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A GENETIC EXAMINATION OF HYBRIDIZATION BETWEEN BLACK-CRESTED AND TUFTED TITMICE ACROSS TWO DIFFERENTLY AGED HYBRID ZONES

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Abstract:	Hybrid zones are powerful natural laboratories for studying the mechanisms that underlie evolutionary processes. Although many hybrid zones are relatively narrow, they often extend across large geographic distances and occur in a variety of ecological contexts. Despite the fact that variation in ecological context can affect hybridization dynamics, the majority of hybrid zone studies make inferences about hybridization and reproductive isolation from a transect across a single location of contact. Here, we explore hybridization between two passerine taxa, the Black-crested and Tufted titmouse, which hybridize in a region that extends from Oklahoma to southern Texas. Previous studies indicated that the northern parts of this hybrid zone formed no earlier than 150 years ago, while hybridization has been occurring for a few thousand years further south. We sampled two transects across the titmouse hybrid zone, one in the north and one in the south, and assessed hybridization dynamics using molecular and morphological markers. We show that the southern (older) part of the hybrid zone is three times wider than northern (younger) region of hybridization which may be attributed to differences in the age of onset of hybridization or to the breadth of the ecotone between titmouse habitats. Despite differences in width, both transects demonstrate similar patterns of hybridization and introgression, suggesting consistent hybridization dynamics. We further report that patterns in both transects fit a scenario of a local hybrid swarm, and potentially an evolutionary collapse between the two taxa. Nonetheless, a few lines of evidence suggest a possible role of ecological or sexual selection against introgression, all of which warrants further investigation.
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Data availability statement

Illumina short read data will be deposited in the National Center for Biotechnology Information Sequence Read Archive as a BioProject upon acceptance. All other data and R code are available on Open Science Framework repository at https://osf.io/5ajyz/ and will be deposited to Dryad upon acceptance.

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ABSTRACT

Hybrid zones are powerful natural laboratories for studying the mechanisms that underlie evolutionary processes. Although many hybrid zones are relatively narrow, they often extend across large geographic distances and occur in a variety of ecological contexts. Despite the fact that variation in ecological context can affect hybridization dynamics, the majority of hybrid zone studies make inferences about hybridization and reproductive isolation from a transect across a single location of contact. Here, we explore hybridization between two passerine taxa, the Black-crested and Tufted titmouse, which hybridize in a region that extends from Oklahoma to southern Texas. Previous studies indicated that the northern parts of this hybrid zone formed no earlier than 150 years ago, while hybridization has been occurring for a few thousand years further south. We sampled two transects across the titmouse hybrid zone, one in the north and one in the south, and assessed hybridization dynamics using molecular and morphological markers. We show that the southern (older) part of the hybrid zone is three times wider than northern (younger) region of hybridization which may be attributed to differences in the age

of onset of hybridization or to the breadth of the ecotone between titmouse habitats. Despite differences in width, both transects demonstrate similar patterns of hybridization and introgression, suggesting consistent hybridization dynamics. We further report that patterns in both transects fit a scenario of a local hybrid swarm, and potentially an evolutionary collapse between the two taxa. Nonetheless, a few lines of evidence suggest a possible role of ecological or sexual selection against introgression, all of which warrants further investigation.

KEYWORDS: Hybridization, introgression, reproductive isolation, Black-crested titmouse, Tufted titmouse

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 three times narrower than southern (older) region, which could reflect the differences in the hybrid zone age or be a result of differences in the breadth of the ecotone between titmice habitats in the north and south

● Our results suggest that Black-crested and Tufted titmice may be in the process of evolutionary collapse, although we cannot rule out a possible role of ecological or sexual selection in limiting gene flow outside of the hybrid zone

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, 1998; Payseur & Rieseberg, 2016; Taylor and Larson, 2019). 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

48 commonly temporal replicates, rather than geographic replicates, and are used to
49 assess hybrid zone spatiotemporal stability or quantify changes in hybridization rates
50 over time (e.g., Mettler & Spellman, 2009; Taylor et al., 2014; Wang et al., 2019;
51 Walsh et al., 2020). Less commonly, multiple transects are sampled across
52 geographically distinct parts of a hybrid zone (but see Rohwer and Wood, 1998;
53 Brelsford & Irwin, 2009; Scordato et al., 2020). Importantly, ecological context
54 matters, and species interactions can vary with geography (Harrison, 1993; Moore
55 and Price, 1993; Harrison and Larson, 2016). As such, comparisons of patterns of
56 hybridization across multiple transects in geographically distinct parts of a hybrid
57 zone have the potential to provide valuable insights into spatial variation of
58 selection regimes and associated reproductive mechanisms. Such information is also
59 crucial for conservation management and might be valuable for clarifying species
60 status for focal taxa.

61 The Black-crested titmouse (*Baeolophus atricristatus*) and Tufted titmouse (*B.*
62 *bicolor*) are sister lineages (Johansson et al., 2013) distributed in the southern and
63 eastern parts of North America (Figure 1). They are non-migratory and differ in

64 several aspects of plumage, most markedly in the amount of melanin of the crest
65 (Figure 1; Dixon 1955), exhibit song differences (Dixon, 1955; Coldren, 1992; Curry &
66 Patten, 2019), and differential habitat preferences, but similar foraging microhabitats
67 (Dixon, 1955). The two taxa have a spatially extensive area of secondary contact,
68 where the presence of intermediate phenotypes (Figure 1) suggests ongoing or
69 historical hybridization. Southern parts of the contact zone in Texas (Dixon, 1955,
70 1990) are older, where the two taxa may have been interbreeding for several
71 thousand years (Dixon, 1978). The two taxa also co-occur farther north in a
72 southwestern Oklahoma contact zone (Figure 1), where evidence suggests they came
73 into secondary contact as a result of shrub invasion within the past century (Nice,
74 1931, 1943; Sutton, 1967; Arnold, 1972; Rising, 1983; Van Auken, 2000, 2009;
75 Seyffert, 2001; Callahan, 2002; Patten & Smith-Patten, 2008). Morphological evidence
76 shows the northern contact zone is geographically narrower than the southern
77 contact zone, which was hypothesized to be due to its more recent origin. Sixty
78 years of phenotypic data from the southern contact zone indicate its width and

79 position are relatively stable (Dixon, 1990; Curry & Patten, 2014), suggesting its
80 boundaries may be maintained by natural selection
81 Despite long-term study of the titmouse hybrid zone, the genetics of
82 hybridization between Black-crested and Tufted Titmice has not yet been
83 characterized in detail (for characterizations of species divergence and phylogenetic
84 placement as sister taxa, see Braun et al.; 1984; Avise and Zink, 1988; Gill and Slikas,
85 1992; Sheldon et al., 1992; Gill et al., 2005; Johansson et al., 2013). In the current
86 study we sampled two geographically distant transects across both the southern
87 (older) and northern (younger) parts of the hybrid zone (Figure 1) and used a
88 genotyping-by-sequencing approach to develop genetic markers and characterize
89 patterns of hybridization. We first explore whether parental populations demonstrate
90 a signal of population structure and quantify admixture in our two sampled areas of
91 range overlap. Next, we assess whether the southern regions of the hybrid zone are
92 broader than the younger northern region – as suggested by previous plumage
93 studies – by comparing the width of ancestry-informative geographic clines and test
94 whether hybrid zone widths are maintained by selection. We further examine

individual genotypes to test for the presence of distinct hybrid classes such as F1s, advanced generation hybrids, and backcrosses, to assess the plausibility of contrasting hybridization scenarios (e.g. hybrid swarm due to unrestricted hybridization versus predominance of parental genotypes due to strong positive assortative mating and/or selection against hybrids). Finally, we compare 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 and Golden-winged Warblers (*Vermivora cyanoptera* and *V. chrysoptera*; Toews et al., 2016)).

METHODS

Permits and site access

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

111 MB148195-2 (issued by the U.S. Fish and Wildlife Service); Scientific Collecting
112 Permits 4716, 4955, 5210, and 5507 (issued by Oklahoma Department of Wildlife
113 Conservation); Scientific Collecting Permit SPR-0310-019 (issued by Texas Parks and
114 Wildlife Department); and University of Oklahoma Institutional Animal Care and Use
115 Committee protocols R09-004 and R12-009.

116 The following landowners and site managers provided access to their land:
117 private landowners (J. and M. Curry, J. and W. Erickson, L. Henard, S. Osborne), Llano
118 River Field Station (Texas Tech University), U.S. Fish and Wildlife Service, Texas Parks
119 and Wildlife Department, Texas Historical Commission, The City of Graham,
120 Oklahoma Department of Wildlife Conservation, Oklahoma Department of Tourism
121 and Recreation, Quartz Mountain Nature Park, and U.S Forest Service.

122 A total of 120 samples was used in this study (Table S1). Samples were
123 collected by <authors> (N = 77) along two geographic transects in the northern
124 contact zone in Oklahoma and northern Texas (northern transect, N = 41) and 200
125 km further south in north-central Texas (southern transect N = 36). Museum-loaned
126 tissue samples were used to characterize allopatric populations (N = 18) (Figure 1,

Table S1). In addition, samples from San Antonio (Texas), adjacent to the southern transect (N = 25), were provided by Troy Murphy. 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

We extracted genomic DNA using Qiagen DNeasy Blood & Tissue kits (Qiagen, cat. no. 69504) from tissue and blood samples. Reduced-representation genome libraries were constructed using the PSTI restriction enzyme following the genotyping-by-sequencing (GBS) method of Elshire et al., (2011). Single-end libraries were sequenced to 100 bp on a single lane of Illumina HiSeq 2000 platform at the Cornell Institute for Genomic Diversity. Each library had ~ 200 Gbp of unfiltered data with an average of 1.7 million reads per individual. Raw data was 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*

143 *major* reference genome (Laine *et al.*, 2016) using Bowtie 2 2.2.6 (Langmead &
144 Salzberg, 2012) with the "sensitive" settings. *Baeolophus* diverged from *Parus* at
145 least 4 mya (Gill et al., 2005), but at the time of data generation *Parus major* was the
146 closest reference genome available. Read alignment produced 66 - 77% of reads
147 aligning per individual. The *ref_map* wrapper script of the Stacks pipeline was used
148 to call genotypes using default settings with the exception that we used the
149 bounded error SNP calling model with a maximum error rate of 0.05 allowed. We
150 then filtered our dataset as follows using vcftools 0.1.14 (Danecek et al., 2011). We
151 filtered the data to remove sites with more than 20% missing data, with greater than
152 the 95th percentile sequencing depth and with observed heterozygosity exceeding
153 0.75 (the latter two filters help eliminate paralogues incorrectly aligned together),
154 with less than 2 copies of the minor allele (only biallelic sites were retained), and
155 which occur less than 10,000 bp apart in the reference genome. Following filtering
156 we had an average per individual depth of coverage of 16.5 for retained genotype
157 calls. This resulted in a dataset of 315 SNPs used for downstream analyzes.
158

159 **Population structure**

160 We used Structure 2.3.4 (Pritchard et al., 2000; Falush et al., 2003) to evaluate
161 patterns of populations structure. We ran structure analysis only with the number of
162 clusters K=2, given that we were interested in detecting individuals with ancestry
163 intermediate between Black-crested and Tufted titmice. We ran five independent
164 chains with burnin of 100,000 iterations and 500,000 iterations parameter sampling
165 using admixture model and correlated allele frequencies. The resulting Q-scores
166 were averaged between replicates. To assess population clustering without a pre-
167 defined number of clusters, we ran PCA on the mean-centered genetic covariance
168 matrix of SNP genotypes using the R v.3.6.1 (RStudio v.1.1.453) function *prcomp*.

169

170 **Geographic clines and individual admixture patterns**

171 We summarized genomic variation using PCA as described above and used PC1
172 scores as a proxy for ancestry. We fit empirical data to five geographic cline models
173 as described in (Semenov et al., 2021) using Metropolis–Hastings MCMC algorithm
174 implemented in *hzar* (Derryberry et al., 2014) v.0.2-5 package in R. To determine

175 whether the hybrid zone is tension zone (i.e. a hybrid zone maintained by the
176 balance between selection and dispersal), we compared the observed zone widths
177 with estimates expected under a scenario of unrestricted hybridization and neutral
178 diffusion following Barton & Hewitt, (1985) and using the formula $w=2.51\sigma\sqrt{t}$, where
179 σ is the post-natal dispersal distance and t is the number of generations since
180 secondary contact. We used a dispersal distance from Rylander et al., (2020) for
181 Black-crested Titmouse of 0.248 km. Although this estimate may appear
182 unreasonably small given typical distances in other Passerines, it is similar to
183 estimates of 0.091–1.097 km (average of 0.343 km) from a sister group (Gill et al.,
184 2005), the Juniper / Oak titmouse complex (*B. ridgwayi* and *B. inornatus*) (Cicero,
185 2000). For generation time, we assumed a length of one year typical for most
186 Passerines (Ehrlich et al.,1988). Previous work suggests that the southern contact
187 zone formed no later than 4,000 years ago based on climatic data (Dixon, 1978),
188 following initial divergence in allopatry with an estimated split around 250,000 years
189 ago (Dixon, 1978; Klicka and Zink, 1997; Patten and Smith-Patten, 2008). For the
190 northern zone we used a range of estimates between 60 and 150 years. The earliest

191 date for habitat changes in Van Auken, (2000) is the 1870s, therefore we assumed
192 150 years as an upper limit. For the lower limit, Sutton, (1967) lists 1963 as the first
193 Black-crested titmouse record in Oklahoma, and Nice, (1931, 1943) did not report
194 Black-crested titmouse in Oklahoma; hence we assumed the most recent date as 60
195 years ago.

196 To evaluate plausibility of contrasting hybridization scenarios, we compared
197 hybrid index and observed heterozygosity of individual genotypes. This analysis
198 requires loci that are fixed or nearly fixed to alternative allelic states in allopatric
199 populations, so we first used vcftools (Danecek et al., 2011) to calculate Weir &
200 Cockerham F_{st} (Weir and Cockerham, 1984) between allopatric samples. We then
201 selected loci with Fst>0.8 (n=8) to perform the analysis. We chose this approach
202 over using STRUCTURE Q-scores or PC1 scores from Principal Component Analysis
203 as the most assumption-free method (Gompert and Buerkle, 2016). We used custom
204 R code to generate hybrid index scores (0 = Tufted titmouse, 1 = Black-crested
205 titmouse) and then plot hybrid index against heterozygosity.

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 & Patten, 2014). Tufted Titmice have a gray crest and black forehead, which is a phenotypic index score of 0. Male Black-crested titmice have 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 & Patten, 2014). We assumed indices to be 0 (Tufted) and 6 (Black-crested) for museum tissue samples which were not located in the known hybrid zone (Figure 1, Dixon, 1955, 1990; Curry & Patten, 2014).

RESULTS

STRUCTURE analysis assigned allopatric Black-crested and Tufted titmice to distinct genetic clusters (Figure 2). The first principal component of the PCA explained 83% of variance and, similar to STRUCTURE, clearly separated allopatric populations

(Figure 2). Individuals spanning both the northern and southern transects possessed a broad range of admixture proportion values as revealed by STRUCTURE and PCA, confirming genetic admixture between the two taxa. Despite apparent hybridization, areas adjacent to the hybrid zone (e.g., San Antonio sampling location, samples within 66-78 km and 29-57 km from geographic cline center in southern and northern transects respectively, Figure 2) consisted of individuals with predominantly parental ancestry scores.

For both transects, the best geographic cline model of *hzar* was a symmetrical sigmoid cline, with no difference in introgression tails. Geographic cline analysis revealed that the northern (younger) hybrid zone is nearly three times narrower (width= 47.1 km, 2 log likelihood limits = 1-74.4 km) compared to the southern (older) hybrid zone (width=149.2 km, 2 log likelihood limits = 109.3-183.2 km) (Figure 3). The expected width of clines under a scenario of unrestricted hybridization and neutral diffusion was 39.4 km in the southern transect, and 4.8-7.6 km in the northern, both narrower than the observed cline widths.

We found very few genotypes that could be classified as first-generation hybrids (Figure 3), except 1-4 individuals with heterozygosity close to or above 0.75 and hybrid indices close to 0.5. Note that because we used loci that are not completely segregated between allopatric populations, the maximum possible heterozygosity estimate is expected to shift downwards, which creates uncertainty in F1 assignment. Further, there was no single location in the hybrid zones where both parental genotypes were present together, and the spatial transition of ancestry scores was continuous (Figure 3), consistent with a "unimodal" model of hybridization. Therefore, it appears that in both transects hybridization patterns are following a scenario of a local hybrid swarm. There was a strong correlation between ancestry and the plumage index (Figure 4), which can be potentially interpreted as whole genomes, and not only loci associated with plumage, are resistant to introgression.

DISCUSSION

We examined patterns of population structure and hybridization between Black-crested and Tufted titmice. Using data from multiple transects across hybrid zones - instead of only a single transect – can provide important insights into the dynamics of hybridization and the consistency of clinal patterns. In the titmouse hybrid zone, the southern transect (w = 149 km) was three times wider than the northern (w = 47 km). However, along both transects we documented advanced-generation hybrids and backcrosses, with no apparent difference between the transects of a specific hybrid class (Figure 3). Further, both transects demonstrated a unimodal distribution of ancestry scores and continuous spatial transition from one ancestry type to the another (Figure 3), sometimes referred to as unimodal model of hybridization (Gay et al., 2008). These findings suggest that hybridization dynamics are similar between the southern and northern transects and that admixture has been ongoing for many generations. Further, we found no evidence of natural selection maintaining the hybrid zone width based on our comparison between the observed and expected width of geographic clines. Interestingly, locations on the edge and adjacent to the

hybrid zone (Figures 2 and 3) are composed predominantly of individuals with non-admixed ancestry – a finding that was not straightforward to interpret. Furthermore, there was strong correlation between ancestry scores and plumage coloration (Figure 4), that could be interpreted in favor of some degree of selection on plumage (e.g., due to assortative mating), as well as an intermediate stage of collapse between two taxa, wherein phenotypic differences have not yet completely homogenized.

Interpreting conflicting signals of titmouse hybridization

Our finding that observed hybrid zone widths were not narrower than expected under the scenario of neutral diffusion suggest that the titmouse hybrid zone is not acting as a tension zone (i.e. a hybrid zone maintained by the balance between dispersal and selection, Barton and Hewitt, 1985). Such a finding is relatively rare in the literature (but see Baldassare 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. In light of this, an observation of prominent population

283 structure outside of the titmouse hybrid zone was intriguing. This pattern could be
284 explained by introgression outside of the hybrid zone being strongly restricted due
285 to some form of selection limiting dispersal of admixed individuals into parental
286 populations. An alternative explanation could be the unusually short dispersal
287 distances of 0.2-0.4 km observed among titmouse species (Cicero, 2000; Gill et al.,
288 2005; Rylander et al. 2020). The effects of small dispersal distances on shaping
289 admixture and patterns of introgression at range boundaries have not been explicitly
290 studied to our best knowledge. However, it is plausible that short dispersal
291 distances, which are about an order of magnitude smaller in titmice compared to
292 the majority of other Passerines (Price, 2008), could potentially create a sharp
293 transition in ancestry at the expanding front of a hybrid zone, even in the absence
294 of selection. As we discuss below, footprints of these alternative processes are likely
295 hard to distinguish.

296 In many taxa, particularly birds, hybridization sometimes results in nearly
297 complete homogenization of genomes with the exception of a few (often very
298 narrow) genomic regions wherein divergent genotypes are maintained by selection

(e.g., Poelstra et al., 2014; Mason and Taylor, 2015; Toews et al., 2016; Semenov et al., 2021; Funk 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 (see Semenov at al., (2021) for an example). Unlike in the above examples, we found a strong correlation between phenotype and genotype in titmice, despite a small genetic dataset compared to other recent studies (Figure 4; note that we only used samples from within or near the hybrid zone for this analysis). This finding could suggest substantial genome-wide restriction of introgression, rather than genomically localized selection on plumage genes. Alternatively, given the evidence for rampant hybridization, the observed genotype-phenotype association may be due to polygenic nature of plumage coupled with a 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 Tufted and Black-crested Titmice, but perhaps on a longer timescale than other avian examples due to their small dispersal distances.

315 Multiple lines of evidence suggest that Black-crested and Tufted Titmice taxa
316 have seemingly unrestricted hybridization. Local patterns of admixture in the hybrid
317 zone center were consistent with a scenario of hybrid swarms. We did not find any
318 first-generation hybrids, individuals with heterozygosity of 1 and hybrid index of 0.5
319 (Figure 3), although our dataset included loci with incomplete segregation between
320 allopatric populations, which likely biased heterozygosity estimates downwards. With
321 this caveat, the observation that parental genotypes never meet in the hybrid zone
322 is likely indicating it has collapsed into a broad cline in which F1 hybrids are now
323 unlikely to commonly be generated. These results suggest that strong selection due
324 to divergent mate choice is unlikely. However, this does not exclude the possibility
325 that hybrids experience reduced fitness due to selection outside of the hybrid zone.
326 Ecological selection appears to be a plausible candidate, as both allopatric
327 populations inhabit different habitats (Dixon, 1955). Both transects we studied are
328 located in an area of habitat transition, where individuals with intermediate
329 genotype or phenotype may not experience reduced fitness, or even have
330 comparatively increased fitness, according to the model of bounded hybrid

331 superiority (Moore, 1977). It is commonly recognized (Price, 2008) that sharp
332 ecological boundaries, as observed in titmice, can act as barriers to gene flow and
333 hence prevent introgression into allopatric populations. We suggest that mark-
334 recapture experiments at the hybrid zone edges and replicate sampling of the
335 hybrid zone over time are needed to disentangle between scenarios of neutral
336 diffusion and sharp ecotone with strong selection for habitat (Pyron et al., 2015;
337 Patten et al. 2021) in the case of Black-crested and Tufted Titmice. While the
338 observed differences in hybrid zone width between the southern and northern
339 transects may reflect differences in the age of onset of secondary contact – as was
340 previously hypothesized – another possibility could be differences in the extent of
341 intermediate habitat, although there is currently no support for this latter hypothesis
342 (Curry & Patten, 2019).

343 Black-crested and Tufted Titmice have marked differences in plumage and
344 some differences in song. Despite not finding a detectable signal of this in our
345 genetic data, there is evidence that the intensity of hybridization may vary between
346 northern and southern parts of the hybrid zone, given that males in the southern

zone respond more strongly to their own song types compared to males in the northern zone (Curry & Patten, 2016), and song structure correlates with environment only in the northern zone (Curry & Patten, 2019). Along with the possibility of divergent mate choice, and particularly female mate choice, female-female or male-male signaling could act as selective agent (Murphy et al., 2009a, 2009b; Tarvin & Murphy, 2012). At least male Black-crested titmice use their crests for signaling (Dixon 1955, 1978; Queller & Murphy, 2017; Borger et al., 2020). Whether female Black-crested and male or female Tufted titmouse use of crests for signaling is, to the best of our knowledge, as yet unstudied. Thus, hybrid crest 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; Curry and Patten, 2016) or differences in competition (Queller & Murphy, 2017) at different regions in the hybrid zone. Juvenile size also affects dispersal distance in the Black-crested titmouse (Rylander et al., 2020), and Black-crested titmice are, on average, smaller than Tufted titmice (Patten & Smith-Patten, 2008). 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 the allopatric populations and warrant further investigation.

Conclusions

Our study highlights that using multiple geographically distant transects across hybrid zones is an essential component of characterizing evolutionary relationships between a pair of species. While studies of temporal changes in hybrid zone structure commonly document 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 potential localized impact on cross-species admixture due to the disappearance of ecological barriers to hybridization (Grabenstein & Taylor, 2018). Black-crested and Tufted titmice may be one such example, where the initial contact between taxa in Oklahoma potentially resulted from shrub invasion due to cattle grazing in the 20th century. We found that extensive hybridization between the two titmouse species

379 has resulted in localized hybrid swarms. Furthermore, the observed width of
380 geographic clines in both transects was wider than expected under neutral diffusion,
381 potentially supporting the hypothesis of evolutionary collapse between the taxa.
382 However, the two titmouse populations have prominent population structure on a
383 broader geographic scale and demonstrate an abrupt transition in ancestry outside
384 of their hybrid zone. This pattern might be a direct result of the unusually short
385 post-natal dispersal distances that have been documented in titmice, which are also
386 non-migratory. The propensity of migratory avian taxa to occasionally engage in
387 long-distance dispersal that can enhance gene flow far outside of hybrid zones
388 might explain why this pattern has not been documented in other avian hybrid
389 zones in North America. Alternatively, this pattern might be indicative of restricted
390 introgression due to ecological or sexual selection which requires further
391 investigation.

392 **FIGURES**

393

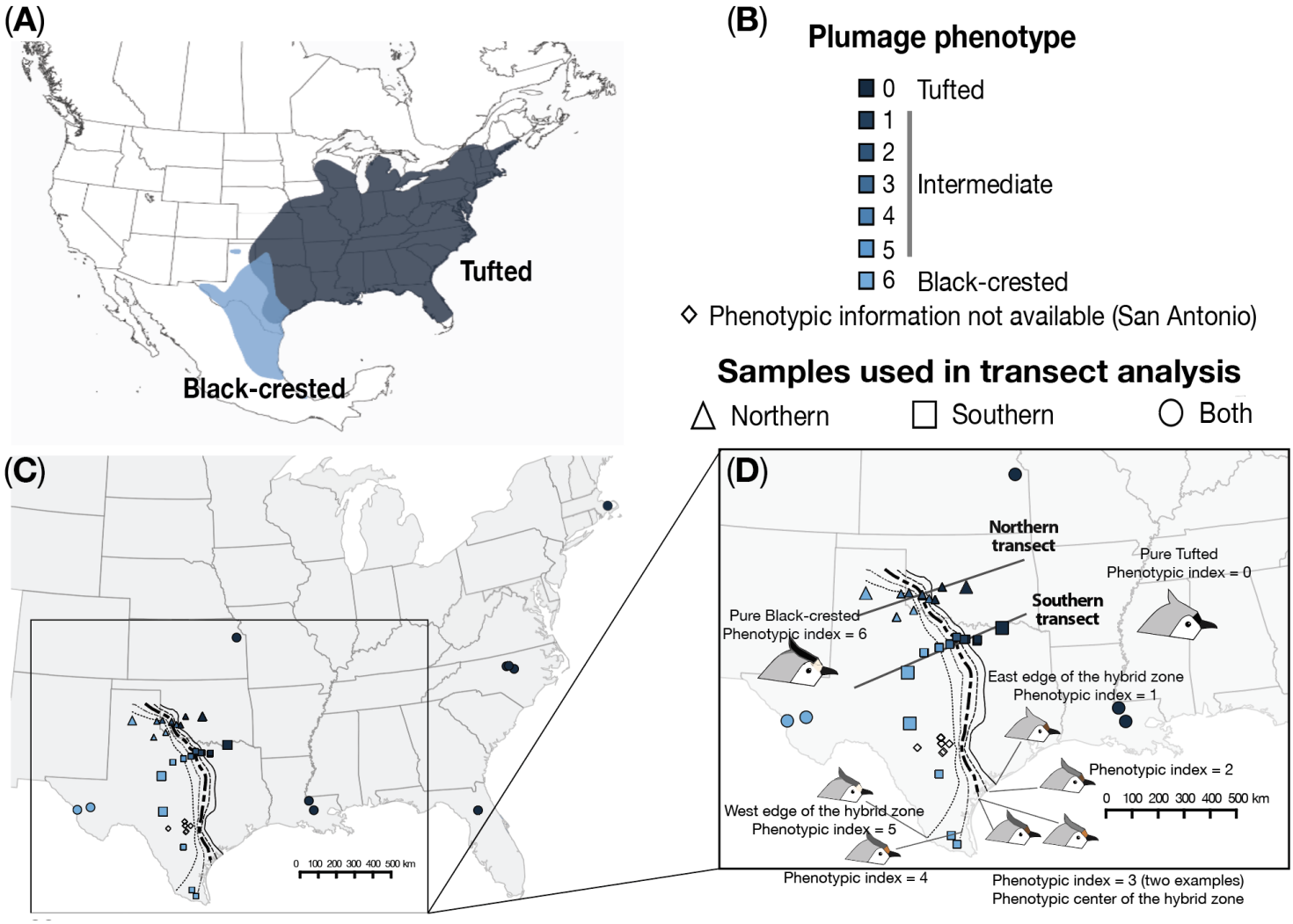

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Figure 1. Distribution, population sampling, transects across hybrid zones and variation in crest color in Black-crested and Tufted titmice. (A) Approximate distributions of Black-crested and Tufted titmice in North America (redrawn from https://www.allaboutbirds.org). (B) Plumage index color scheme used throughout this study. (C) and (D) Population sampling and examples of plumage phenotypes and corresponding phenotypic indices. In (D), predominant phenotypes are shown for some key areas. Larger and smaller symbols (squares for southern transect, triangles for northern 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 northern and southern transect analyses. Solid lines indicate transect directions.

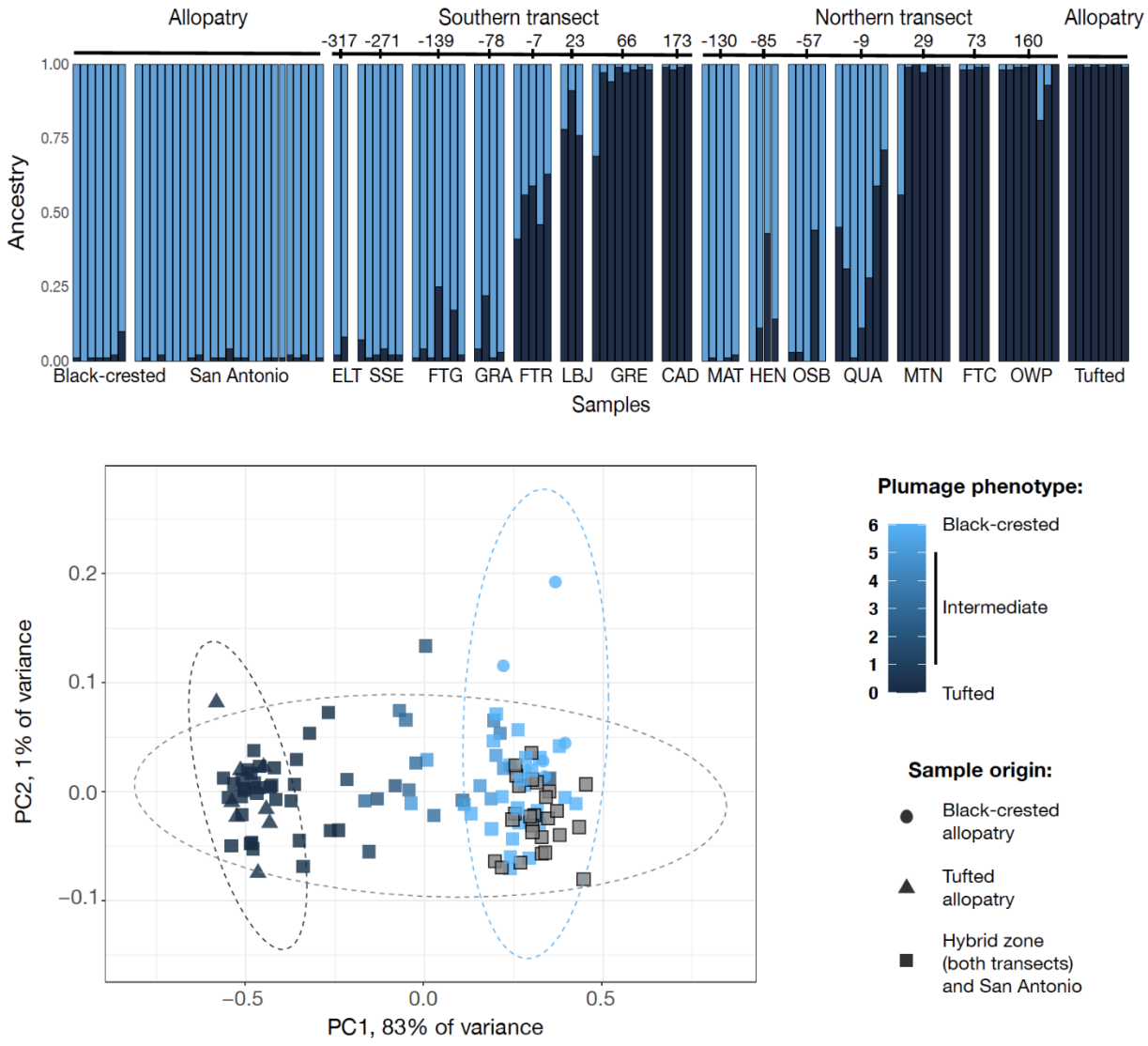

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Figure 2. Population structure in Black-crested and Tufted titmice. Top: Results of STRUCTURE analysis with the number of clusters K=2. Individuals are arranged by sampling location (see supplementary Table S1 for location codes). Locations follow the direction of transects across hybrid zones with distances from geographic cline center indicated on the top. Bottom: Results of Principal Component Analysis performed on mean-centered covariance matrix of genotypes. Gray symbols with black outline are individuals from San Antonio (allopatric Black-crested titmouse adjacent to southern hybrid zone) with missing phenotypic data. Dashed lines indicate then 95% confidence interval. Note that both methods 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 titmice arrived in winter and stayed to breed in 1886-1887 (Dixon 1990), so the samples are of interest to see if any traces of genetic admixture remain.

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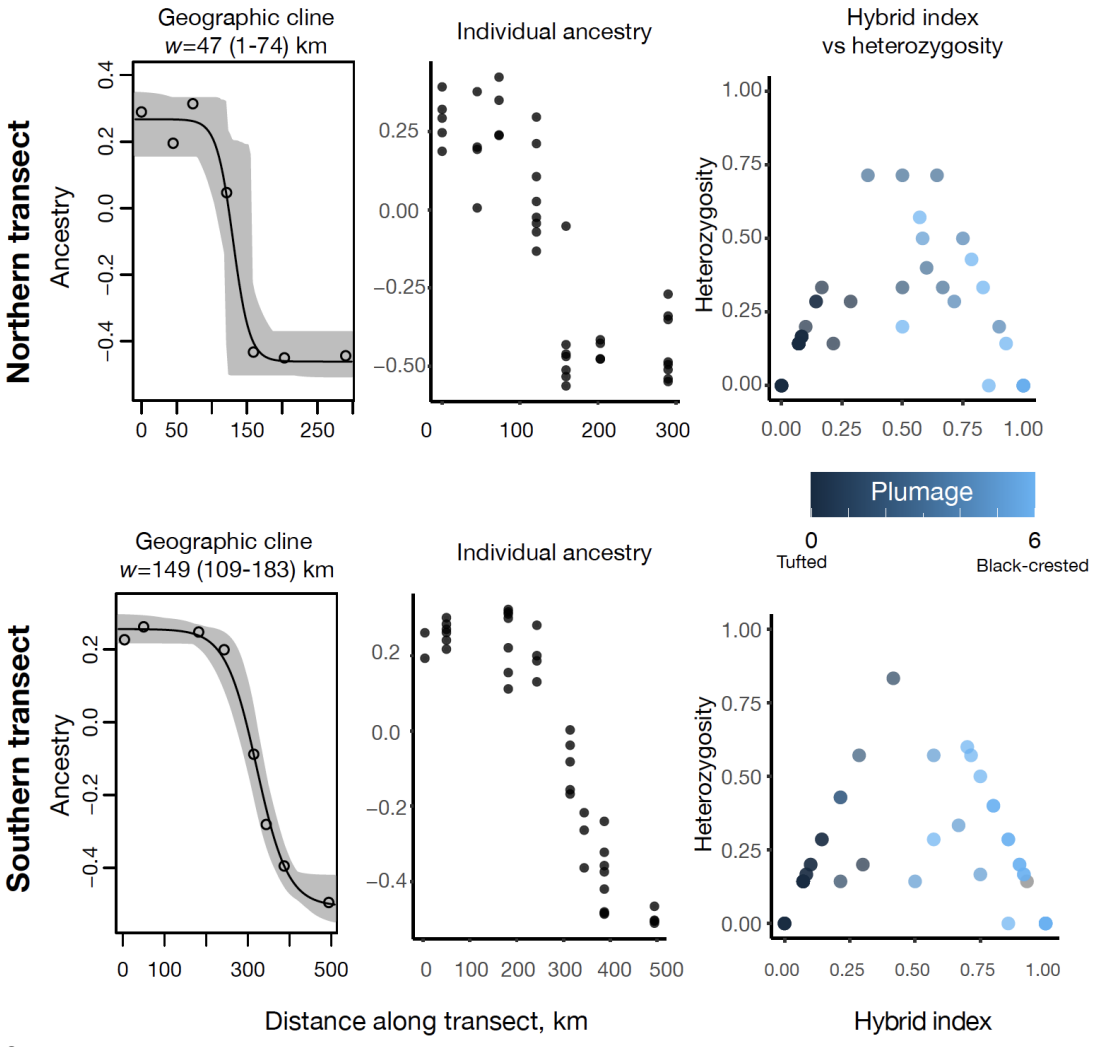

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Figure 3. Patterns of hybridization in Black-crested and Tufted titmice in the northern (top) and southern (bottom) transects. Left: Results of geographic cline analysis. Note that scale is different between north and south, and that the geographic cline for the southern transect is about three times wider than the northern transect. Middle: distribution of individual ancestries across transects. Right: Heterozygosity plotted against hybrid index. The individuals with hybrid index of 0 and 1, and low heterozygosity correspond to parental Tufted and Black-crested genotypes respectively. Recent-generation hybrids have hybrid index close to 0.5 and heterozygosity close to 1, while later-generation hybrids have lower levels of heterozygosity and variable hybrid indices due to recombination and segregation of alleles. Backcrosses have hybrid indices closer to parental genotypes but higher heterozygosity (sides of the "triangle"). Note that there are various types of hybrids and backcrosses found in both transects, suggesting that hybridization and introgression are ongoing. Also note that there is no single location where both parental genotypes are present together, consistent with a "unimodal" model of hybridization (i.e. a local hybrid swarm).

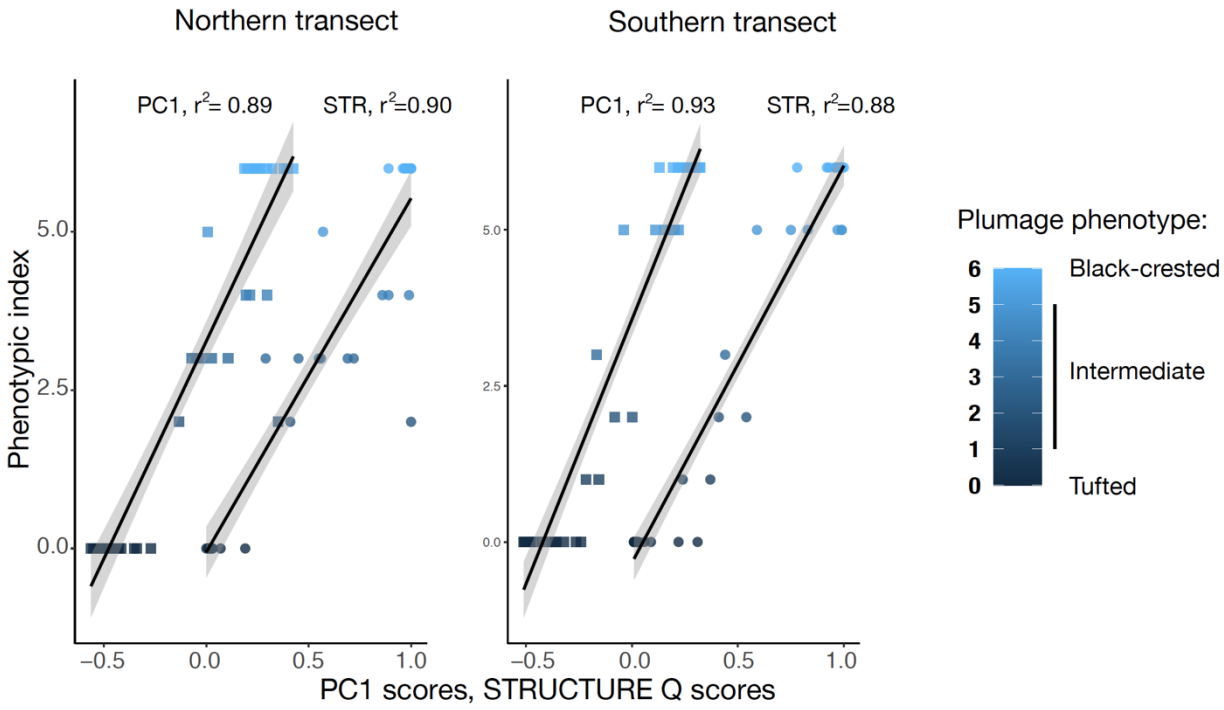

Figure 4. Association between plumage phenotype (crest color) and ancestry (PC1 and STRUCTURE Q-scores) for the northern and southern transect.

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