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Title: Limited movement of a hybrid zone in relation to regional variation in magnitude of climate change

Running title: Variable movement of chickadee hybrid zone

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Abstract:

Hybrid zones can provide clear documentation of range shifts in response to climate change and identify loci important to reproductive isolation. Using a deep temporal (36-38 years) comparison of the black-capped (*Poecile atricapillus*) and Carolina (*P. carolinensis*) chickadee hybrid zone, we investigated movement of the under-sampled western portion of the zone (western Missouri) as well as investigating whether loci and pathways underpinning reproductive isolation were similar to those from the eastern portion of the hybrid zone. Using 92 birds sampled along the hybrid zone transect in 2016, 68 birds sampled between 1978 and 1980, and 5 additional reference birds sampled from outside the hybrid zone, we generated 11,669 SNPs via ddRADseq. We used these SNPs to interpolate spatially and assess the movement of the hybrid zone interface through time, and to assess variation in introgression among loci. We demonstrate that the interface has moved approximately 5-8 km to the northwest over the last 36-38 years, i.e., at only one-fifth the rate at which the eastern portion of the hybrid zone (e.g. Pennsylvania, Ohio) has moved. Temperature trends across the last 38 years reveal that eastern areas have warmed 50% more than western areas in terms of annual mean temperature, possibly providing an explanation for the slower movement of the hybrid zone in Missouri. Using genomic cline analyses, we detected four genes that showed restricted introgression in both Missouri and Pennsylvania, including *Pnoc*, a gene involved in metabolism, learning and memory, concordant with previous physiological and behavioral findings on hybrids and the parental species. Overall, our results suggest differing impacts on hybrid zone movement due to climate change varying between areas in broadly distributed species. In addition, our study provides further evidence for how crucial museum collections are in assessing the impacts of climate change.

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Introduction

Hybrid zones are fundamental for understanding the mechanisms underpinning reproductive isolation (Taylor & Larson, 2019) and speciation (Gompert, Parchman, et al., 2012). However, they can also provide valuable and robustly documented evidence of range shifts in response to anthropogenic impacts, including deforestation (Thurman et al., 2019) and climate change (Arntzen, 2019; Ryan et al., 2018; Taylor et al., 2015). One of the most tractable ways to document temporal shifts in hybrid zones is the comparison of the spatial position of a hybrid zone based on contemporary samples to that of previous sampling, and museum collections are invaluable in this regard (Thurman et al., 2019; S. Wang et al., 2019). Birds have been a frequent subject of hybrid zone studies, because their ease of observation facilitates broad characterization of hybrid zone geography over continental scales. Many avian hybrid zones studied in North America are roughly north-south in orientation: e.g. meadowlarks (Rohwer, 1972), buntings (Carling et al., 2010; Carling & Brumfield, 2008; Emlen et al., 1975), orioles (Carling et al., 2011; Rising, 1970; Sibley & Short Jr., 1964), phoebes (Schukman et al., 2011), and pewees (Manthey & Robbins, 2016), limiting their applicability for assessing the impacts of climate change. In contrast, the largely east-west orientation of the black-capped (*Poecile atricapillus*)/Carolina (*P. carolinensis*) chickadee hybrid zone (except for extreme western Missouri/southeastern Kansas), makes it particularly relevant in a climate change context. Indeed, this contact zone has been sampled and analyzed extensively (Braun & Robbins, 1986; Brewer, 1963; Bronson et al., 2005; Bronson, Grubb, & Braun, 2003; Bronson, Grubb, Sattler, et al., 2003; Curry, 2005; Johnston, 1971; Merritt, 1978; Reudink et al., 2007; Rising, 1968; Robbins et al., 1986; Tanner, 1952; Taylor, Curry, et al., 2014; Taylor, White, et al., 2014; Wagner et al., 2020; Ward & Ward, 1974).

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Although the black-capped/Carolina chickadee hybrid zone ranges from southeastern Kansas to

the Atlantic coast in New Jersey (AOU, 1998), most research has focused on the eastern portion

(Bronson, Grubb, & Braun, 2003; Bronson, Grubb, Sattler, et al., 2003; Curry, 2005; Reudink et

al., 2007; Taylor, Curry, et al., 2014; Taylor, White, et al., 2014; Wagner et al., 2020). It has

been proposed that the hybrid zone location may be determined by winter temperatures, limiting

the northward range of Carolina chickadees (Taylor, White, et al., 2014). This limitation is

potentially mediated by differences in metabolism and competitive ability between the two

species (McQuillan & Rice, 2015; Olson et al., 2010). In addition, the hybrid zone is relatively

narrow (Taylor, White, et al., 2014), likely caused by reduced reproductive success of hybrids

(Bronson et al., 2005; Bronson, Grubb, & Braun, 2003). Learning and memory impairment (e.g.,

recall ability for location of stored food caches) in hybrid chickadees may contribute to this

reduced reproductive success (McQuillan et al., 2018).

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Genetic and morphological studies in Pennsylvania and Ohio have demonstrated that the hybrid

zone has moved northward at >1 km/year for over 100 years (Bronson, Grubb, Sattler, et al.,

2003; Harr & Price, 2014; Taylor, White, et al., 2014) and this northward movement of the

hybrid zone has been correlated with climate change (Bronson, Grubb, Sattler, et al., 2003;

Reudink et al., 2007; Taylor, White, et al., 2014). However, movement of the zone has been

predicted to differ geographically, with ecological niche models indicating a retraction of

suitable habitat for Carolina chickadees in the western portion of their range (McQuillan & Rice,

2015). Analysis of song data in Illinois supports these models, with little hybrid zone movement

detected (Enstrom & Bollinger, 2009), but song and morphology are less sensitive indicators of

92   hybridization than genetic markers owing to extreme similarities in plumage morphology and

93   heterospecific song learning between these species (Bronson, Grubb, Sattler, et al., 2003;

94   Johnston, 1971; Kroodsma et al., 1995; Robbins et al., 1986; Sattler et al., 2007; Sattler & Braun,

95   2000; Shackleton & Ratcliffe, 1993; Tanner, 1952). In spite of early analyses (Braun & Robbins,

96   1986; Robbins et al., 1986), data are lacking on the current position of the hybrid zone in the

97   farthest western portions of the range (e.g. Missouri and Kansas) (McQuillan & Rice, 2015).

98

99   In addition to movement of hybrid zones as a whole, the influence of localized selective

100   pressures on the introgression of genes linked to reproductive isolation is of interest when

101   species come into contact (Gompert et al., 2017; Harrison & Larson, 2016; Moran et al., 2020;

102   Taylor & Larson, 2019). Comparisons of transects in different portions of broadly distributed

103   contact zones, such as that of black-capped/Carolina chickadees, are of particular interest.

104   Previous genetic analyses of the black-capped/Carolina chickadee hybrid zone in eastern

105   Pennsylvania has identified genes underpinning metabolic and neural signaling pathways as

106   being subject to temporally consistent restriction in introgression across the hybrid zone (Taylor,

107   Curry, et al., 2014; Wagner et al., 2020). In addition, these studies affirmed that SNPs associated

108   with sex chromosome Z are particularly resistant to introgression (Taylor, Curry, et al., 2014;

109   Wagner et al., 2020), a pattern seen in other avian systems (Battey, 2020; Bourgeois et al., 2020)

110   and analogously in systems involving chromosome X (Carneiro et al., 2014; Janoušek et al.,

111   2012; Maroja et al., 2015). These temporally-consistent specific genes resistant to introgression

112   support observations about differences in metabolic capability between black-capped and

113   Carolina chickadees, and of memory deficiency in hybrids (McQuillan et al., 2018). However,

114   are these specific genes and associated metabolic pathways spatially consistent? That is, are the

same regions of the genome resistant to introgression 1,500 km to the west in Missouri, in an

area subject to different local selective pressures?

In 2016, we resampled a segment of the hybrid zone in west-central Missouri that had been

sampled intensively by one of us in 1978-1980 (Braun & Robbins, 1986; Robbins et al., 1986).

At 36-38 years apart, these samples provide not only the deepest temporal genetic comparison of

the chickadee hybrid zone interface, but indeed one of the deepest of any avian contact zone in

North America. We demonstrated northwest movement of the hybrid zone in Missouri, although

this movement is limited compared to other areas of the USA. A comparison with climate data

for the same time period suggests that eastern areas of the USA have warmed 50% more than

Missouri in terms of annual mean temperature, providing an explanation for the slower

movement of the hybrid zone in Missouri. Our results suggest the specific impacts of climate

change on broadly distributed species will manifest at local scales and provide further illustration

of how crucial museum collections are in assessing the impacts of climate change.

Materials and Methods

Field work and selection of historical samples

The same west-central Missouri transect that was sampled in 1978 and 1980 (Fig. 1 in Robbins

et al., 1986) was sampled again by Robbins in March-April 2016 (**Table S1**). Of the 92

chickadees collected in 2016, 17 were obtained from parental populations classified as "pure"

(non-admixed) during sampling in 1978-1980 based on morphological and vocal variation

(Robbins et al., 1986). For the Carolina chickadee, these 10 "pure" samples were taken from the

Bird Song Conservation Area, St. Clair County (Site 50 in top panel of **Fig. 1/Fig. S1**; equivalent

138 to Site 20-22 in bottom panel of **Fig. 1/Fig. S1** and Site 4 in Robbins et al., 1986). For the black-

139 capped chickadee, $n = 7$ "pure" samples were taken from the Miami Creek drainage northwest of

140 Butler, Bates County (Sites 1-4 in top panel of **Fig. 1/Fig. S1**, equivalent to Site 1-2 in bottom

141 panel of **Fig. 1/Fig. S1** and Site 1 in Robbins et al., 1986). We also included a further five

142 reference birds (three black-capped and two Carolina) sampled from well outside the putative

143 contact zone (locations in **Table S1**) just in case the hybrid zone was wider than it appeared in

144 Robbins et al. (1986).

145

146 The remaining 75 samples from 2016 were taken in the contact zone, which was more

147 intensively sampled than in 1978-1980, including several additional sites. For both sampling

148 periods, when possible, chickadees were audio-recorded, then collected, and immediately frozen

149 on dry ice. The protocol and procedures employed during collection were reviewed and approved

150 by the University of Kansas Institutional Animal Care and Use Committee. Samples were

151 archived in either -80°C freezers (1978-80 samples) or in liquid nitrogen (2016 samples).

152 Voucher study skins (n=92) and genetic material from the 2016 work are deposited at the

153 University of Kansas Biodiversity Institute. Specimen data (including links to audio recordings)

154 for all 2016 samples are accessible via VertNet (vertnet.org). Audio recordings from both 1978-

155 1980 and 2016 are deposited at the Macaulay Library, Cornell Lab of Ornithology, Ithaca, New

156 York. The 1978-1980 genetic samples are deposited at the United States National Museum,

157 Smithsonian Institution, whereas associated voucher specimens are deposited at Louisiana State

158 University of Natural Science, Baton Rouge, Louisiana.

159

A total of 68 genetic samples was included from the 1978-1980 study. We included 10 of 17 and

10 of 21 total birds available from Miami Creek (Site 1-2 in bottom panel of **Fig. 1/Fig. S1**) and

Collins (Sites 20-22 in bottom panel of **Fig. 1/Fig. S1**), respectively, to reflect more closely the

numbers of samples taken from those locations in 2016 ($n = 7$ birds across Sites 1-4, and $n = 10$

at Site 50, respectively, top panel of **Fig. 1/Fig. S1**).

DNA extraction

DNA was extracted from approximately 15 mg of tissue using a Blood DNA kit and

manufacturer protocols on a Maxwell® RSC instrument (Promega), with the following

modifications: before loading into the cartridge, samples were lysed for 24 hours with 32 μL of

proteinase K and 180 μL of tissue lysis buffer (Promega) in a 1.5 mL tube on a heat block at

56°C before being spun for 2 minutes at maximum speed to pellet any remaining tissue at the

bottom of the tube. The supernatant was then transferred to Well 1 of the cartridge. The volume

of elution buffer used was 100 μL. DNA was quantified using the QuantiFluor® dsDNA System.

Laboratory methods for ddRADseq

We used a double-digest RADseq protocol (Peterson et al., 2012), pooling sets of 8-16 samples

(distinguished using internal barcodes), with pools distinguished by external barcodes (**Table

S2**). Briefly, 500 ng of DNA for each sample (except for two pools of 16 samples where 250 ng

was used as input) was digested with 20 U each of *SbfI*-HF and *MspI*, and 1× CutSmart buffer

(New England Biolabs®: NEB), made up to a total volume of 50 μL with PCR-grade H$_2$O. A ¼

reaction was run with Lambda DNA (NEB) for each pool of chickadee samples as a positive

182   control. Following digestion for at least five hours at 37°C, samples were purified with 1.5×

183   volume of Agencourt® AMPure XP® beads (Beckman Coulter), using two washes of 200 μL

184   fresh, cold 70% ethanol. Following the final wash, samples were eluted in 40 μL PCR-grade

185   H_2O and quantified using Qubit^{TM} (Invitrogen).

186

187   To ligate adaptors, we set up reactions with up to 32 μL of cleaned, digested sample

188   (standardized to the sample with the lowest concentration within the pool); 100 nM of sample-

189   specific "P1" *SbfI* cut-site adaptor (with an internal barcode; **Table S2**); 1 μM of "P2" *MspI* cut-

190   site adaptor (not sample-specific; **Table S2**); 400 U of T4 DNA ligase (NEB); and 1× T4 DNA

191   ligase buffer (NEB), made up to a total volume of 40 μL with PCR-grade H_2O. We also set up

192   ligation reactions for our positive lambda controls. Samples were incubated at 23°C for 1 hour,

193   heat killed at 65°C for 10 min, and then cooled by 2°C every 90 s until reaching room

194   temperature (20°C). Following ligation, samples with unique P1 adaptors were pooled (8-16 per

195   pool) and purified with 1.5× volume of Agencourt® AMPure XP® beads using two washes of

196   200 μL of fresh, cold 70% ethanol. Following the final wash, samples were eluted in 50 μL

197   PCR-grade H_2O, and a second round of purification carried out with 1.5× volume of Agencourt®

198   AMPure XP® beads, using two washes with 200 μL of fresh, cold 70% ethanol. Following this

199   final wash, pools were eluted in 35 μL of Buffer EB (QIAGEN). This process was conducted

200   separately for our positive lambda controls.

201

202   To confirm that digestion and adaptor ligation were successful for our samples, we set up a test

203   PCR with 400 nM each of "common" Primer 1 and pool-specific Primer 2 (**Table S2**), 1×

Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB) and 1.5 μL of cleaned, pooled,

post-ligation product, made up to a total volume of 25 μL with PCR-grade H_2O. An initial

denaturation step of 98°C for 30 s was followed by 11 cycles of 98°C for 10 s, 65°C for 30 s,

72°C for 60 s, followed by a final extension of 72°C for 10 min. This test PCR was also

conducted separately for our positive lambda controls. Cleaned post-ligation products and test

PCR products for our sample pools/positive lambda controls were run against undigested lambda

DNA and 100 bp DNA ladder (Promega) for reference. Following successful digestion and

adaptor ligation, no high-molecular weight crowns were observed, and post-ligation reactions

were broad smears, potentially with some laddering for our pooled samples. PCR reactions

produced narrower and brighter (than the post-ligation) smears for the sample pools. For the

positive lambda control, fragments of the following size were expected following the test PCR:

154, 199, 495, 558, 1610 bp.

After confirming that the pools represented samples that were successfully digested and had

adaptors ligated, fragments between 200-500 bp were selected using a 2% DNA Gel Cassette

with Internal V1 marker on a BluePippin (Sage Science) following the manufacturer instructions.

Size-selected sample pools were quantified with Qubit (the optional step of using Dynabeads®

Invitrogen to select against fragments with P1 adaptors ligated to both ends was used

successfully on only two pools before being dropped owing to difficulties with library loss

during this step), before the final enrichment PCR that added pool-specific external barcodes.

This PCR consisted of 400 nM each of "common" Primer 1 and pool-specific Primer 2 (**Table**

S2), 1× Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB), and 21 μL of the size-

selected sample pool, made up to a total volume of 50 μL with PCR-grade H_2O. The

227 thermoprofile used was the same as for the test PCR. The final enrichment PCR was then

228 purified with 1.5× volume of Agencourt® AMPure XP® beads, using two washes of 200 μL

229 fresh, cold 70% ethanol. Following this final wash, pools were eluted in 40 μL of Buffer EB and

230 quantified using Qubit. An initial set of eight samples was sequenced on 5% of a HiSeq 3000

231 paired-end 150 bp lane at the Oklahoma Medical Research Foundation (OMRF). Following this

232 successful test run, the remaining 157 samples were prepared and combined in pools of 15-16

233 individuals. After combining the pools at equimolar concentrations, the final library (of 191

234 individuals, including 34 samples unrelated to this project) was sequenced on a paired-end 150

235 bp HiSeq3000 run.

236

237 ***ddRADseq data analysis and identification of genetic clusters***

238 Our SNP data set was assembled to the black-capped chickadee genome (Wagner, Curry, Chen,

239 Lovette, & Taylor, 2020; BioSample: SAMN13264372; BioProject: PRJNA589043; Assembly

240 accession: GCA_011421415.1) through ipyrad v.0.9.51 (Eaton & Overcast, 2020). To be

241 included in the final dataset, loci were required to be found in at least one of the reference black-

242 capped and one of the reference Carolina samples. Specific code/parameters used for the analysis

243 are detailed in **Fig. S2**. From this dataset, we selected one variable site per locus, and used

244 custom R code (**Fig. S3)** to filter out singletons as per the recommendations of Linck & Battey

245 (2019) for running STRUCTURE (Falush et al., 2003; J. K. Pritchard et al., 2000). We used this

246 dataset as input into the program STRUCTURE v 2.3.4 run via Structure_threader v 1.3.0 (Pina-

247 Martins et al., 2017). We carried out an initial run at $K = 1$ to infer lambda, using 50,000 burn-in

248 steps, followed by 100,000 steps. We fixed lambda at its inferred value and then carried out five

249 replicates for $K = 1$ to $K = 5$ under the ancestry admixture model and allowing for correlated

allele frequencies. All code used for implementing STRUCTURE through Structure_threader is

detailed in **Fig. S3**. The Evanno method (Evanno et al., 2005) was used to assess the best fitting

K through structure harvester (Earl & vonHoldt, 2012), and individual structure assignments to

each cluster for the best fitting *K* averaged across the five replicates.

Movement of hybrid zone based on ddRADseq data

Sampling locations were plotted using program R (R Core Team, 2017), along with the dplyr

(Wickham et al., 2018), ggmap (Kahle & Wickham, 2013), ggplot2 (Wickham, 2016), ggrepel

(Slowikowski, 2017), readr (Wickham et al., 2017), and scatterpie (Yu, 2018) packages (**Fig.

S4**). The plot function of tess3R (Caye et al., 2016; Caye & Francois, 2016) (**Fig. S5**) was used

to interpolate STRUCTURE assignments spatially for the area of overlapping sampling effort

between the modern and historical sampling periods to assess hybrid zone movement

qualitatively. Analyses were restricted to the overlapping area between the two sampling periods

(yellow background in labels on **Fig. 1/Fig. S1**) to restrict the influence of sampling sites that

were not well matched between the temporal samples (e.g., sites 5-9 in 2016 sample; sites 11, 12,

15-17 in 1978-1980 sample, **Fig. 1/Fig. S1**). Map tiles for **Fig. 1 and Fig. S1** provided by

Stamen Design, under CC BY 3.0 with data by OpenStreetMap, under ODbL. After confirming

that the hybrid zone interface ran from the southwest to the northeast during the previous

analysis, we calculated the distance to each of our samples from a southwest-northeast line

centered on the southeast portion of the study area shown in **Fig. 2**. We then used the

STRUCTURE assessments of genomic admixture to conduct a geographic cline analysis using

HZAR v.0.2.5 separately for the 2016 and 1978-1980 samples (Derryberry, Derryberry, Maley,

& Brumfield, 2014; analysis code in **Fig. S6**).

Variation in patterns of introgression by locus

We identified loci putatively involved in reproductive isolation between the black-capped and Carolina chickadee by carrying out a genomic cline analysis in BGC v1.0.3 (Gompert & Buerkle, 2012), following the approach of Taylor et al. (2014). Putative parental populations were defined as individuals who showed ≥ 99% assignment to either the black-capped or Carolina genetic clusters based on the previous STRUCTURE analysis, with the admixed population including all of the remaining individuals. Given the limited geographic extent of the Missouri hybrid zone that we studied, nested population effects were not included in our model; instead, the hybrid zone was considered a single population following Gompert and Buerkle, (2011). The analysis was conducted across the combined temporal samples (as well as the 5 parental reference samples), because the shared ancestry of the temporal populations means they cannot be considered independent replicates (Taylor, Curry, et al., 2014). All samples were included, i.e., we did not limit the samples to just those from the more concentrated overlapping region used in the geographic cline analysis. The loci that were included were restricted to those found in at least 90% of our samples, to limit the total number of loci in view of computational constraints. We implemented the genotype uncertainty model of Gompert et al., (2012). Parameter estimates were based on the median and 95% tails of the marginal posterior probability distribution (applying a Bonferroni correction for the 6,748 loci compared) across our 50,000 MCMC state chain (sampling every fifth state), which followed a 25,000-iteration burn-in. We confirmed convergence of parameter estimates by running a second shorter chain (25,000 MCMC stats, 12,500 burn-in). Code for implementing these analyses is available in **Fig. S7**.

Loci where 95% posterior probability intervals did not overlap 0 were classified as outliers: positive α outliers have an increase in the probability of black-capped ancestry in comparison to that predicted by the hybrid index (i.e. more black-capped than expected); negative α have an increase in the probability of Carolina ancestry; positive β outliers have excess ancestry-based linkage disequilibrium (i.e. locus-specific ancestry restricted to matching genomic background, potentially indicating loci that are less free to introgress across the hybrid zone); negative β outliers have ancestry less strongly associated with genomic background than in other loci (i.e. loci are more free to introgress). We investigated significant differences in how these outlier loci were distributed across chromosomes using G-tests, with a Bonferroni correction to account for multiple comparisons across the 34 chromosomes/scaffolds. Because positive β outliers (less freely introgressing loci) could be associated with reproductive isolation between the species (Gompert, Parchman, et al., 2012), we focused on these loci for additional comparisons. First, we identified consecutive SNPs that were positive β outliers, potentially indicative of broader regions (e.g., inversions/non-recombining areas of chromosomes) of reduced introgression. We used a cut-off of five consecutive loci, because this was unlikely to occur by chance if positive β outliers were randomly distributed across our dataset. We extracted sequence from these regions using seqtk v1.3 (Li, 2020), and used Magic-BLAST v1.5.0 (Boratyn et al., 2019) to match these regions to nucleotide sequence from black-capped chickadee coding sequences (CDS) identified using a different black-capped reference genome (GCA_013398625.1_ASM1339862v1_cds; Bird 10,000 Genomes [B10K] Project - Family phase). A direct comparison to the reference genome that we used for the rest of our analyses (GCA_011421415.1) was not possible as annotations are not yet available for this genome (however, GCA_011421415.1 had higher contiguousness than GCA_013398625.1, making it more suitable for the reference-based steps of

our analyses). Based on the genes identified as mapping to our regions of interest, we carried out

an analysis of biological pathways enriched among these genes using gene ontology (GO)

annotation through http://geneontology.org/ (PANTHER Overrepresentation Test [Released

20200728]; GO Ontology database DOI: 10.5281/zenodo.4081749 Released 2020-10-09; *Homo*

sapiens reference list. *Homo sapiens* was selected as the reference list was more complete than

the avian genomes available), with a Fisher's Exact test, and a False Discovery Rate for multiple

comparisons. We then repeated these analyses (extracting sequence, Magic-BLAST to identify

whether SNPs were near/within CDS regions, GO term enrichment) for all significant positive β

outlier SNPs, following Wagner et al. (2020) in using 5,000 bp of flanking sequence on each side

of the SNP. Finally, we compared the positive β outliers (and associated genes) identified in our

analyses with previous genetic investigations of the black-capped/Carolina chickadee hybrid

zone (Taylor, Curry, et al., 2014; Wagner et al., 2020). All code used to run these analyses is

available at **Fig. S7.**

Song/morphological analyses

During field work in 2016, 59 birds were audio recorded, including 38 birds also genetically

characterized with ddRADseq. Individual male songs were classified as two notes (black-capped

like, "*fee-bee"*), four notes (Carolina like, "*fee-bee-fee-bay*"), or three notes ("aberrant"). Birds

were classified as singing only black-capped song, only Carolina song, a mix of parental songs

(both black-capped and Carolina), or a mix of aberrant and parental songs (aberrant plus either

black-capped or Carolina song). No birds were recorded singing only aberrant songs. Although

no birds were *recorded* singing aberrant songs as well as singing both parental songs, these birds

were present during sampling based on observations made during song collection

342   (KU132081/ML523592, **Table S3**). Frequency (pitch) was not used in characterizing song

343   because there was considerable variation among individuals, even within sites singing only a

344   single song type, as well as a large variance even within individuals not genetically characterized

345   as hybrids (i.e., within a single bout of song e.g., KU132048, **Table S3**).

346

347   Given the relatively small sample sizes of song (numbers of bouts/individual), and reliance on

348   only number of notes to characterize song (e.g. see Robbins et al., 1986), caution is warranted

349   about individual-level characterization of birds based on song. In some of the longer sequences

350   of song, birds switched between parental song types, suggesting that characterization based on

351   shorter bouts of song could incorrectly classify the bird's repertoire. This ability for individuals

352   within the hybrid zone to switch between black-capped, Carolina, and 'aberrant' song types has

353   been noted previously (Robbins et al., 1986), and likely reflects social interactions being

354   important for the song learning process (Kroodsma et al., 1995; Shackleton & Ratcliffe, 1993).

355   In addition, observations made even well away from the contact zone (e.g., > 130 km) within the

356   range of the black-capped chickadee demonstrate that "pure" black-capped birds give single-

357   noted, double-noted (the typical black-capped song) and even three-noted "aberrant" song

358   (personal observation, MBR). These points suggest that the presence of "aberrant" song is not

359   associated with a bird's genetic makeup nor exposure to the other conspecific taxon. Given these

360   issues, here we present song data classified only by the presence/absence of song types

361   considered, not the abundance of each song type (as this could be affected by the differing

362   recording times for each bird). Due to conflicting evidence on the importance of three-noted

363   aberrant song, with this observed well away from the hybrid zone yet used as indication of

364   hybridization in previous research (Brewer, 1963; Enstrom & Bollinger, 2009), we took a

365   conservative approach and restricted our comparisons to birds recorded singing only two-noted

366   and/or four-noted song. Finally, we did not analyze these data in an explicitly spatial context but

367   restricted ourselves to comparing genetic assignment of birds singing either two-noted, four-

368   noted or both two and four-noted song.

369

370   A subset of 23 birds collected in 1978-1980 and characterized with ddRADseq was remeasured

371   for wing (chord) and tail length. All 92 birds collected in 2016 and genotypically characterized

372   with ddRADseq were weighed, with wing and tail length measured for a subset ($n = 34$). We

373   examined correlations between mass, wing/tail ratio and genetic STRUCTURE assignments

374   (correlations for mass based on 2016 birds only, as mass was not available for the 1978-1980

375   sample). Again, as for song, given the limitations of morphological measurements in reliably

376   distinguishing even pure black-capped vs Carolina chickadees (Robbins et al., 1986), we did not

377   analyze these data in an explicitly spatial context.

378

379   ***Climate analyses***

380   To provide an environmental context for the genetic analyses, annual precipitation and mean

381   annual temperature data were downloaded from PRISM (2017). All data for 1976-1980, 1998-

382   2002, 2008-2012, and 2012-2016 were downloaded in *.bil format. These date ranges were

383   selected to correspond to the five years prior to the start and end dates of the studies in Missouri

384   (1980-2016) and Pennsylvania (2002-2012). We derived two estimates of the rate of change of

385   temperature and precipitation: one based on the 1980-2016 interval, and the other on the 2002-

386   2012 interval. We averaged each climate dimension over the appropriate 5-year range. We

387   calculated the change in temperature as the average of conditions during the end of the interval

388   minus the average of the five years preceding the beginning of the interval. We then calculated

389   the rate of change by dividing change by the number of years covered by this period (e.g. for

390   Missouri, 2016-1980 = 36 years).

391

392   To examine the consistency in the rates of change between 1978-2014 and 2000-2010, we

393   examined correlations in the rates of change between these two time periods. Following this

394   exploratory analysis, we examined longer-term (38 years i.e. the duration of our Missouri study)

395   and shorter-term (10 years i.e. the duration of the Pennsylvania study Taylor, White, et al., 2014)

396   trends at each of the sites (**Table S4**). Overall, we conducted two separate contrasts, 1998-2002

397   versus 2008-2012 (corresponding to the Pennsylvania study time frame), and 1976-1980 versus

398   2012-2016 (corresponding to our study in Missouri). We generated frequency histograms of rates

399   of realized change in each environmental dimension within the 0.5° (~55 km) buffers shown as

400   dashed lines in **Fig. 3**.

401

402   **Results**

403   ***Summary of ddRADseq dataset and initial structure runs***

404   The number of reads obtained across the initial test set of 8 samples ranged from 2,188,989 to

405   3,332,843 (mean = 2,760,725; s.e. = 145,973). After excluding one of the historical samples

406   (catalog number: 99788; tissue number: 649257) that had extremely low sequencing coverage

407   (8,855 reads in total), the number of reads among the remaining 156 samples ranged from

408   280,350 to 1,146,532 (mean = 663,743; s.e. = 11,303). This difference in sequencing coverage

409   between the test and main samples was also reflected both in the number of clusters found in

410   each individual (test mean = 36,065; s.e. = 1,924, vs. main mean = 25,210; s.e. = 356), and also

411   in the number of loci found in the final data set for each bird (test mean = 10,211; s.e. = 30, vs.

412   main mean = 9,750; s.e. = 62) (**Table S5**).

413

414   A lack of variation among the estimated log likelihood of data for our K 3–5 replicates negated

415   the use of the Evanno et al. (2005) method to confirm the number of underlying clusters in our

416   STRUCTURE analyses. However, a K of 2 had the highest average likelihood among our K 1–3

417   STRUCTURE runs; given that our samples span two separate species, we focused our analyses

418   on a K of 2. Four of the five reference samples we included were inferred to belong to the "pure"

419   populations they were purported to represent (99.9% assignment to respective genetic clusters,

420   **Table S1**). The remaining black-capped chickadee reference sample (Catalog number: 95776),

421   showed an assignment of 93.38% to the black-capped chickadee cluster. Despite high levels of

422   missing data in our ddRADseq data set (**Fig. S8**), the 8,056 SNPs in our final STRUCTURE

423   dataset demonstrated a strong gradient of genomes ranging from "pure" black-capped

424   (northwest) to "pure" Carolina chickadees (southeast) (**Fig. 1; Fig. S1**), with patterns of missing

425   data more consistent with variation in sequencing coverage than erosion of restriction enzyme

426   sites as a function of phylogenetic distance (Eaton et al., 2017; Lee et al., 2018; Pante et al.,

427   2015) (**Fig. S9**). Overall, these results suggest that we have the ability to distinguish between the

428   unadmixed parental species. The five reference samples were then excluded from downstream

429   analyses, except for the genomic cline analyses.

430

431   ***Movement of hybrid zone based on ddRADseq data***

432   Spatial interpolation of the STRUCTURE assignments of birds sampled in 1978-1980 in

433   comparison with samples from 2016 showed that the contact zone has moved approximately 8

434   km to the northwest over the last 36-38 years (top panel **Fig. 2**). To quantitatively estimate the

435   movement of the hybrid zone, we assumed the hybrid zone interface had strictly moved to the

436   northwest. We found the hybrid zone had moved 5.6 km based on this geographic cline analysis

437   (bottom panel **Fig. 2**). This pattern of approximately 5-8 km of movement was also supported by

438   changes in the STRUCTURE assignments of birds in the locations with fine-scale sampling

439   overlap between both periods: Appleton City and Rockville. Across the 12 birds sampled from

440   Appleton City sites in 1978-1980 (Sites 5, 9, 10, 13 and 14 in bottom panel of **Fig. 1/Fig. S1,** top

441   right of top left panel of **Fig. 2**), 33.3% were black-capped, 25% were Carolina, and 41.7% were

442   hybrids (defined as less than 95% assignment to either parental genetic cluster). In 2016, the 10

443   birds sampled from Appleton City sites (Sites 21, 24, 29, 30, 32, 33 and 36 in the top panel of

444   **Fig. 1/Fig. S1**, top right of **Fig. 2**) were 40% Carolina and 60% hybrid, with no black-capped

445   birds identified. The average genomic proportion assigned to the black-capped cluster decreased

446   between the two sampling periods at this location (average assignment to the black-capped

447   cluster in 1978-1980 sample = 56%; average assignment in 2016 sample = 22%), albeit this

448   difference was not statistically different according to a Mann-Whitney U test (p-value = 0.1377).

449   Across the 28 birds sampled in the Rockville area in 1978-1980 (Sites 3, 4, 6, 7, 8, and 18 in

450   bottom panel of **Fig. 1/Fig. S1**, left of top of **Fig. 2**), 39.3% were black-capped, 21.4% Carolina,

451   and 39.3% hybrids. Across the 31 birds sampled in the Rockville area in 2016 (Sites 10, 11, 13,

452   14, 16, 19, 20, 22, 23, 31, 34, 35, 37, 41, 43 and 45 in top panel of **Fig. 1/Fig. S1**, left of top right

453   of **Fig. 2**), 6.45% were black-capped, 54.8% were Carolina, and 38.7% were hybrids. A Mann-

454   Whitney U test indicated a significant decrease in the average genomic proportion assigned to

the black-capped cluster between the two sampling periods at this location (average assignment

to the black-capped cluster in 1978-1980 sample = 64%; average assignment in 2016 sample =

32%; p-value = 0.01017; assuming unequal variance between samples; code for statistical

calculations **Fig. S10**).

However, despite detecting a temporal movement of the hybrid zone, our results indicate that the

zone in west-central Missouri has not moved at the same pace during the past 36-38 years as in

the eastern portion of the chickadee contact zone in southeastern Pennsylvania and Ohio

(Bronson et al., 2005; Bronson, Grubb, Sattler, et al., 2003; Taylor, White, et al., 2014; Wagner

et al., 2020). Even at the fastest potential pace suggested by our data – assuming that the zone

moved from northwest of Rockville (see previous section), to the Pleasant Gap area (sampled

only in 2016; Sites 6-9 top panel of **Fig. 1/Fig. S1**) – the distance is only ca. 8-9 km in the

intervening 36-38 years (~ 0.2 km/year), well below the rate of >1 km/year recorded in

southeastern Pennsylvania and Ohio (Bronson, Grubb, Sattler, et al., 2003; Taylor, White, et al.,

2014; Wagner et al., 2020).

Variation in patterns of introgression by locus

Although we fully acknowledge the limitations of using RADseq markers to detect selection,

given limitations in marker density relative to blocks of linkage disequilibrium (Lowry et al.,

2017), we conducted a genomic cline analysis in an attempt to identify loci showing restricted

movement across the hybrid interface using BGC. Based on inspection of the BGC chains, we

removed an additional 1,500 states as well as the defined burn-in, before (successfully)

477   confirming convergence. Of the 6,748 loci included in this analysis, 1,825 outlier loci (27.05%

478   of total loci) were identified (**Table S6A; Fig. S11A**). Outliers were classified as a locus being

479   "more black-capped" than expected based on genomic background [+α: 3.40% of total loci],

480   "more Carolina" than expected based on genomic background [-α: 0.04% of total loci], less

481   capable of introgressing across the hybrid zone [+β: 9.94% of total loci], more capable of

482   introgressing across the hybrid zone [-β: 16.91% of total loci], and combinations of these

483   categories (**Table S6A; Fig. S11A**). These outlier categories were not evenly distributed across

484   the chromosomes (**Fig. 4**). The five chromosomes most distinct from the underlying distribution

485   shown by the total genome (**Fig. 4**) were Chromosome Z, 3, 2, 4, and 18. Chromosome 18 had

486   significantly fewer outlying loci in any category compared to the genomic background.

487   Chromosomes 2, 3, and 4 all had a larger percentage of 'freely introgressing' loci (-β).

488   Chromosome Z showed a pattern that strongly contrasted, with a large excess of loci that appear

489   to introgress less freely (+β), even after accounting for the total number of loci mapping to this

490   chromosome (**Fig. S11B**).

491

492   For the remainder of our analyses, we focused on the significant positive β outliers as regions of

493   the genome potentially involved in reproductive isolation. Most positive β outliers (486 of 671

494   loci) were not within 5 kbp of black-capped CDS regions. The proportion of our outlying

495   positive β SNPs within 5 kbp of genic regions (185 of 671) was lower than that of the outlying

496   loci identified by Wagner et al. (2020) (452 of 470, Fisher's exact test, p < 0.0001), potentially

497   owing to the different restriction enzymes used influencing the targeted regions of the genome

498   (*SbfI/MspI* in our study, *PstI* in Taylor et al. 2014/Wagner et al. 2020), and/or the ability of

499   Wagner et al. (2020) to use the annotations that they developed for the genome rather than the

23

500   CDS mapping approach we performed. However, of the positive β outliers identified within 5

501   kbp of a gene; 169 were within 5 kbp of a single gene; 14 were within 5 kbp of 2 genes; and 2

502   outliers were within 5 kbp of 3 genes. Among the 191 CDS regions represented across the185

503   positive β outliers within 5 kbp of a gene, we found no significant enrichment for GO terms.

504   Among the 11 CDS regions found within 5 kbp of multiple positive β outlier SNPs (**Table S6B**),

505   again, no GO pathways were found to be significantly enriched. We then compared the genes or

506   genomic locations of our positive β outlier loci to previous genomic characterizations of outlying

507   loci from the black-capped/Carolina chickadee hybrid zone, depending on the information

508   available from these previous studies (genes: Taylor et al. 2014; genomic location: Wagner et al.

509   2020). None of the genes associated with the 13 outlier loci in Taylor et al. (2014) were

510   identified in our current analyses. However, 5 out of our 671 positive β outlier loci were within 5

511   kbp of at least one of the 1,850 total outlying Wagner et al. (2020) loci (the number of outlying

512   Wagner et al. 2020 loci in close association with any given one of our outliers ranged between 2

513   and 32: **Table S6B**). Three of these loci were on Chromosome 3, one on LGE22, and one on

514   Chromosome Z. Two of the loci on Chromosome 3 were also within 5 kbp of genes: *Otof_1* and

515   *Pnoc* (**Table S6**). The number of positive β outlier loci overlapping between our studies (5) was

516   not significantly greater than expected by chance (p = 0.055, binomial test on 5/671 against

517   0.0030 of the chickadee genome calculated to be covered by outliers from Wagner et al. 2020

518   and their 5 kbp flanking regions).

519

520   We then searched for stretches of consecutive significant positive β loci (potentially indicative of

521   inversions/regions of reduced recombination), finding these for Chromosome 1A (1 region), and

522   Chromosome Z (10 total regions) (**Table S6B; Fig S11C**). Each of these regions contained

multiple genes, even if the positive β outlier SNPs within the regions were not found within 5 kbp of a gene. One of the regions on Chromosome Z (genomic coordinates 26730718:34172229) was significantly enriched for the following GO pathways: oncostatin-M-mediated signaling; leukemia inhibitory factor signaling; and cytolysis (**Table S6C**). Apart from this region, no significant enrichment for GO terms was found for any region, or the combined regions from either chromosome. However, across the total 'consecutive loci gene' dataset, the following pathway was significantly enriched: 'cellular nitrogen compound metabolic process' (**Table S6C**). Among the 13 outlier loci mapping to nearby genes in Taylor et al. (2014), 2 were also found among regions defined by the bounds of consecutive significant positive β loci in our study (Chromosome Z: *Ptprd* from 15113305:26027709 and *Ndufs4* from 26730719:34172229).

Analysis of morphology and song

As reported previously (Bronson, Grubb, Sattler, et al., 2003; Sattler & Braun, 2000), morphometric variation (wing length, tail length, and mass) is largely concordant with patterns of hybridization revealed by genetic variation, but less sensitive at detecting the extent of hybridization and introgression than genetic markers (**Fig. 5**). This is not surprising given the difficulties in distinguishing these morphologically similar species (Johnston, 1971; Robbins et al., 1986; Tanner, 1952), complicated by sexual dimorphism (Desrochers, 1990; James & Rising, 1985).

In addition to examining patterns in the genetic and morphological datasets, we obtained song for 38 birds in the 2016 sample that also had genetic data available. A comparison of song data

545   and genetic assignment based on STRUCTURE showed that birds that sang only Carolina song

546   were on average more genetically Carolina than birds that sang black-capped song were

547   genetically black-capped (t-test assuming unequal variance between samples, *p*-value = 0.0003;

548   **Fig. 6**). Although our sampling was relatively limited and may have therefore missed sampling

549   black-capped individuals, we also observed the continued presence of black-capped chickadee

550   song in areas that now consist predominantly of Carolina chickadees and hybrids (e.g., Appleton

551   City, **Fig. 6**).

552

553   *Correlation of hybrid zone movement with climate change*

554   Rate of temperature change was highly consistent between our study period of 1978-2014 and

555   the study period of Taylor et al. (2014) of 2000-2010, with an r^2 of 0.22 between the two time

556   spans based on 10,000 random points distributed across the lower 48 states of the United States

557   (**Fig. S12**). In contrast, precipitation had far less consistency between sampling periods, with an

558   r^2 of 0.0063 (**Fig. S12**). Given the low consistency of trends in the precipitation data, we focused

559   our analyses on temperature. Comparing climatic trends between Missouri and Pennsylvania, we

560   found that contrasts of climate based on the 10-year time period of the previous Pennsylvania

561   study (Taylor, White, et al., 2014) failed to detect climate warming at all in Missouri, although

562   warming is indeed present in Missouri in the long-term 38-year contrast (**Fig. S13A**). Over the

563   longer-term contrast, Pennsylvania has warmed ~50% more than Missouri (**Fig. 3, Fig. S13A**),

564   which is strongly evident when plotting the rates of change within 50 km of the Missouri and

565   Pennsylvania transects (**Fig. S14**). In terms of precipitation, Missouri has become wetter,

566   whereas Pennsylvania has not changed (**Fig. S13B**).

567

Limitations of hybrid zone width assessment

When examining the STRUCTURE assignment of the 1978-80 birds characterized with ddRADseq, the contact zone appeared to extend further northwest than originally defined based on vocalizations, plumage morphology, and allozyme data (Robbins et al., 1986). For example, based on song, morphology, and allozyme data, Site 4 in the 1980 sample (bottom panel of **Fig. 1/Fig. S1**, equivalent to Robbins et al. 1986 Site 2) was considered outside the hybrid zone in an area where only black-capped chickadees were thought to occur. However, STRUCTURE analyses inferred that 5 of 12 birds collected at this site were hybrids (defined as having less than 95% of their genome assigning to any given parental species cluster), with the remainder classified as black-capped chickadees (**Fig. 1/Fig. S1**). In contrast to these genetic results, only black-capped vocalizations were heard and recorded at that site in 1980 (Robbins et al., 1986).

In addition to the proposed repositioning of the 1978-1980 hybrid zone based on genetic data, spatial interpolation of STRUCTURE assignment of birds from the 2016 sample suggested that the current hybrid zone extends to the northwest of our dense spatial sampling regime (e.g., failure to observe dark red contour; **Fig. 2** right panel). For this reason, we focused our hybrid zone movement analyses on the position of the black-capped/Carolina chickadee interface as inferred through tess3R, and do not comment on changes in the potential extent of hybridization (i.e., hybrid zone width) across this zone through time.

Discussion

589   Using one of the deepest temporal comparisons of any avian contact zone in North America (also

590   see S. Wang et al., 2019), we demonstrated northwest movement of the black-capped and

591   Carolina chickadee hybrid zone in Missouri between 1978-1980 and 2016. The movement of this

592   zone, in context of the results from other studies at the eastern end of this contact zone, appears

593   to be consistent with contrasts in the degree of climate change (Bronson, Grubb, Sattler, et al.,

594   2003; Harr & Price, 2014; Taylor, White, et al., 2014). We identified pathways and genes that

595   are potentially involved in reproductive isolation across the entire length of the chickadee hybrid

596   zone; however, we did not find that outlying loci/regions between the studies overlapped more

597   than expected by chance.

598

599   *Movement of the black capped and Carolina chickadee hybrid zone*

600   The west-central Missouri hybrid zone we characterized in this study has moved at only ~0.2×

601   the rate of other locations; eastern studies have documented rates of 1.2 km/year (Pennsylvania:

602   Harr & Price, 2014; Taylor, White, et al., 2014) and 1.6 km/year (Ohio: Bronson, Grubb, Sattler,

603   et al., 2003), but our western transect moved at only 0.19 km/year. Analyzing temperature trends

604   across the region over the last 38 years, we found that eastern areas have warmed 50% more than

605   the Osage Plains and surrounding areas in southwestern Missouri. Our climate data analysis also

606   suggests little movement of the Illinois hybrid zone is expected, consistent with the stability of

607   chickadee song types in this area (Enstrom & Bollinger, 2009). However, given the issues with

608   song data (presented below), genetic data will be needed to clarify the rate of movement of the

609   Illinois hybrid zone.

610

Morphological measures (mass and wing/tail ratio) showed positive correlations with genetic

assignment, but with low resolution reflecting difficulty in distinguishing even pure parental

black-capped and Carolina chickadees with these measures. Song showed a conflicting pattern,

with the 'ghost' of black-capped chickadee song remaining in areas that now consist

predominantly of Carolina chickadees and hybrids based on our genetic analyses (e.g. Appleton

City). Remnant black-capped song has also been detected in areas that are now predominantly

genetically Carolina in Pennsylvania (Reudink et al., 2007), suggesting this pattern is widespread

across the hybrid zone. This mismatch between genetics and song-type is consistent with

chickadees responding more strongly to the song type that is most frequent in the local

population (Robbins et al., 1986). Although the numbers of birds with morphological

measurements were too limited in our study to make generalizations, in other areas, birds in

hybrid zones appear more phenotypically similar to Carolina than black-capped chickadees

(Johnston, 1971), potentially reflecting the predominant direction of genetic introgression across

the hybrid zone (south to north, Carolina into black-capped). Our genomic cline results were

consistent with this idea: we found far more loci that were "more black-capped" than expected

based on genomic background (i.e., $+\alpha$ outliers: 'remnant' black-capped alleles remaining

against the background of predominant Carolina ancestry due to the direction of introgression),

in comparison with loci that were "more Carolina" ($-\alpha$) than expected.

However, even though climate is likely important, other factors probably influence the

movement and width of the hybrid zone. Despite being on average smaller (Rising, 1968), male

Carolina chickadees tend to be dominant in heterospecific interactions, and females of both

species appear to show a preference for them (Bronson, Grubb, Sattler, et al., 2003), particularly

as extrapair partners (Reudink et al., 2006) and observations suggest that assortive mating of

"black-capped-like" and "Carolina-like" birds is not occurring within the hybrid zone (Robbins

et al., 1986). Also, studies have documented no consistent differences in habitat preferences

between parental species other than elevation in sky island populations of black-capped

chickadees (Johnston, 1971).

Given the overall reduction in the average assignment of chickadees to the black-capped genetic

cluster through time, it is somewhat surprising that F1 hybrids continued to be present at

Appleton City (**Fig. S1**), especially as selection against hybrids has been demonstrated

previously in eastern areas of the hybrid zone (Bronson et al., 2005; Bronson, Grubb, & Braun,

2003; McQuillan et al., 2018; Olson et al., 2010). One potential explanation could be that black-

capped chickadees are present at low frequencies at these sites, which is why we failed to detect

any in our sample. Continued interbreeding of these presumed black-cappeds with Carolinas

could lead to the production of F1 hybrids and facilitate learning of black-capped song by

Carolina/hybrid chickadees. A potential alternative explanation is that selection against hybrids

is weaker in the Missouri hybrid zone, or that differences exist in genomic architecture of the

chickadees between Missouri and Pennsylvania.

Genetic architecture of the black-capped and Carolina chickadee hybrid zone

We compared the genomic location of the outlying loci identified in our study of the Missouri

transect, with the previous studies of Taylor et al. (2014) and Wagner et al. (2020), who

examined birds from the Pennsylvania hybrid zone (Wagner et al. 2020 reanalyzed the data of

Taylor et al. 2014 using a reference genome, so we focus on comparing to the reference-guided

results here). Broadly (i.e., at chromosomal level), our results were very similar. The

chromosomes that contained the largest number of loci significantly resistant to introgression

(i.e., positive β outliers) in our study were (from greatest to least), Chromosome Z, 1A, 2, 1, and

5. Chromosome Z also had the largest number of tracts of consecutive positive β outliers,

potentially indicative of inversions/regions of reduced recombination. Wagner et al. (2020)

found similar results, with the exception of Chromosome 2. The importance of Chromosome Z

in both studies is consistent with reduced introgression due to Haldane's rule and the large X(Z)

effect (Irwin, 2018; Runemark et al., 2018).

However, at a finer scale, we were unable to confirm that the outlying regions found in our study

of the Missouri transect overlapped more than that expected by chance with the outliers

identified by either Taylor et al. (2014) or Wagner et al. (2020) in the Pennsylvania transect. This

outcome is not inconsistent with the results from at least some other hybrid zones where multiple

transects have been sampled (**Table 1**). However, like previous studies that examined patterns of

introgression of specific genes between different geographic transects of the same hybrid system,

we used reduced representation sequencing (**Table 1**). Given the limitations of reduced

representation sequencing for detecting underlying loci under selection, it is likely that these

studies, including our own, are underestimating the number of regions resistant to introgression

that are concordant between different transects (Janoušek et al., 2012; Lowry et al., 2017). In

addition, variation in recombination landscapes among locations (e.g., Burri et al. 2015) could

further impact the ability to identify underlying regions resistant to selection that are concordant

among locations. Examining the consistency across multiple hybrid-zone transects of

679 introgression patterns using whole genome resequencing data will allow the field to use

680 quantitative assessments of the proportion of shared versus unique loci, rather than the somewhat

681 subjective assessments currently captured in **Table 1** (e.g., the column "Patterns of introgression

682 across different transects"). The use of whole genome sequencing will also allow comparison

683 across different hybrid systems of the factors influencing consistency between multiple transects,

684 including the influence of local population ancestry or selective pressures on the outcome of

685 introgression across hybrid zones (Gompert et al., 2017; Harrison & Larson, 2016; Teeter et al.,

686 2010). However, even with whole genome sequencing, where the loci under selection are

687 targeted directly, the detection rate of loci resistant to introgression will not be 100% (Gompert

688 & Buerkle, 2011).

689

690 This broad comparison across species (**Table 1**) suggests a need to standardize laboratory

691 methodology (i.e., whole genome sequencing), the method of identifying outliers, and the

692 threshold for deciding whether concordant patterns of introgression have been found between

693 transects, before it can be concluded that variation in patterns of introgression could impact

694 differential speed of movement of the chickadee hybrid zone. Currently, variation in climate is

695 the most parsimonious explanation for the differences observed between Missouri and

696 Pennsylvania. However, we identified a number of genes associated with outliers/outlying

697 regions in our study, and also presented as outlying regions in Taylor et al. (2014) or Wagner et

698 al. (2020). Previous genomic evidence from the hybrid zone in Pennsylvania suggests that

699 genomic regions resistant to introgression in this area may be involved in metabolic breakdown

700 (e.g., Olson et al. 2010), and spatial memory and problem solving (e.g., McQuillan et al., 2018),

701 based on gene ontology category enrichment. We found a number of pathways enriched over

subsets of loci associated with our outlying markers and/or consecutive regions of outlying

markers including oncostatin-M-mediated signaling, leukemia inhibitory factor signaling,

cytolysis, and cellular nitrogen compound metabolic process. These pathways are involved in

regulation of cellular processes and metabolism, consistent with the previous findings of Taylor

et al. (2014) and Wagner et al. (2020). At a finer scale, the genes *Ptprd* and *Ndufs4* were found

among regions on Chromosome Z defined by bounds of consecutive outlying loci in our study as

well as by Taylor et al. (2014), and *Otof_1* and *Pnoc* were near outlying loci in both our study

and Wagner et al. (2020). *Ptprd* has been in found in chickens to be involved "in promoting

neurite growth, and regulating neurons axon guidance"

(https://www.ncbi.nlm.nih.gov/gene/5789); *Ndufs4* mutations in humans can be associated with

neurological disorders (https://www.ncbi.nlm.nih.gov/gene/4724); *Otof_1* mutations in humans

are associated with deafness (https://www.ncbi.nlm.nih.gov/gene/9381); and *Pnoc* is involved in

pain sensitivity, and additionally potentially regulating body temperature, learning and memory,

and hunger (https://www.ncbi.nlm.nih.gov/gene/5368). Although dysregulation of any these

genes in hybrid individuals could be expected to impact their neurological performance (e.g.,

McQuillan et al., 2018), *Pnoc* is a particularly promising target for future studies, as based on its

inferred functions in humans, it could be involved in both the metabolic impacts shown in hybrid

chickadees (Olson et al., 2010), as well as the deficiencies they show in learning and memory

(McQuillan et al., 2018). Future work could focus on evaluating the expression of these genes in

the hippocampus, a brain region previously implicated in adaptation to the harsher winter

climates inhabited by black-capped chickadees (Roth et al., 2012). Focusing on transcriptomes

and/or methylomes will also be important in identifying other (epi)genetic mechanisms that

impact on hybrid performance, as not all adaptation/dysregulation due to hybridization is likely

725   to be reflected in genomic sequence (Moran et al., 2020). An additional future avenue of research

726   will be examining the degree to which the microbiome influences the reduced fitness of hybrids,

727   as observed in hybrid zones of other species (J. Wang et al., 2015).

728

729   *Conclusion*

730   Comparison of levels of admixture in contemporary and historical samples is a powerful method

731   of documenting the impact of climate change. Using museum samples, we documented

732   movement of the black-capped and Carolina chickadee hybrid zone in Missouri; however, the

733   rate of movement in this area was less than in previously studied areas of the hybrid zone,

734   consistent with a slower rate of warming in Missouri than in Pennsylvania. Human-caused

735   climate change has influenced distributions, abundances, and the likelihood of extinction of

736   many taxa (Thomas et al., 2004). Although it can be tempting to make broad characterizations

737   about how climate change will affect species with large distributions, geographic variation in

738   hybrid zone movement rates suggests that the specific impacts on broadly distributed species will

739   need to be assessed at local scales. As climate change phenomena continue to manifest, detailed

740   characterization of their variation will be key in assembling a predictive view of their

741   implications, with museum collections critical in this endeavor (Billerman et al., 2019; Lopez et

742   al., 2020; Ryan et al., 2018; Schmitt et al., 2018).

743

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1036 **Data accessibility and benefit-sharing statement:** Demultiplexed sequence data for each

1037 individual has been deposited in the NCBI SRA (accession no: XXX-XXX). All other data are

1038 available in the main text, the supplementary material, dryad and/or at

1039 https://github.com/laninsky/chickadees. A lay summary of the results has been provided to the

1040 Kaskaskia [Peoria] and Osage peoples as traditional custodians of the area the study was

1041 conducted in.

1042

1043 **Author contributions:** MR conceptualized study and carried out field work. MR, AA, and JH

1044 carried out lab work. MR, AA, and ATP carried out analyses. AA and ATP visualized results.

1045   AA and MR were responsible for data curation and wrote manuscript. All authors reviewed and

1046   edited manuscript. MR, AA, RM and ATP acquired or provided funding.

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Table 1: Summary of studies that have compared locus-specific patterns of introgression at multiple geographic transects for a given hybrid zone system, ordered by Taxa. Studies where patterns of introgression across different transects are largely consistent/congruent, have their entry for this column bolded. Potential factors that may influence the recovery of consistent introgression patterns are also given (method for identifying introgression outliers, subdivisions between transects, and whether the hybrid zone is natural or human-mediated e.g. Kane et al. 2009).

Species system	Taxa	Method of identifying introgression outliers	Patterns of introgression across different transects	Subdivisions in taxa examined between transects	Natural hybrid zone	Marker type	Reference
Helianthus annuus and *H. petiolaris*	plant	Frequency of individuals who had "*petiolaris*" band	**"Striking congruence of marker introgression patterns between widely separated hybrid zones in Nebraska and southern California"**	Yes, morphological differences	No[†]	RAPD markers (n = 61)	Buerkle and Rieseberg (2001)
Pinus contorta and *P. banksiana*	plant	(Gompert & Buerkle, 2009, 2010)	**"Patterns of introgression were more similar between the zones than expected by chance, but there were significant differences between these regions at specific loci"**	No	Yes	SNPs (n = 29)	Burns et al. (2019)
Gryllus pennsylvanicus and *G. firmus*	invertebrate	(Gompert & Buerkle, 2009)	**"Consistent patterns of introgression for individual loci"**	No	Yes	Sequenom MassARRAY (n = 110 SNPs)	Larson et al. (2014)
lineages of *Tigriopus californicus*	invertebrate	(Gompert & Buerkle, 2009, 2010)	**"we observe blocks of linked markers with similar introgression patterns"**	No	Yes[§]	Sequenom MassARRAY (n = 54 SNPs)	Prichard and Edmands (2013)
Cottus perifretum and *C. rhenanus*	fish	(Gompert & Buerkle, 2009)	"Patterns observed at individual loci show little correlation between zones"	No	No[‡]	Microsatellites (n = 168)	Nolte et al. (2009)
Bufo bufo and *B. spinosus*	amphibian	(Gompert & Buerkle, 2011, 2012)	**"Twenty-six barrier markers are shared between transects [...]which is more than would be expected by chance."**	Genetic substructure within *B. bufo*	Yes	3RAD (n = 10,535 to 39,750 SNPs)	van Riemsdijk et al. (2020)
Lissotriton montandoni and *L. vulgaris*	amphibian	(Gompert & Buerkle, 2011, 2012)	"We found limited overlap of cline outliers between transects"	Two lineages of *L. vulgaris*	Yes	Molecular Inversion Probes (n = 1,233 loci)	Zieliński et al. (2019)
lineages of *Podarcis muralis*	reptile	(Gompert & Buerkle, 2011, 2012)	"Putative barrier loci were enriched in genomic regions that were highly differentiated between the two lineages and showed low concordance between the transects. The exception was a consistently low genetic exchange around ATXN1, a gene that modulates social behavior"	No (population structure present, but paired across transects)	Yes	ddRADseq SNPs (n = 1029)	Yang et al. (2020)
Pipilo maculatus and *P. ocai*	bird	(Gompert & Buerkle, 2011)	**"Results are consistent with a history in which reproductive isolation has been influenced by a common set of loci in both hybrid zones, but where local"**	Population structure within *P. ocai*	Yes	GBS (n = 41,000 SNPs)	Kingston et al. (2017)

			environmental and stochastic factors also lead to genomic differentiation"				
Poecile atricapillus and *P. carolinensis*	bird	(Gompert & Buerkle, 2011, 2012)	"The number of positive β outlier loci overlapping between our studies was not significantly greater than expected by chance"	No	Yes	GBS/RADseq, with different enzymes between studies (This study, n = 6,784 SNPs; Wagner et al. 2020: n = 76,883 SNPs)	This study; Taylor et al. (2014); Wagner et al. (2020)
Mus domesticus and *M. musculus*	mammal	(Gompert & Buerkle, 2009, 2010)	"Different patterns of introgression in the two transects highlight the challenge of using hybrid zones to identify genes underlying isolation and raise the possibility that the genetic basis of isolation between these species may be dependent on the local population genetic make-up or the local ecological setting"	No	Yes	TaqMan probes (n = 41 SNPs)	Teeter et al. (2010)
Mus domesticus and *M. musculus*	mammal	(Gompert & Buerkle, 2009, 2010)	"Markers shared between transects is a relatively small subset of the markers identified in the two transects separately"	No	Yes	n = 1401 SNPs	Janoušek et al. (2012)
Mus domesticus and *M. musculus*	mammal	(Gompert & Buerkle, 2009, 2010)	**"There is some evidence of common architecture of reproductive isolation."**	No	Yes	PCR (n = 24 X-chromosome markers)	Macholán et al. (2011)

1052 † *H. petiolaris* introduced to California from Great Plains, however, *H. annus* and *H. petiolaris* occur in sympatry in the Great Plains
1053 § mimicked with laboratory crosses
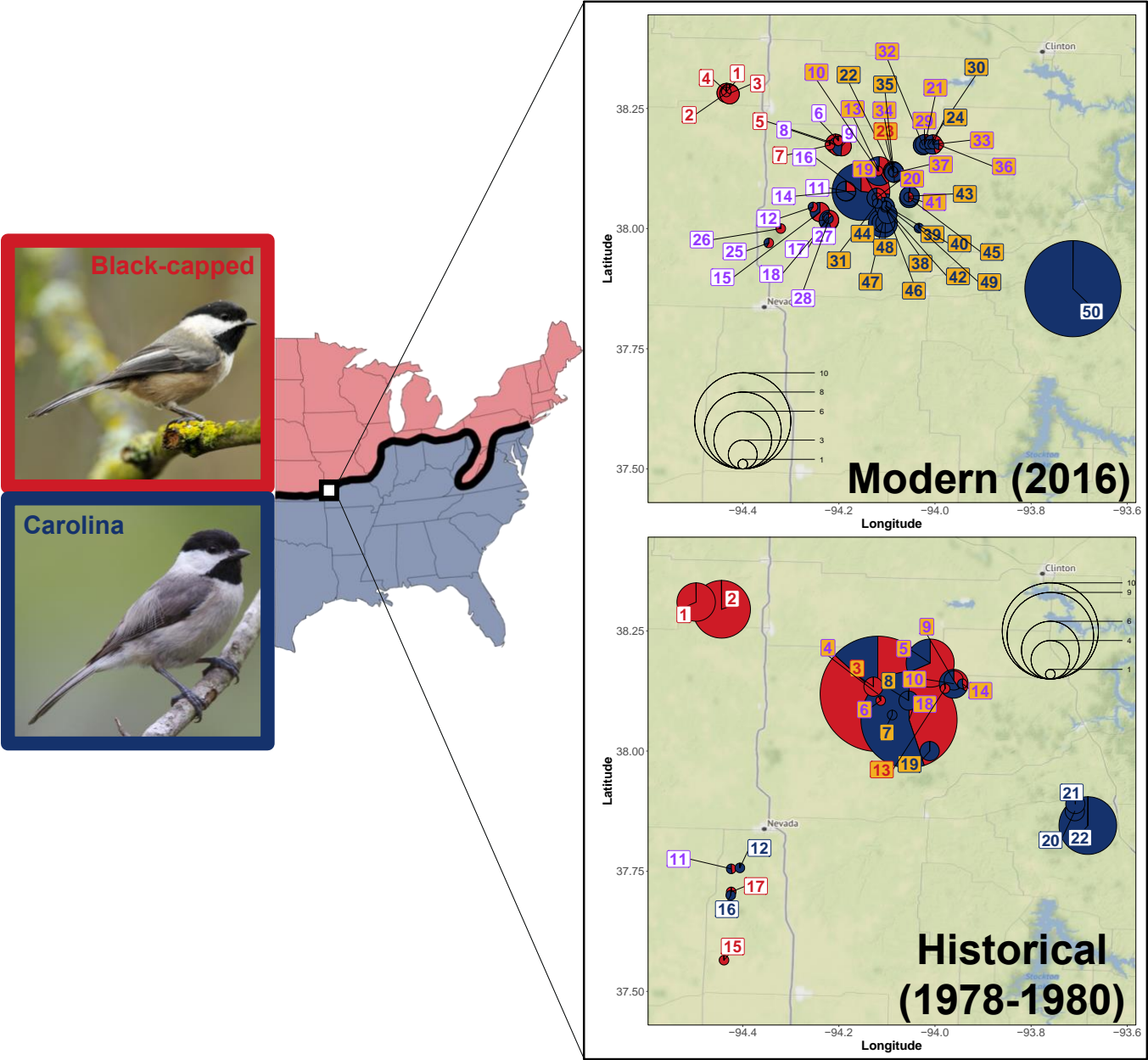1054 ‡ *C. perifretum* is considered invasive

Fig. 1: Total proportion of genomes across all birds sampled at each site assigned to black-capped (red) or Carolina chickadee (blue) genetic clusters via STRUCTURE. Size of pie is proportional to the number of birds sampled at each site. Sampling site labels have red font if only black-capped birds present (individual assignment >95% to black-capped cluster), blue if only Carolina present (individual assignment >95% to Carolina cluster), and purple if hybrids and/or mix of parental species present. Sample sites highlighted in yellow used for spatial interpolation of hybrid zone movement (**Fig. 2**). Map tiles provided by Stamen Design, under CC BY 3.0. Map data by OpenStreetMap, under ODbL. Code for generating **Fig. 1** is given in **Fig. S4**. Individual chickadee assignments to black-capped and Carolina genetic clusters are given in **Fig. S1**. Images via Wikimedia Commons (black-capped chickadee: Minette Layne, Carolina chickadee: Dan Pancamo).
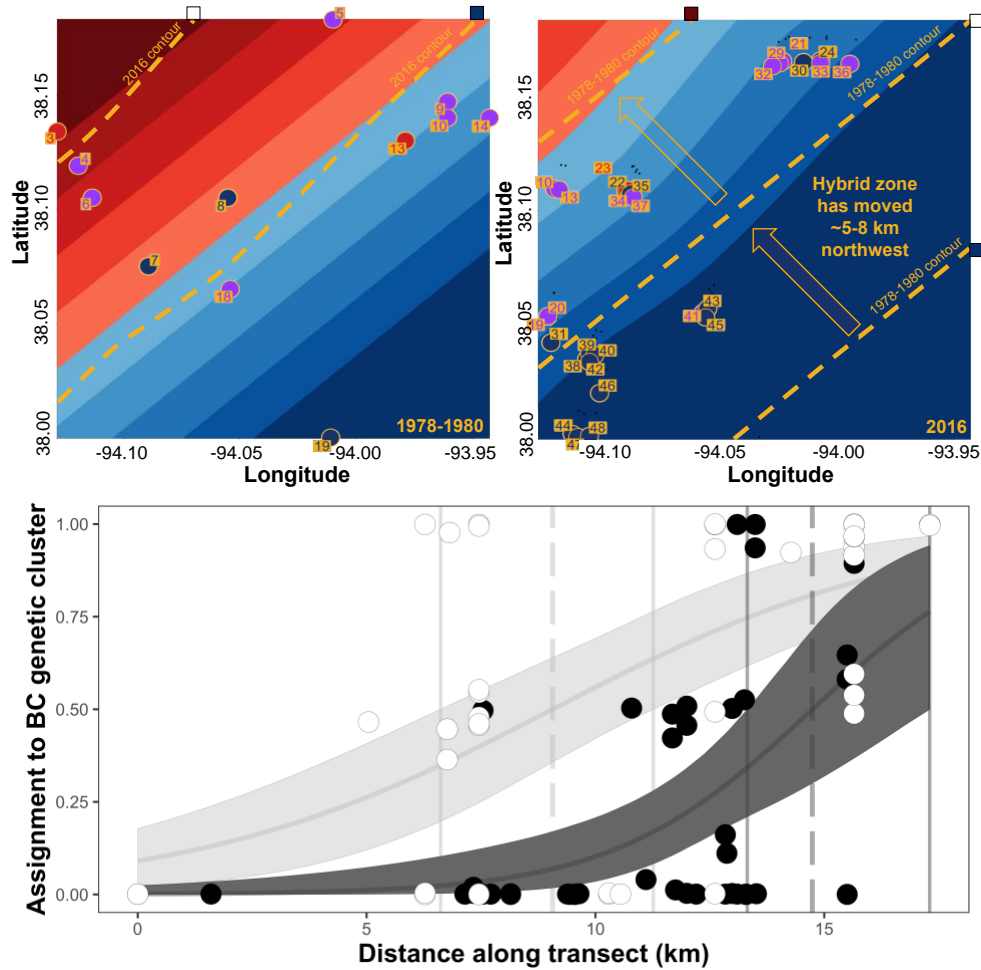
45

Fig. 2: Movement of Missouri hybrid zone through time.

Top panel: Spatial interpolation of 1978-1980 samples shown on left, 2016 samples shown on right. To demonstrate movement, contours corresponding to spatial interpolation of average genome proportions ranging from predominantly black-capped (dark red), to predominantly Carolina (dark blue), with the interface between bird genomes being on average black-capped and on average Carolina (the interface between light red and light blue) from 2016 are overlaid in the 1978-1980 plot (left), and vice-versa (right). Note, dark red contour not observed across 2016 sites so analyses of hybrid zone movement are restricted to the position of the black-capped/Carolina interface (the red/blue interface), rather than considering width of hybrid zone. Numbered sample sites correspond to those given in **Fig. 1/Fig. S1**. Code for generating top panel is given in **Fig. S5**.

Bottom panel: Geographic cline analysis of the change in black-capped (BC) chickadee ancestry with distance along transect, assuming a strict southwest (left) to northeast (right) direction. Light grey ribbon gives the 95% confidence interval of the geographic cline estimated for the 1978-1980 samples (shown as white points). Dark grey ribbon gives the 95% confidence interval of the geographic cline for the 2016 samples (shown as black points). The line in the center of ribbons is mean estimated geographic cline. Solid vertical lines correspond to minimum and maximum 95% confidence intervals of the center of the genomic cline, with dashed lines giving the estimated center (estimates for 1978-1980 shown in light grey, and 2016 in dark grey). Code for generating bottom panel is given in **Fig. S6**.
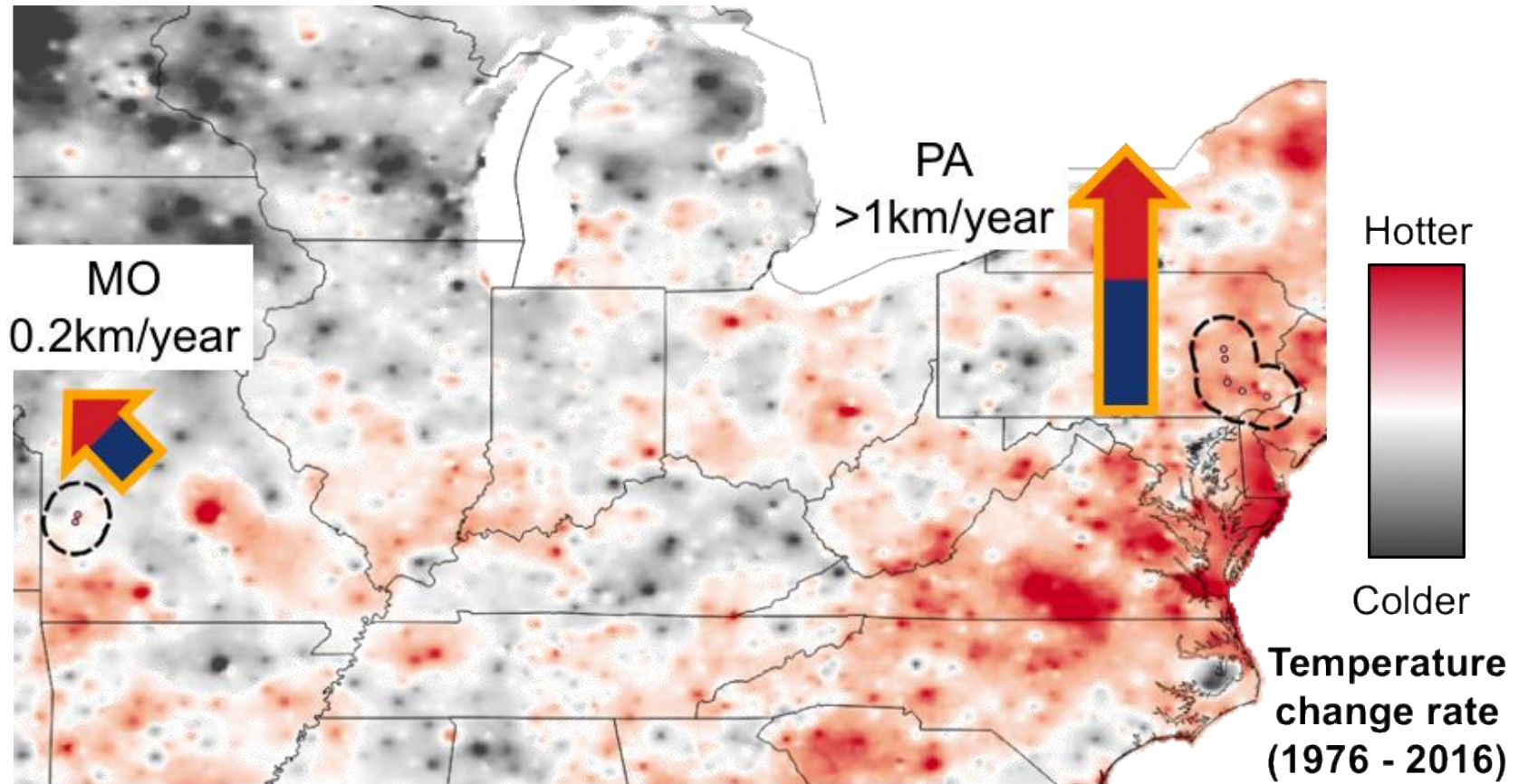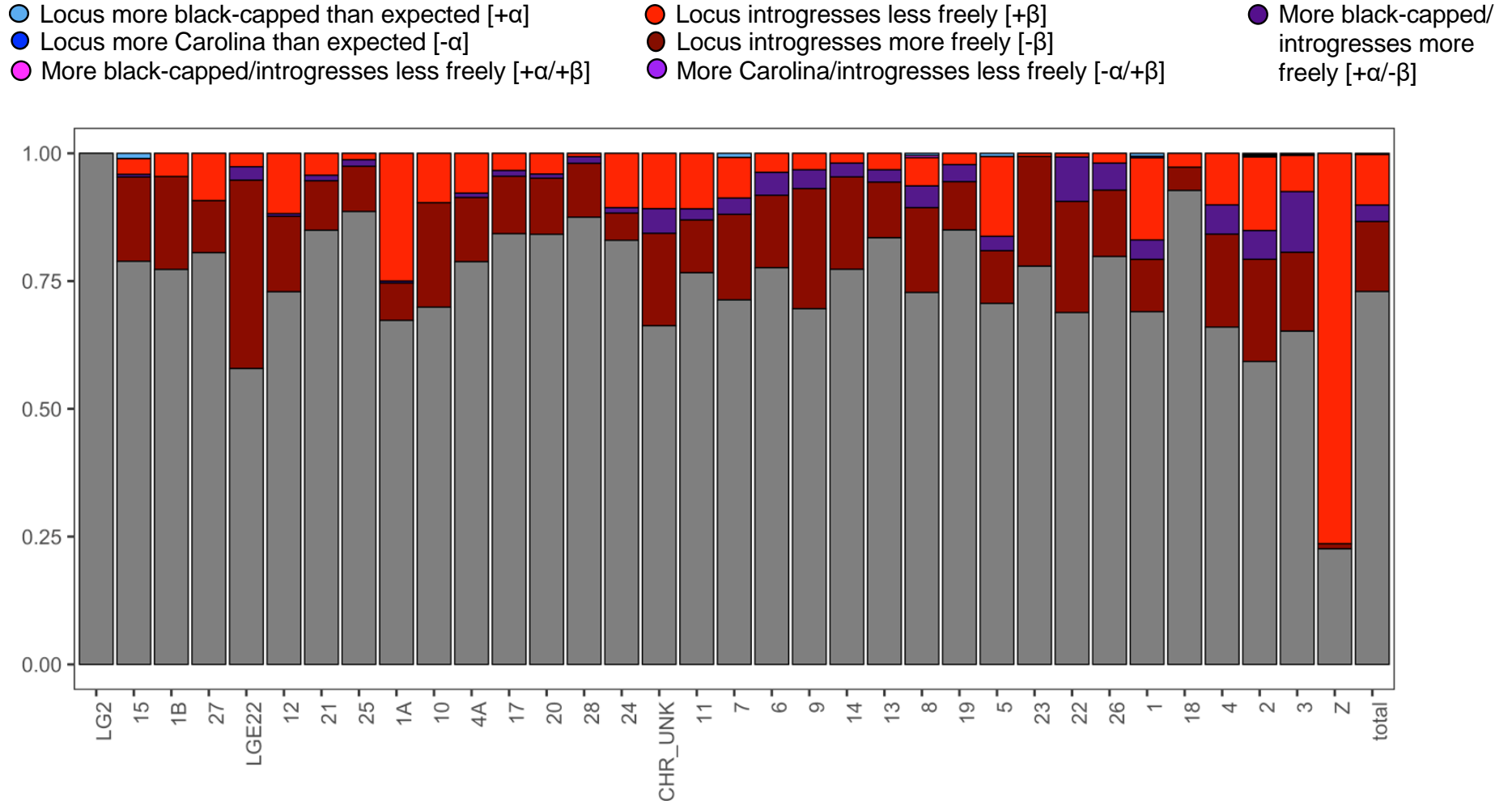
Fig. 3: Slower movement of the black-capped and Carolina chickadee hybrid zone is associated with less temperature change in Missouri (MO), compared with Pennsylvania (PA). Rate of temperature change between 1976-1980 and 2012-2016 is based on five-year means. Sample sites used to infer climatic trends at each location are listed in **Table S4**. Temperature change rates range from -0.00703 °C/year in black, through +0.0261 °C/year in white, to +0.0946 °C/year in dark red.

○ Locus more black-capped than expected [+α] ○ Locus introgresses less freely [+β] ● More black-capped/
○ Locus more Carolina than expected [-α] ● Locus introgresses more freely [-β] introgresses more
○ More black-capped/introgresses less freely [+α/+β] ○ More Carolina/introgresses less freely [-α/+β] freely [+α/-β]

Fig. 4: Proportion of outlying loci categories (as identified by BGC) for each chromosome. Chromosomes ordered by G-test statistic on whether their outlier loci composition differed significantly from the background total genome composition (which is shown on far right). Ordered from left (not significantly different to background genome composition) to right (Chromosome 11 and all chromosome/scaffolds to the right of it were significantly different from the background genome composition). Non-outlying loci are indicated in grey. Specific values for the numbers of loci in each outlier category by chromosome are available at https://github.com/laninsky/chickadees/blob/master/output/Table_S7_outlier_by_chrom.csv
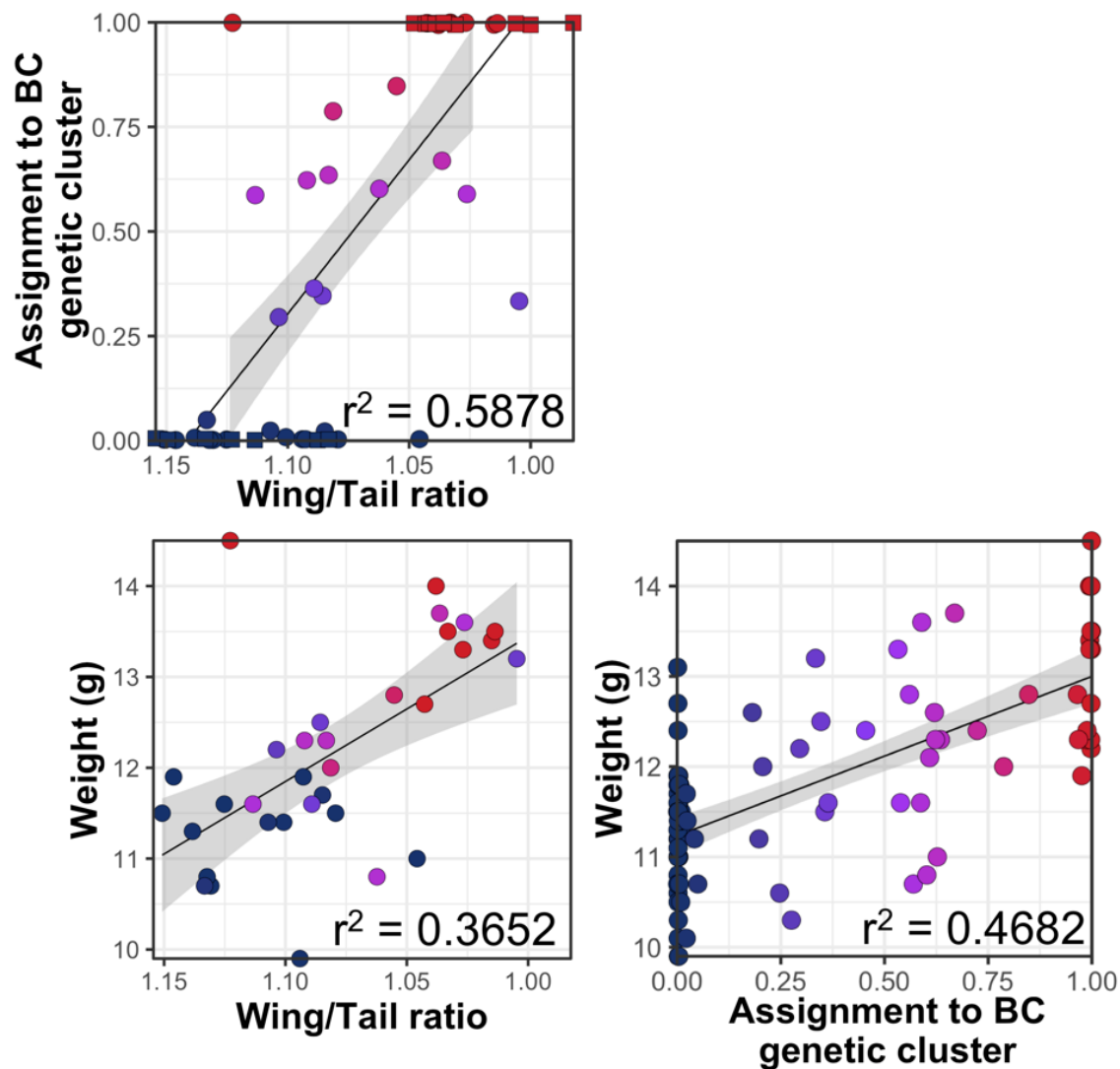
Fig. 5: Correlations between morphological measurements and STRUCTURE assignment. Each point represents an individual bird sampled in 2016 (circles) or 1978-1980 (squares – weight data not available for these samples, so featured in top plot only), color coded by their genomic assignment to the black-capped structure cluster (red = 100% assignment to black-capped cluster through to dark blue = 100% assignment to Carolina cluster). Best fit line for each plot component calculated using a linear model. Note overlap in morphological characteristics between birds strongly assigning to black-capped and Carolina genetic clusters. Code for generating components of this plot is given in **Fig. S15**.
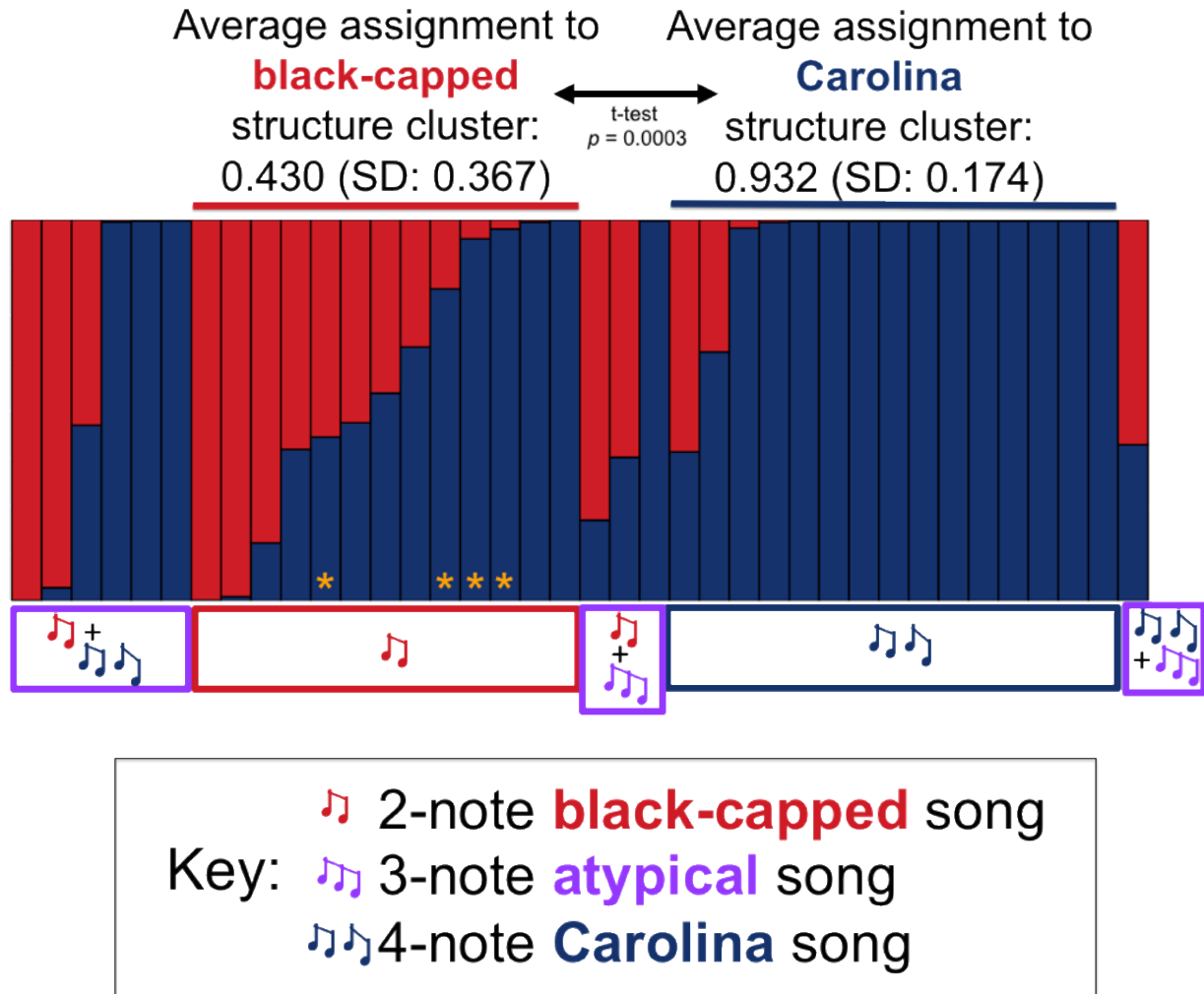
Fig. 6: Song type and genomic make-up based on structure assignments (**Fig. S1**) for the 38 birds sampled in 2016 for both song and genetic loci. Birds that were recorded singing only black-capped song are denoted by the red box with the red notes inside. Birds recorded singing only Carolina song are denoted by the blue box with blue notes inside. Birds recorded singing both parental songs are denoted by the purple box to the left of the image with both red and blue notes. Birds recorded singing atypical song are denoted by the purple notes. Caution is warranted about individual-level characterization of birds based on song. In some of the longer sequences of bird song, birds switched between parental song types, suggesting that characterization based on shorter bouts of song could incorrectly classify the bird's repertoire. However, when contrasting birds recorded singing either only black-capped song or only Carolina song, birds singing only Carolina song look more genetically Carolina than birds singing only black-capped look genetically black-capped. Birds sampled at Appleton City, referenced in the main text for being composed of Carolina/hybrid chickadees in our sample yet singing black-capped song, denoted by asterisks (birds from Sites 21, 24 and 33 **Fig. 1/Fig. S1**, no audio recorded at Appleton City Sites 29, 30, 32 and 36). Code for generating components of this plot is given in **Fig. S16**.