged [*Vermivora cyanoptera*] and Golden-winged [*Vermivora chrysoptera*] warblers, Toews *et al.* 2016).

METHODS

Permits and Sampling

Our samples were obtained under Federal Bird Banding Permit 23215H (issued by the U.S. Geological Survey Bird Banding Laboratory), Federal Fish and Wildlife Permit MB148195-2 (issued by the U.S. Fish and Wildlife Service); Scientific Collecting Permits 4716, 4955, 5210, and 5507 (issued by Oklahoma Department of Wildlife Conservation); Scientific Collecting Permit SPR-0310-019 (issued by Texas Parks and Wildlife Department).

We used 120 samples in this study (Supplementary Material Table S1). Birds were captured with feeder traps (Bacon 1987) and mist nets (Curry 2014). Blood samples were collected by C.M.C. ($n = 80$) along two geographic transects in the younger contact zone in Oklahoma and northern Texas (hereafter younger transect, $n = 41$) and 200 km farther south in north-central Texas (hereafter older transect, $n = 39$) and preserved in Queen's lysis buffer (Seutin *et al.* 1991). Ethanol-preserved museum-loaned tissue samples were used to characterize allopatric populations ($n = 15$) (Figure 1, Supplementary Material Table S1). In addition, samples from San Antonio (Texas), adjacent to the older transect ($n = 25$) (preserved in Queen's lysis buffer), were provided by Troy G. Murphy (Trinity University, One Trinity Place, San Antonio, Texas, USA). San Antonio is within the range of Black-crested Titmouse, but in 1886–1887 a few Tufted titmice arrived in winter and stayed to breed (Dixon 1990), so the samples are of interest to determine if there is any evidence of historical genetic admixture in the San Antonio titmouse population.

Laboratory Methods

DNA was extracted using Qiagen DNeasy Blood and Tissue kits (Qiagen, cat. no. 69504). We modified the default Qiagen protocol in order to increase DNA concentration: we used 2 final elutions of 50–100 μ L (default was 200 μ L) with 10 mM Tris: 0.1 mM EDTA buffer (default was AE buffer). Reduced-representation genome sequencing libraries were constructed using the *Pst*I restriction enzyme following the genotyping-by-sequencing (GBS) method of Elshire *et al.* (2011). Single-end libraries were sequenced to 100 base pairs (bp) on a single lane of the Illumina HiSeq 2000 platform at the Cornell Institute for Genomic Diversity and produced ~200 Gbp of unfiltered data with an average of 1.7 million reads per individual. Raw data were filtered using the *process_radtags* module of the Stacks 1.44 pipeline (Catchen *et al.* 2011, 2013) following Barrera-Guzmán

et al. (2018). Filtered reads were next aligned to the *Parus major* reference genome (Laine *et al.* 2016, Release 101, https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Parus_major/101/) using Bowtie 2 2.2.6 (Langmead and Salzberg 2012) with the “sensitive” settings. *Baeolophus* diverged from *Parus* at least 4 mya (Gill *et al.* 2005), but at the time of data generation, *Parus major* was the closest reference genome available. Read alignment produced 66–77% of reads aligning per individual. The *ref_map* wrapper script of the Stacks pipeline was used to call genotypes using default settings with the exception that we used the bounded-error single nucleotide polymorphism (SNP) calling model with a maximum error rate of 0.05. We set the Stacks *m*-parameter at 10 for the populations step that deletes any genotype with <10 reads. We then filtered our dataset as follows using *vcftools* 0.1.14 (Danecek *et al.* 2011). We filtered the data to remove sites with more than 40% missing data, with greater than the 95th percentile sequencing depth and with observed heterozygosity exceeding 0.75 (the latter 2 filters help eliminate paralogues incorrectly aligned together), with less than 2 copies of the minor allele (only biallelic sites were retained), and which occur less than 10,000 bp apart in the reference genome. While 40% of missing data per locus might be considered higher than normal for genomic studies, our comparison with a dataset with more stringent filtering criteria of 20% missing data per locus showed minimal effects of missingness on estimates of interest (e.g., principal component analysis [PCA], STRUCTURE, and geographic cline analysis) (Supplementary Material Figure 1). The vast majority of genotypes in our dataset had less than 50% missing data with the exception of 4 individuals (A_O_43224, B_O_18858, B_Y_18907 and B_US_5306), 2 of which were sampled in the older transect, 1 was in the younger transect, and 1 was an allopatric Tufted Titmouse (see Supplementary Material Table 1).

Population Structure and Differentiation

We used STRUCTURE 2.3.4 (Pritchard *et al.* 2000, Falush *et al.* 2003) to evaluate patterns of population structure. We ran STRUCTURE analysis only with the number of clusters set to $K = 2$, given that we were interested in detecting individuals with ancestry intermediate between the Black-crested and Tufted titmouse. We ran 5 independent chains with burn-in of 100,000 iterations and 500,000 iterations parameter sampling using an admixture model and correlated allele frequencies. STRUCTURE analysis was conducted for all samples simultaneously. The resulting Q-scores were averaged between replicates. To assess population clustering without a pre-defined number of clusters, we ran PCA on the mean-centered genetic covariance matrix of SNP genotypes using the R v.3.6.1 (RStudio Team 2020) function *prcomp*.

We estimated Weir and Cockerham F_{ST} (Weir and Cockerham 1984) using *vcftools* 0.1.14 (Danecek *et al.* 2011) between allopatric Black-crested and Tufted titmouse and visualized the Manhattan plot using R package *qqman* (Turner 2018).

Geographic Clines and Individual Admixture Patterns

We summarized genomic variation using PCA as described above and used PC1 scores as a proxy for ancestry. We fit empirical data to 5 geographic cline models with or without allowing asymmetric introgression (“free none”, “free both”, “free right”, “free left”, and “free mirror”; see Semenov *et*

al. 2021 for details) using Metropolis–Hastings MCMC algorithm implemented in *hzar* v.0.2-5 (Derryberry *et al.* 2014) package in R. To determine whether the hybrid zone is a tension zone (i.e., a hybrid zone maintained by the balance between selection and dispersal), we compared the observed zone widths with estimates expected under a scenario of unrestricted hybridization and neutral diffusion following Barton and Hewitt (1985) and using the formula $w=2.51\sigma\sqrt{t}$, where σ is the post-natal dispersal distance and t is the number of generations since secondary contact. We used a dispersal distance from Rylander *et al.* (2020) for a Black-crested Titmouse of 0.248 km. Although this estimate may appear unreasonably small given typical distances in other Passerines, it is similar to estimates of 0.091–1.097 km (average of 0.343 km) from a sister group (Gill *et al.* 2005), the Juniper and Oak titmouse complex (*Baeolophus ridgwayi* and *Baeolophus inornatus*) (Cicero 2000). For generation time, we assumed a length of one year typical for most Passerines (Ehrlich *et al.*, 1988). Previous work suggests that the older contact zone formed no later than 4,000 years ago (Dixon 1978), following initial divergence in allopatry with an estimated split around 250,000 years ago (Dixon 1978, Klicka and Zink 1997, Patten and Smith-Patten 2020). For the younger zone, we used a range of estimates between 60 and 150 years. The earliest date for habitat changes in Van Auken (2000) is the 1870s; therefore, we assumed 150 years as an upper limit. For the lower limit, Sutton (1967) lists 1963 as the first Black-crested Titmouse record in Oklahoma, and Nice (1931 1943) did not report a Black-crested Titmouse in Oklahoma; hence, we assumed the most recent date as 60 years ago.

To evaluate contrasting hybridization scenarios, we compared the hybrid index and observed inter-specific heterozygosity of individual genotypes (Gompert and Buerkle 2016). This analysis requires loci that are fixed or nearly fixed to alternative allelic states in allopatric populations, so we first used *vcftools* 0.1.14 (Danecek *et al.* 2011) to calculate Weir and Cockerham F_{ST} between allopatric samples as described above and to select loci with $F_{ST} = 1$ ($n = 68$). We applied additional filtering to only retain loci with <30% missing data in order to decrease the uncertainty of estimates, resulting in 24 SNPs in this analysis. We chose to use a hybrid index over using STRUCTURE Q-scores or PC1 scores from PCA as the most assumption-free method (Gompert and Buerkle 2016). We used custom R code to generate hybrid index scores and then plotted the hybrid index against inter-specific heterozygosity. In this analysis, individuals with the hybrid index of 0 and 1, and inter-specific heterozygosity of 0 will correspond to parental Tufted and Black-crested genotypes, respectively. First-generation hybrids are expected to have a hybrid index of 0.5 and inter-specific heterozygosity of 1, while F2s and later-generation hybrids have lower levels of inter-specific heterozygosity and variable hybrid indices due to recombination and segregation of alleles. Backcrosses have hybrid indices closer to parental genotypes but have higher inter-specific heterozygosity.

To assess the accuracy of the hybrid class assignment given the relatively high frequency of missing data in our dataset, we used simulated data for parental genotypes, recent-generation hybrids, and backcrosses. We used a set of 24 fixed loci described above and allopatric Black-crested ($n = 7$) and Tufted ($n = 8$) titmouse (Supplementary Material Table 1) to simulate 100 genotypes of each parental population,

F1s, F2s, and first-generation backcrosses using NewHybrids (<https://github.com/eriqande/newhybrids>). We then introduced missing data at a 30% rate using the R function *delete_MCAR()* of the *missMethods* (<https://github.com/torockel/missMethods>) and estimated hybrid index and inter-specific heterozygosity.

To assess patterns of genotypic variance across the transect, we estimated variance and its 95% confidence intervals (CIs) using the hybrid index based on the above set of 24 fixed loci and the same sample grouping as in our geographic cline and STRUCTURE analyses (Supplementary Material Table 1, Figure 2). For genotypic variance analysis, we excluded sample ELT from the older transect due to the small sample size ($n = 2$).

For plumage variation, individuals were categorized via Dixon's hybrid index (Dixon 1955) (which we further refer to as phenotypic index to avoid confusion with our genetic hybrid index) and correlated with colorimeter data on the same birds from which blood samples were taken (for details about colorimeter measurements, see Curry and Patten 2014). The Tufted Titmouse has a gray crest and black forehead, which is a phenotypic index score of 0. Male Black-crested Titmouse has a black crest and pale forehead, which is an index score of 6. Hybrids show intermediate combinations, often with a chestnut forehead (Figure 1) (Curry and Patten 2014). We assumed indices to be 0 (Tufted) and 6 (Black-crested) for museum tissue samples that were not located in the known hybrid zone (Dixon 1955, 1990, Curry and Patten 2014; Figure 1). The plumage hybrid index was then compared to 3 metrics of ancestry (STRUCTURE Q-scores, hybrid index, and PC1) to assess the strength of the relationship between phenotypic and genetic variation.

RESULTS

Following filtering we had an average per individual depth of coverage of 17.7 reads for retained genotype calls. Applying all filters to the initial dataset of 391,522 SNPs produced 3,691 SNPs for downstream analyses. STRUCTURE analysis assigned allopatric Black-crested and Tufted titmouse to distinct genetic clusters (Figure 2A). The first principal component of the PCA explained 90% of the variance and, similar to STRUCTURE, clearly separated allopatric populations (Figure 2B, Supplementary Material Figure 1). Individuals spanning both the younger and older transects possessed a broad range of STRUCTURE admixture proportion values and PC1 scores, confirming genetic admixture between the 2 taxa. There was a moderate amount of population differentiation between allopatric Black-crested and Tufted titmouse (average $F_{ST} = 0.11$, 3,691 loci) and no apparent clustering of highly differentiated loci on any chromosome (Figure 2C).

For both transects, the best geographic cline model was a symmetrical sigmoid cline with no difference in introgression tails. Geographic cline analysis revealed that the younger (northern) hybrid zone is nearly four times narrower (width = 43.8 km, 2 log likelihood limits = 25.5–73.3 km) compared to the older (southern) hybrid zone (width = 178.9 km, 2 log likelihood limits = 121.5–218.8 km) (Figure 3A). The expected width of clines under a scenario of unrestricted hybridization and neutral diffusion was 4.8–7.6 km in the younger and 39.4 km in the older transect. These expected estimates were both narrower than the observed cline widths and their 95% confidence intervals, suggesting that the titmouse hybrid

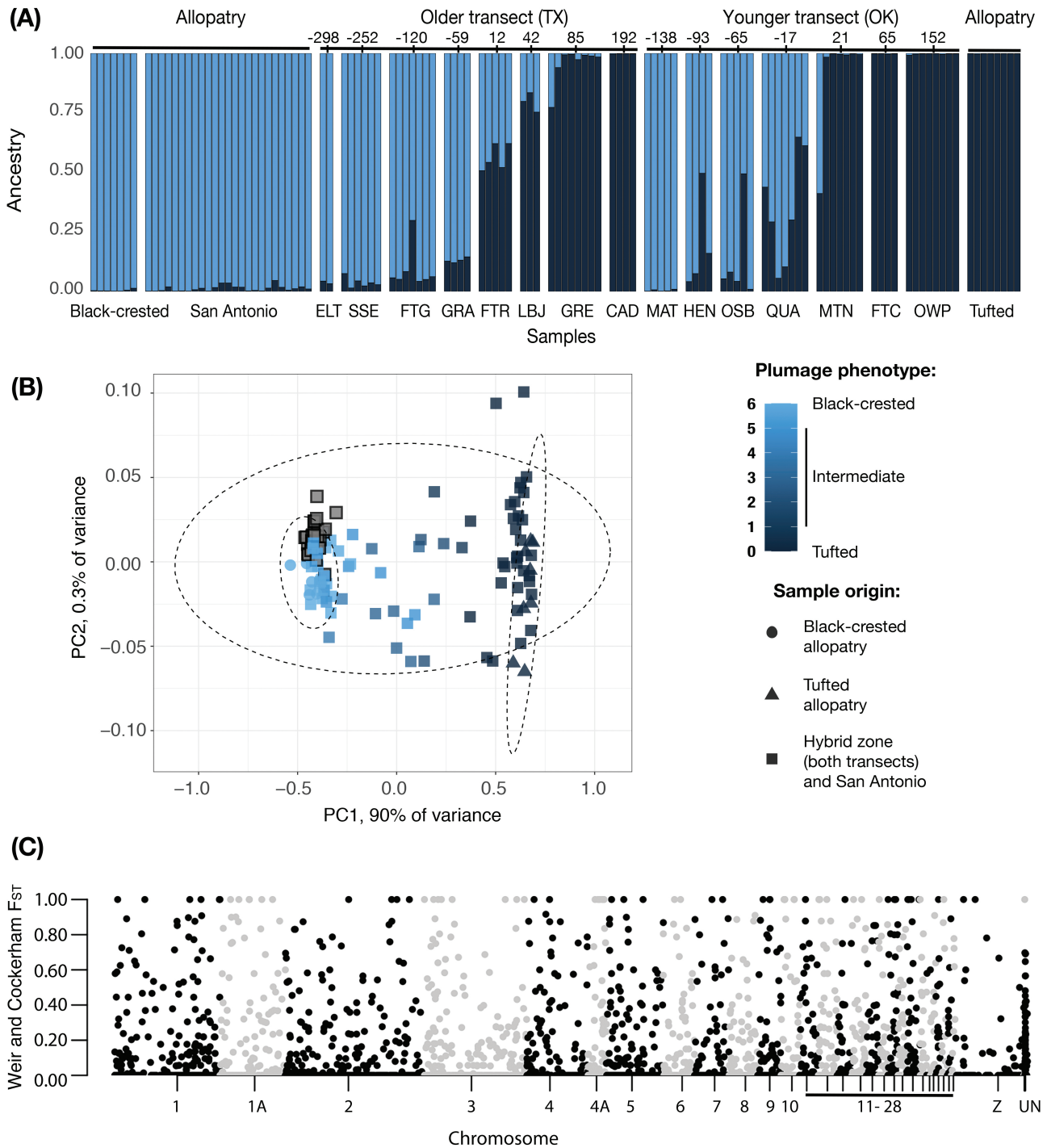


FIGURE 2. Population structure and differentiation between the Black-crested and Tufted titmouse based on 3,691 loci. (A) Results of STRUCTURE analysis with the number of clusters $K = 2$. Individuals are arranged by sampling location (see [Supplementary Material Table S1](#) for location codes). Locations follow the direction of transects across hybrid zones with distances from the geographic cline center indicated on the top. (B) Results of principal component analysis (PCA) performed on mean-centered covariance matrix of genotypes. Gray symbols with black outline are individuals from San Antonio (allopatric Black-crested Titmouse adjacent to older hybrid zone) with missing phenotypic data. Dashed lines indicate the 95% confidence interval. For presentation purpose, PC1 was flipped by multiplying PC1 scores by negative one. (C) Weir and Cockerham F_{ST} between allopatric Black-crested and Tufted titmouse. Note that STRUCTURE and PCA clearly separate allopatric populations of the two taxa and indicate a range of intermediate ancestries in areas of hybridization. Note that while San Antonio is within the range of allopatric Black-crested Titmouse, a few Tufted Titmouse arrived in winter and stayed to breed in 1886–1887 (Dixon 1990), and genetic traces of such events appear to remain.

zone is not consistent with a tension zone model. There was no single location in either hybrid zone where both parental genotypes were present together (Figure 3B), suggestive of low dispersal rates (and/or short dispersal distances) of par-

ental genotypes in the hybrid zone. There was no apparent increase in genotypic variance towards the hybrid zone center in the older transect, and only a slight increase in the younger transect (Figure 3C), providing support for the “unimodal”

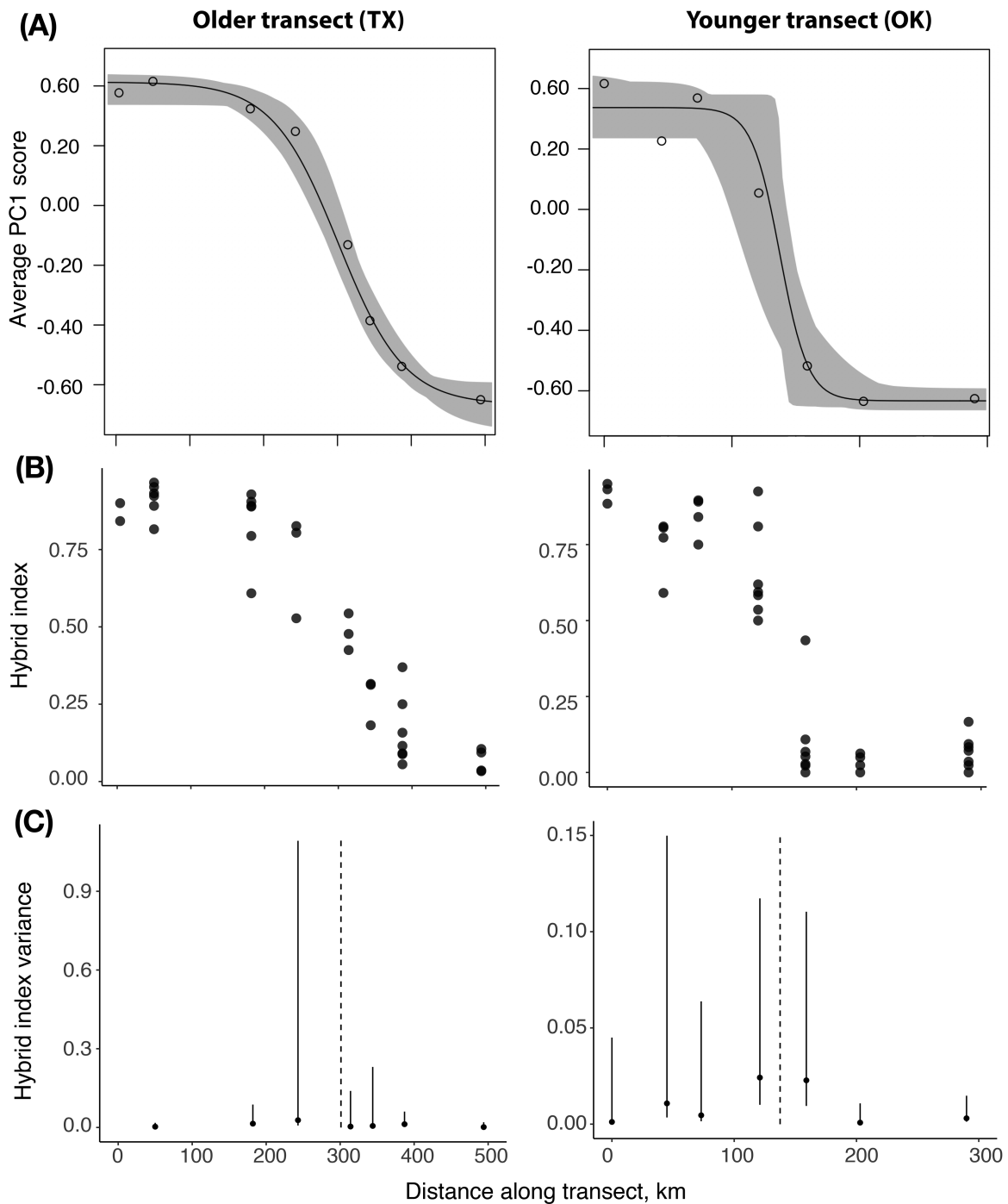


FIGURE 3. Geographic patterns of hybridization in the Black-crested and Tufted titmouse in the older (left) and younger (right) transects. (A) Results of geographic cline analysis. Each dot represents average value of PC1 scores of genome-wide PCA for a given sampling location. A sigmoid line is the most parsimonious geographic cline that approximates the transition in ancestry and gray shading is the 95% CI. Note that scales are different between plots. (B) Distribution of hybrid indices along transects. Each dot is a hybrid index for an individual in a given sampling location. (C) Variance in genotypes for sampling locations and the 95% confidence intervals for one sample in the older transect was excluded due to small sample size. Dashed lines indicate hybrid zone centers. Note that there are no samples where distinct parental genotypes (i.e., individuals with hybrid indices close to 0 and 1) coexist at a given location. Also note that there is no apparent increase of genotypic variance toward zone center in the older zone, and only slight increase in the younger zone. These patterns are consistent with the “unimodal” model of hybridization, which is a localized hybrid swarm. The complete set of 3,691 loci was used in geographic cline analysis and 24 fixed loci were used for estimating hybrid index.

(i.e., a situation with so much admixture and backcrossing that most individuals are similarly admixed) rather than “bimodal” or “trimodal” genotype distribution models.

Our simulations using 24 SNPs fixed to alternative states between allopatric titmouse populations revealed that we

can confidently differentiate parental genotypes and F1s from other genotypic classes (Figure 4A). Although there was a slight overlap between the distributions of F2s and both the Black-crested and Tufted backcrosses, the majority of the simulated genotypes could be confidently assigned to each

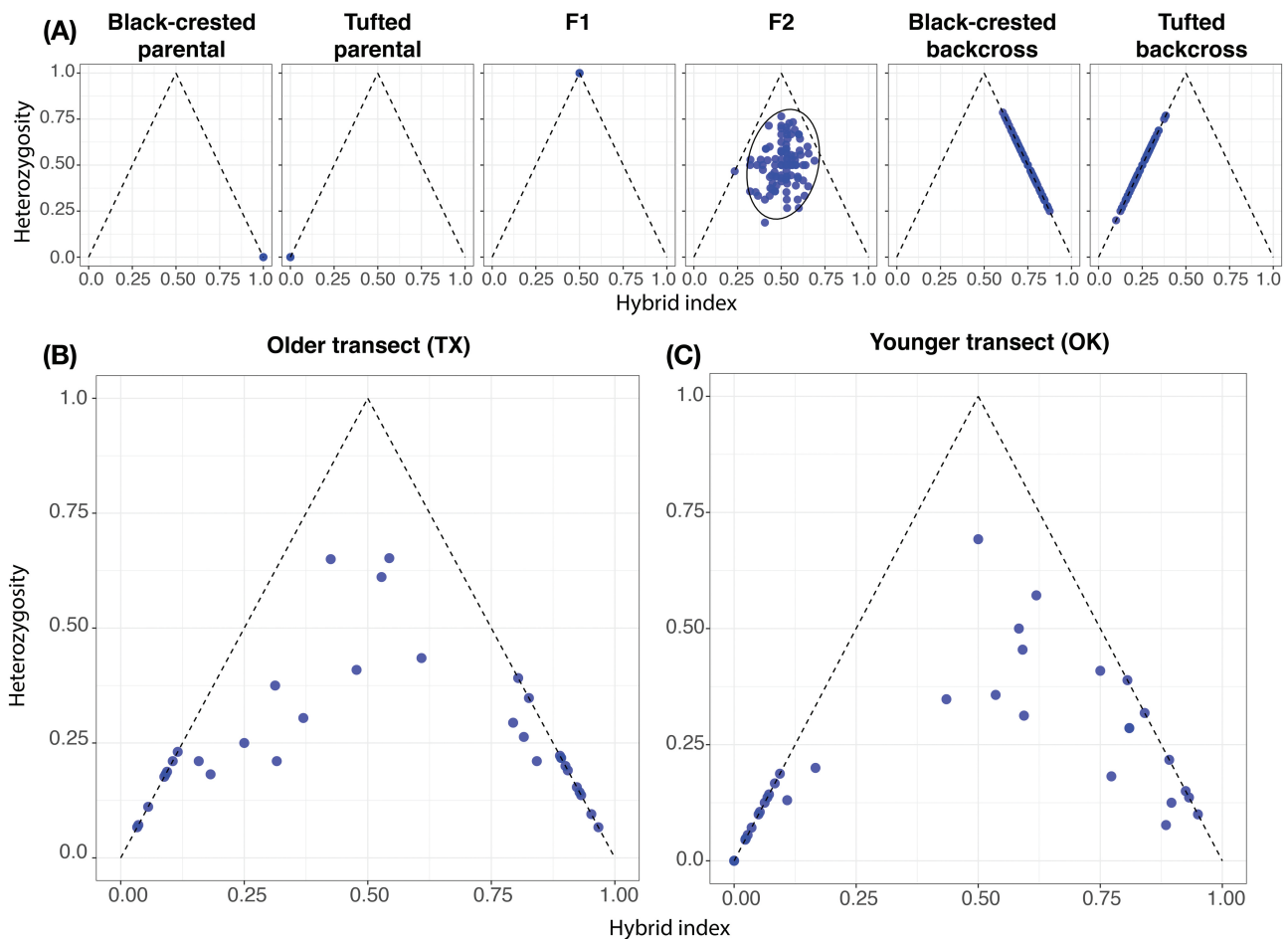


FIGURE 4. Comparison of hybrid index and inter-specific heterozygosity (24 fixed loci). (A) Top panel illustrates simulated genotypes of 100 of each parental Black-crested and Tufted titmouse, first- and second-generation hybrids, and both types of backcrosses, and their 95% confidence intervals. Bottom: empirical data for the older (B) and younger (C) transects. Individuals with hybrid index of 0 and 1, and inter-specific heterozygosity of 0 correspond to parental Tufted and Black-crested genotypes, respectively. First-generation hybrids have hybrid index of 0.5 and inter-specific heterozygosity of 1, while F2s and later-generation hybrids have lower levels of inter-specific heterozygosity and variable hybrid indices due to recombination and segregation of alleles. Backcrosses have hybrid indices closer to parental genotypes, but higher inter-specific heterozygosity (sides of the “triangle”). Note that there are only few parental genotypes found in the hybrid zone (three Tufted in the younger transect and none in the older), and no F1s, while the majority of individuals appearing to be F2s or more advanced generation hybrids and backcrosses. Note that the observed patterns are similar between older and younger transects, suggesting that, despite differences in contemporary zone widths, the dynamics of hybridization are geographically consistent.

respective class as well (Figure 4A). Given these simulation results, the analysis of hybrid index and inter-specific heterozygosity shows that there are few (8% in the younger transect) to none (older transect) parental genotypes found in the hybrid zone (Figure 4B and C). We also did not observe any genotypes that could be categorized as F1s, with the vast majority of individuals appearing to be F2s or more advanced generation hybrids and backcrosses.

There was a significant correlation between ancestry and the plumage index ($r^2 = 0.89\text{--}0.93$, $P < 2.2e\text{--}16$, Figure 5A–C), suggesting that extensive hybridization and introgression do not result in substantial decoupling between phenotypic and genomic variation.

DISCUSSION

We examined patterns of population structure and hybridization between the Black-crested and Tufted titmouse using 2 transects across the hybrid zone with presumably an order of magnitude difference in the timing of the onset of range con-

tact and hybridization. Our results revealed that the older part of the hybrid zone was nearly four times wider ($w = 179$ km) than the younger ($w = 44$ km), consistent with the findings of Curry and Patten (2014) and supporting the previous hypothesis that the zone width might be expanding over time. We found several lines of evidence (composition of hybrid classes and their spatial transition, along with variance analysis) of extensive admixture and introgression between titmouse taxa. Further, we found that the observed width of geographic clines in both transects is wider than the widths expected under a scenario of neutral diffusion, suggesting that there is little to no selection in maintaining the titmouse zone width.

Interpreting Patterns of Black-crested and Tufted Titmouse Hybridization

Our finding that observed hybrid zone widths were not narrower than expected under a scenario of neutral diffusion suggests that the titmouse hybrid zone is not a tension zone (i.e., a hybrid zone maintained by the balance between dispersal and selection; Barton and Hewitt 1985). Such a finding is

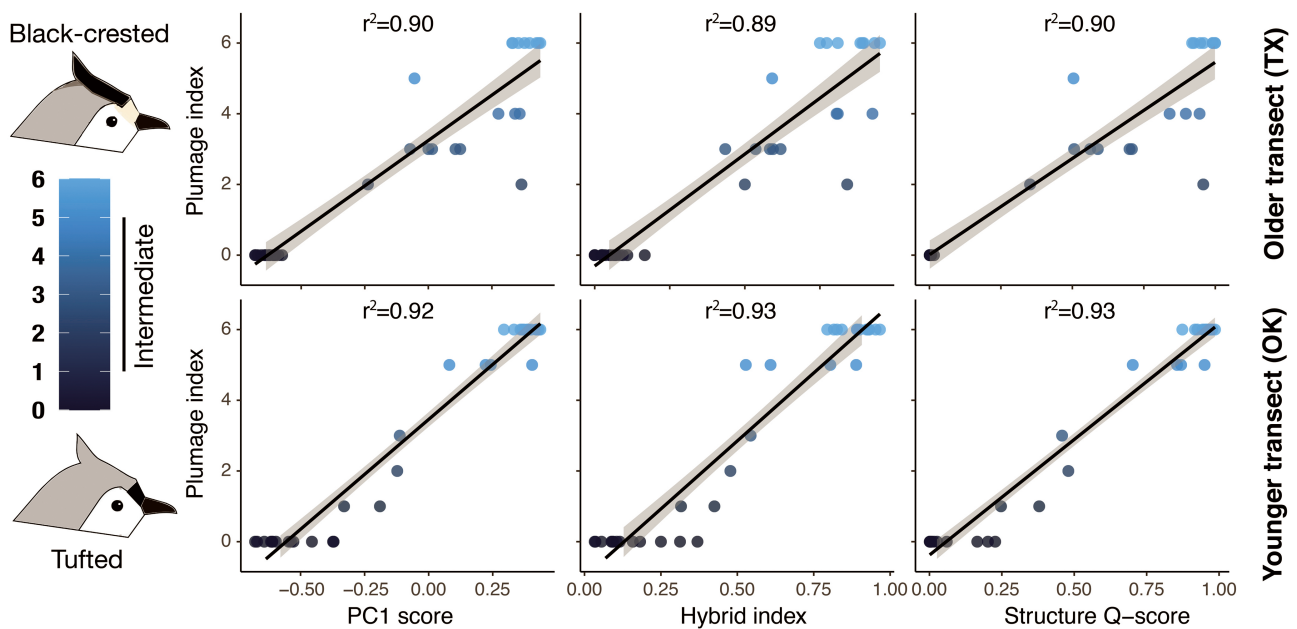


FIGURE 5. Association between plumage phenotype (crest color) and ancestry (PC1) (3,691 loci), hybrid index (24 loci) and STRUCTURE Q-scores (3,691 loci) for the older and younger transects.

relatively rare in the literature (but see [Baldassarre et al. 2014](#), [Wang et al. 2019](#), [Del-Rio et al. 2022](#)) as the vast majority of hybrid zones (particularly in birds), are consistent with the tension zone model. One interesting natural history aspect of titmice (Black-crested and Tufted titmice and Juniper and Oak titmouse) is their unusually short dispersal distance of 0.25–0.35 km ([Cicero 2000](#), [Rylander et al. 2020](#)). In other temperate Passerine species, average dispersal distances are typically estimated on a scale of several kilometers ([Tittler et al. 2009](#)). If the true dispersal distance of the Black-crested and Tufted titmouse is substantially underestimated in previous studies (i.e., larger than the current known estimate of 0.25 km), it raises a possibility that the cline width expected under neutral diffusion may be wider than our estimate. However, if we assume that the dispersal distance in Black-crested and Tufted titmice is similar to the maximum estimate known for Juniper and Oak titmice (1.097 km, [Cicero 2000](#)), the cline widths under neutral diffusion would be 21–34 km for the younger and 174 km for the older transect. Both of these estimates fall within the limits of the observed zone widths (26–73 km for the younger transect, and 122–219 km for the older transect), supporting a model of neutral diffusion.

Other pieces of evidence are also consistent with low dispersal distances in the titmouse taxa. Our finding of a gradual transition in hybrid indices along both transects ([Figure 3B](#)) and the lack of elevated genotypic variance towards the hybrid zone center for either transect ([Figure 3C](#)), both support a “unimodal” model of titmouse hybridization. This model implicates low rates of dispersal and weak selection against admixture ([Gay et al. 2008](#)). Further, the observation of few parental genotypes in the hybrid zone, and the complete absence of F1 hybrids in our dataset, also suggests a low dispersal of allopatric (unadmixed) individuals into the hybrid zone. A plausible explanation for this pattern is short dispersal distances compared to the contemporary geographic scale of admixture.

It is important to note that the estimate of hybrid zone width under a scenario of neutral diffusion relies on precise know-

ledge about the number of generations since secondary contact as well as dispersal distance. If the number of generations since the onset of hybridization was severely underestimated in our study (which is particularly plausible for the older parts of the hybrid zone), there is a possibility that the titmouse zone would deviate from the model of neutral diffusion. In addition, introgression rates are often highly heterogeneous across the genome ([Harrison and Larson 2016](#)). It is possible that some loci in the titmouse hybrid zone are resistant to introgression despite the fact that we did not find support for this in our analyses: we only considered genome-average estimates of ancestry and we were working with a relatively small genomic dataset. These considerations mean that our finding of a pattern consistent with neutral diffusion in the titmouse hybrid zone should be taken with some caution.

The observation that parental genotypes never meet in the titmouse hybrid zone suggests that even if some degree of assortative mating existed historically, it does not contribute substantially to reproductive barriers and that postzygotic isolation is likely weak (consistent with theoretical findings of [Pulido-Santacruz et al. 2018](#), [Cronemberger et al. 2020](#), [Irwin 2020](#); and predictions of [Curry and Patten 2016](#)). However, this does not exclude the possibility that hybrids experience reduced fitness due to selection outside of the hybrid zone. Ecological selection is plausible because both allopatric populations occur in different habitats ([Dixon 1955](#)). Both transects we studied are located in regions of habitat transition, where individuals with intermediate genotype or phenotype may not experience reduced fitness, or even have comparatively increased fitness, according to the model of bounded hybrid superiority ([Moore 1977](#)). It is commonly recognized that sharp ecological boundaries, as observed in the titmouse hybrid zone, can act as barriers to gene flow and prevent introgression into allopatric populations ([Price 2008](#)). We suggest that mark-recapture experiments at the hybrid zone edges and replicate sampling of the hybrid zone over time are needed to disentangle between scenarios of neutral diffusion

and strong selection for habitat (Pyron *et al.* 2015, Patten *et al.* 2021).

While the observed differences in hybrid zone width between the older and younger transects may reflect differences in the age of onset of secondary contact, another possibility could be differences in the extent of intermediate habitat, although there is currently no support for this latter hypothesis (Curry and Patten 2019). Several other avian species pairs (e.g., *Melanerpes* woodpeckers, *Passerina* buntings, and *Colaptes* flickers), along with some species of reptiles and mammals have zones of contact and hybridization in southern parts of the Great Plains (Rising 1983). This further increases the possibility that habitat factors (along with historical-biogeography factors) may play a role in the distribution and location of the Black-crested and Tufted titmouse hybrid zone.

In many taxa, particularly birds, hybridization sometimes results in nearly complete homogenization of differentiated genomes with the exception of a few (often narrow) genomic regions wherein divergent genotypes are maintained by selection (e.g., Poelstra *et al.* 2014, Toews *et al.* 2016, Semenov *et al.* 2021). In such instances, selectively neutral genotypes across the majority of the genome will show little to no association with phenotypes and, hence, demonstrate no correlation between genome-wide ancestry scores and plumage (e.g., Semenov *et al.* 2021). Unlike in the above examples, we found no clustering of highly differentiated loci in the genome (Figure 2) and a strong correlation between phenotype and genotype in the Black-crested and Tufted titmouse, despite a small genetic dataset compared to other recent studies (Figure 5; note that we only used samples from within or near the hybrid zone for this analysis). This finding could potentially suggest substantial genome-wide restriction of introgression, rather than genomically localized selection on plumage genes. More plausibly, given the evidence for rampant hybridization, the above patterns may be due to the polygenic nature of plumage coupled with the selectively neutral hybridization dynamic. If this is the case, we can expect that hybridization will ultimately lead to the homogenization of plumage and genetic differences between the Tufted and Black-crested titmouse, but perhaps on a longer timescale than other avian examples due to their small dispersal distances.

The Black-crested and Tufted titmouse have marked differences in plumage and some differences in song. Despite not finding a detectable signal of this in our genetic data, there is evidence that the intensity of hybridization may vary between the older and younger parts of the hybrid zone, given that males in the older zone respond more strongly to their own song types compared to males in the younger zone (Curry and Patten 2016), and that song structure correlates with the environment only in the younger zone (Curry and Patten 2019). Along with the possibility of divergent mate choice, and particularly female mate choice, female-female or male-male signaling could act as a selective agent (Murphy *et al.* 2009a, 2009b, Tarvin and Murphy 2012, Brooks and Wimberger 2023). At least male Black-crested Titmouse use their crests for signaling (Dixon 1955, 1978, Queller and Murphy 2017, Borger *et al.* 2020); anecdotally female Black-crested Titmouse do not (Borger *et al.* 2020). Whether male or female Tufted titmouse uses crests for signaling is, to the best of our knowledge, as yet unstudied, although foreheads in Tufted titmouse are related to status signaling (Moses and Ritchison 2000).

Thus, hybrid crest color and forehead color could be linked to either intrasexual signaling alone or additionally linked to the environmental selection due to contrast (Davis *et al.* 2022) with background vegetation (Dixon 1978 and Curry and Patten 2016) or differences in competition at different regions in the hybrid zone (Queller and Murphy 2017). Thus, while strong effects of behavioral isolating factors in the hybrid zone itself are unlikely as we discuss above, they could still play a role in limiting introgression into allopatric populations and warrant further investigation.

Conclusions

Our study shows that the Black-crested and Tufted titmouse demonstrate high levels of hybridization and introgression that have resulted in localized hybrid swarms. Furthermore, the observed width of geographic clines in both transects was wider than expected under neutral diffusion, potentially supporting the hypothesis of evolutionary homogenization between the taxa. In addition to presumably weak or absent selection against hybrids and backcrosses, short post-natal dispersal distances may play a key role in generating the observed patterns of hybridization.

Broadly, our study suggests that we may be observing the slow expansion of the titmouse hybrid zone. Using reversed calculations with the formula for estimating neutral diffusion cline width, expansion of the titmouse hybrid zone is expected to proceed at a rate of 10–80 meters per year. At this rate, it would take hundreds of thousands to millions of years to fully homogenize the genetic pools of the Black-crested and Tufted titmouse. That said, we emphasize that we cannot rule out a possible additional role of ecological factors in constraining zone widths, as well as the potential (although most likely, weak) role of intra- and inter-sexual selection against hybrids outside of the hybrid zone. We also acknowledge that small sample sizes in some parts of our transects could affect our confidence in the variance analysis (and to a lesser extent, the geographic cline analysis).

Our study highlights that using multiple geographically distant transects across hybrid zones is useful for characterizing evolutionary relationships between a pair of taxa. While studies of temporal changes in hybrid zone structure commonly document the movement of hybrid zones over time, it is almost always unknown whether patterns of local hybrid zone movement are consistent across a broader spatial scale. This question becomes particularly important because of the influence of anthropogenic habitat disturbance observed in many species, and the potential localized impact on interspecific admixture due to the disappearance of ecological barriers to hybridization (Grabenstein and Taylor 2018). The Black-crested and Tufted titmouse may be one such example, where the contact between taxa (at least in some portions of their ranges) coincided with shrub invasion due to cattle grazing and increased overlap of ecological niches. Whether anthropogenic habitat transformation played a role in initiating secondary contact or facilitated hybridization that had been already ongoing for thousands of generations remains an open question. Comparing transects across two regions of the titmouse hybrid zone allowed us to document its dynamic nature, to find stronger support for a scenario of an expanding hybrid zone—a relatively rare finding in the hybrid zone literature—and setting the stage for future examinations of the role of demographic factors and possibly ecology in titmouse hybrid zone dynamics.

Supplementary material

Supplementary material is available at *Ornithology* online.

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Ethics statement

All sampling procedures were approved by University of Oklahoma Institutional Animal Care and Use Committee (protocols R09-004 and R12-009).

Conflict of interest statement

The authors declare they have no conflicts of interest.

Author contributions

Claire M. Curry and Michael A. Patten conceived the idea and design. Claire M. Curry conducted sampling. Georgy A. Semenov, Claire M. Curry and Jason T. Weir analyzed the data. Georgy A. Semenov wrote the manuscript with input from all co-authors.

Data availability

Illumina short read data are available in the National Center for Biotechnology Information Sequence Read Archive as a BioProject PRJNA949949. The R code is available on Open Science Framework repository at <https://osf.io/5ajyz/> (DOI 10.17605/OSF.IO/5AJYZ). VCF table and corresponding metadata are available at Dryad doi:10.5061/dryad.1jwstqk0g.

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