WHOLE GENOMES REVEAL CONTINUED NORTHWARD MOVEMENT OF A HYBRID ZONE MAINTAINED BY STRONG POSTZYGOTIC SELECTION

by

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Whole genomes reveal continued northward movement of a hybrid zone maintained by strong postzygotic selection

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Hybridization—the interbreeding of individuals from populations that differ by one or more heritable characters—can be studied to better understand the evolutionary processes associated with speciation (e.g., the mechanisms that maintain reproductive isolation between closely-related species), as well as the way species are responding to environmental change. Black-capped and Carolina Chickadees hybridize in a narrow hybrid zone that extends from New Jersey to Kansas, and that is moving northwards at a rate of ~1 km per year (Taylor et al. 2014; Wagner et al. 2020). Hybrid chickadees have deficient learning and memory abilities compared to either parental species (McQuillan et al. 2018), suggesting that cognition may be a postzygotic reproductive isolating barrier—where hybrids with deficient cognitive abilities experience negative selection. To determine if cognition is acting as a post-zygotic reproductive isolating barrier in chickadees, we used high-resolution, whole-genome data to examine patterns of introgression across the hybrid zone and determined the biological processes associated with loci experiencing reduced introgression. We also used whole genome data to determine chickadee ancestry across our hybrid zone transect and compared hybrid zone position to previous studies. We found 1) that chickadee ancestry and geographic cline analysis indicated continued northward movement of the chickadee hybrid zone in Pennsylvania (Taylor et al. 2014; Wagner et al. 2020), 2) that genomic cline analysis revealed reduced introgression of loci on the Z chromosome, and 3) that loci experiencing reduced introgression across the hybrid zone are related to cognitive and metabolic function.

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INTRODUCTION

Hybridization—the interbreeding of individuals from populations that differ by one or more heritable characters—is important for understanding the evolutionary processes associated with speciation (Vázquez-Miranda et al. 2009; Harrison and Larson 2014; Payseur and Rieseberg 2016). In particular, hybridization can provide insight into the mechanisms that maintain reproductive isolation between closely related species (Abbott et al. 2013; Urbanelli et al. 2014; Xie et al. 2017). Hybrid zones can also be used to understand how species distributions are changing in response to climate change (Taylor et al. 2015). Among hybridizing taxa, the exchange of genetic material between distinct populations may lead to: (1) novel combinations of genetic material in hybrid individuals that contribute to adaptive divergence and reproductive isolation, leading to speciation of the hybrid population (i.e., hybrid speciation), (2) the strengthening of barriers that inhibit gene flow, leading to reinforcement of reproductive isolation, (3) the breakdown of barriers that inhibit gene flow, leading to the degradation of reproductive isolation and loss of genetic differentiation (i.e., despeciation or lineage fusion) and, (4) hybridization and speciation may establish an equilibrium where regions of the genome introgress between species, but reproductive isolating barriers prevent loss of differentiation (Abbott et al. 2013).

Hybrid speciation—where novel combinations of genetic material result in adaptive divergence or reproductive isolation of the hybrid species—can occur due to genome duplication (i.e., allopolyploid speciation) or from novel genetic combinations that result in reproductive isolation from the parental species (i.e., homoploid speciation; Taylor and Larson 2019). In *Heliconius* butterflies (*H. melpomene* and *H. cydno*), an intermediate wing morphology in the hybrid lineage (*H. heurippa*) reproductively isolated hybrids from the parental species, leading to

homoploid hybrid speciation (Mavárez et al. 2006). While hybridization may lead to the speciation of a single hybrid lineage, the admixing of parental genomes may provide substantial genetic variation that leads to adaptive radiation and subsequent speciation of many lineages. In *Haplochromine* cichlid fishes, hybridization generated extensive allelic variation in the opsin gene—genetic variation that led to an adaptive radiation of >700 cichlid species (Meier et al. 2017). Numerous studies have suggested a role of hybrid speciation in generating the extant biodiversity, but few have demonstrated all of the criteria for identifying a hybrid species (Taylor and Larson 2019). Although evidence of hybrid speciation has primarily been limited to multicellular species, such as plants and animals, hybrid speciation also occurs in single-celled organisms (e.g., yeast; Leducq et al. 2016). It is increasingly clear that novel combinations of genetic material can lead to reproductive isolation of a hybrid lineage, however, admixture of parental genomes can also provide genetic variation that promotes adaptive divergence without leading to reproductive isolation.

In cases where hybridization does not lead to hybrid speciation, the interbreeding of distinct populations can provide genetic variation that facilitates evolutionary change in the hybridizing species (Grant et al. 1996). In *Drosophila* (*D. yakuba* and *D. santomea*), reproductive isolating barriers evolved despite gene flow, reinforcing species boundaries and leading to speciation (Matute 2010). While the evolution of species barriers can be observed in short-generation organisms, observing the development of reproductive isolation is difficult in most species. Therefore, to determine the evolutionary history of distinct populations, high-throughput sequencing can be used to identify ancient hybridization and subsequent speciation (Taylor and Larson 2019). In Eastern Wolves (*Canis lycaon*) and Red Wolves (*Canis rufus*), whole-genome sequencing provided evidence that these now distinct populations are likely the

product of hybridization between Gray Wolves (*Canis lupus*) and Coyotes (*Canis latrans*) (vonHoldt et al. 2016). Further evidence of ancient hybridization in numerous extant species suggests that hybridization is widespread and of evolutionary importance for many species (Marcet-Houben and Gabaldón 2015; Toews et al. 2016; Svardal et al. 2017).

Hybridization can be important for generating diversity either through speciation or adaptive divergence, but the interbreeding of previously allopatric species and loss of reproductive isolation can also erode species barriers by allowing extensive gene flow between hybridizing species. Without reproductive isolating barriers limiting gene flow, once distinct populations may evolve into a single population due to loss of genetic differentiation. In the *Montastraea annularis* species complex, ecologic and morphologic analyses of fossil data suggest ancient hybridization and lineage fusion of coral species (Budd and Pandolfi 2004). Likewise, in the Small Skipper (*Thymelicus sylvestris*), discordance in the nuclear and mitochondrial genomes—where the nuclear genome is more homogenized than the mitochondrial genome—suggests a history of lineage fusion events (Hinojosa et al. 2019).

The majority of well-studied hybrid zones appear to be at some sort of equilibrium—where reproductive isolating barriers maintain species boundaries despite ongoing gene flow. For example, in Brown Lemurs (*Eulemur rufifrons* and *E. cinereiceps*), the hybrid zone is maintained by environmental selection favoring hybrids in novel habitats (Delmore et al. 2013). In stable hybrid zones, where distinct populations regularly interbreed but are not undergoing speciation or lineage fusion, a variety of morphological, physiological and behavioral traits may maintain reproductive isolation in the hybridizing species through prezygotic or postzygotic isolation. In European flycatchers (*Ficedula hypoleuca* and *F. albicollis*), female mate choice is a prezygotic reproductive isolating barrier—females choose conspecific males based on plumage color,

limiting the frequency of hybridization (Saetre et al. 1997). In *Primula* flowering plants (*P. secundiflora* and *P. poissonii*), reduced F_1 hybrid fitness is a postzygotic reproductive isolating barrier—hybrids develop fewer seeds, have lower germination rates, and have higher seed-embryo developmental failure and seed inviability rates (Xie et al. 2017). The plethora of prezygotic and postzygotic barriers influencing reproductive isolation makes stable hybrid zones an excellent resource for studying anthropogenic climate change (Taylor et al. 2015), the effects of parasites on speciation (Cozzarolo et al. 2018), mating patterns (Randler 2008), and more.

Cognition, compared to physiology or hybrid sterility, has received little attention for its role in maintaining postzygotic isolation in hybridizing species. While primarily studied in humans and non-human primates, numerous studies have estimated heritability of various cognitive traits, such as spatial memory or the ability to learn (Croston et al. 2015). Cognition is a known target of selection in a number of species—previous research has identified genomic signatures of selection on cognitive traits, and has documented possible introgression of genes related to cognition in hybrids. For example, in the Great Tit (*Parus major*), regions of the genome under positive selection are primarily associated with neuronal functions, learning and cognition (Laine et al. 2016). In European wildcat (*Felis silvestris silvestris*) and free-ranging domestic cat (*Felis silvestris catus*) hybrids, an overrepresentation of introgressed genes in the hybrid genome are associated with cognition, possibly because they are adaptive in human-dominated landscapes (Mattucci et al. 2019).

Given that learning and memory are heritable characteristics and hybrid cognitive abilities may differ from parental species, genomic regions associated with cognitive function may either introgress (e.g., if hybrids have superior cognitive abilities) or experience reduced introgression (e.g., if hybrids have inferior cognitive abilities). There is some evidence that

hybrids can possess superior cognitive abilities from the novel gene combinations they inherit (heterosis; Baranwal et al. 2012). In mules (i.e., *Equus. caballus* and *E. asinus* hybrid) and inbred mouse strains, hybrids performed better at a visual discrimination learning and water-escape test, respectively, than the parental species (Winston 1964; Proops et al. 2009). Because cognition may influence hybridization, our aim is to determine whether cognitive breakdown in hybrids acts as a postzygotic, reproductive isolating barrier in a rapidly moving avian hybrid zone.

In hybridizing species, hybrid individuals may inherit genotype combinations that lead to lower cognitive abilities, but the underlying mechanism is less clear (McQuillan et al. 2018). Cognition may breakdown due to incompatible alleles inherited from the parental species in a variety of pathways, which could include neuron signaling and / or neuron growth and development (Orr 1996). Hybrids could also experience reduced cognitive capacity due to carryover effects from inefficient metabolism—the brain is one of the most metabolically active organs, and brain growth and development might be affected by breakdown (Wagner et al. 2020). Despite the potential for cognition to act as a postzygotic, reproductive isolating barrier, previous research has been limited to the role of cognition in premating isolation (Rice and McQuillan 2018), and studies focused on how hybridization influences cognition have been lacking (Rice 2020). To address this gap in the literature, we expanded our previous examination of the role of cognition in maintaining the species barrier between two hybridizing songbirds (Wagner et al. 2020) using high resolution whole genome data.

Black-capped (*Poecile atricapillus*) and Carolina (*Poecile carolinensis*) Chickadees form a well-studied, narrow hybrid zone that stretches from New Jersey to Kansas (Kershner and Bollinger 1999; Bronson et al. 2005; Reudink et al. 2007; McQuillan et al. 2017; Wagner et al. 2020). The chickadee hybrid zone is an excellent system for determining whether cognition is a

postzygotic, reproductive isolating barrier. Chickadees are non-migratory songbirds that cache seeds for recovery in the winter when food resources are scarce. Because successful retrieval of seeds from caches is necessary for winter survival (Sonnenberg et al. 2019), and finding caches is dependent on cognitive ability (Yi et al. 2016), natural selection will act on cognitive abilities—individuals with poor spatial memory fail to find enough caches to survive the winter months (Pravosudov and Roth 2013; Sonnenberg et al. 2019). In Black-capped and Carolina Chickadees, hybrid individuals have deficient learning and memory abilities compared to either parental species (McQuillan et al. 2018), and regions of the genome related to neuron signaling and metabolism appear to resist introgression (Wagner et al. 2020), suggesting that cognition in hybrids is selected against and limits gene flow between Black-capped and Carolina Chickadees. Our previous examinations of this hybrid zone were limited to low-resolution reduced representation genomic data. To more comprehensively determine whether cognition may be a postzygotic, reproductive isolating barrier in Black-capped and Carolina Chickadees, we collected blood or tissue samples from 117 chickadees across the hybrid zone in eastern Pennsylvania for whole-genome analyses. We used high resolution, whole genome data to develop a comprehensive understanding of chickadee ancestry, advance our knowledge of hybrid zone movement, identify patterns of introgression, and determine the biological processes underlying signatures of reproductive isolation across the Black-capped and Carolina Chickadee hybrid zone.

METHODS

Hybrid Zone Sampling

From 2013 to 2018, we captured 81 Black-capped (*Poecile atricapillus*) and Carolina (*Poecile carolinensis*) Chickadees from three sympatric populations in eastern Pennsylvania: 23 chickadees from Jacobsburg State Park (JSP; 40°47'03.7"N 75°17'37.8"W), 11 chickadees from DeSales University (DSU; 40°32'26.2"N 75°22'41.8"W), and 47 chickadees from Lehigh University (LHU; 40°35'47.6"N 75°22'05.9"W). We captured 10 Black-capped Chickadees from Hickory Run State Park, Pennsylvania (HRP; 41°01'21.0"N 75°43'15.6"W) from 2015 to 2018, 4 Black-capped Chickadees from DeRuyter, New York (DNY; 42°45'31.7"N 75°53'11.1"W) in 2019, and 11 Black-capped Chickadees from Ithaca, New York (INY; 42°28'48.0"N 76°27'04.0"W) in 2012 for a total of 25 allopatric Black-capped Chickadees. We captured 6 Carolina Chickadees from East Carolina University, North Carolina (ECW; 35°37'51.4"N 77°28'55.4"W) in 2019, and 5 Carolina Chickadees from Baton Rouge, Louisiana (LSU; 30°27'07.2"N 91°11'11.0"W) in 2013 for a total of 11 allopatric Carolina Chickadees (Figure 1).

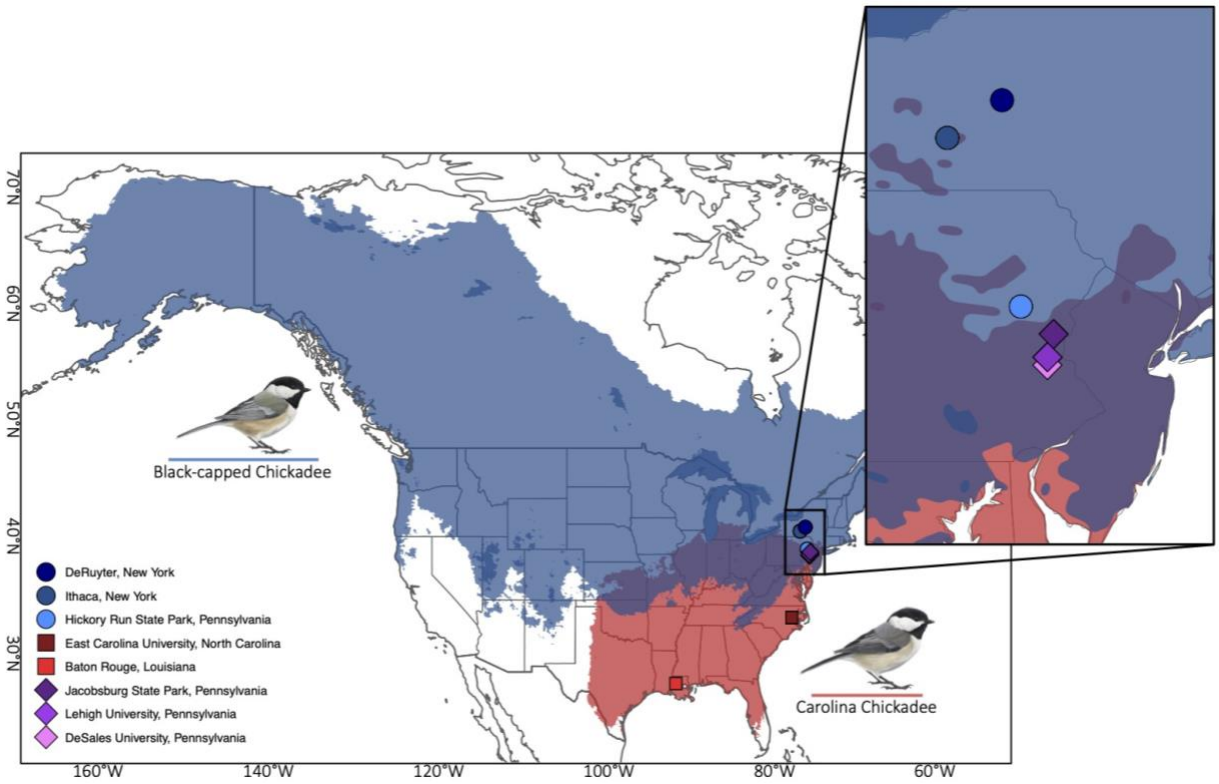

Figure 1: Map of the Black-capped (blue) and Carolina (red) Chickadee range distributions. The hybrid zone, represented by the area of range overlap (purple), spans from New Jersey to Kansas. Points represent the populations sampled: allopatric Black-capped Chickadees (DNY, INY and HRP; blue circles), allopatric Carolina Chickadees (ECW and LSU; red squares), and sympatric populations (JSP, LHU and DSU; purple diamonds).

During the breeding season, we banded and collected blood samples from the brachial vein of chickadees captured at artificial nesting tubes, or using mist nests in conjunction with song playback and a chickadee model from all sympatric, allopatric Black-capped Chickadee, and the ECW allopatric Carolina Chickadee populations. We stored blood samples on dry ice in the field and kept the samples at -80°C until DNA extraction. For the Baton Rouge allopatric Carolina Chickadee population, we collected tissue samples from the pectoral muscle of specimens that were deposited in the Louisiana State University Museum of Natural Science. All protocols were approved by the Cornell University, Louisiana State University and Lehigh

University IACUC panels, and all methods in this study were performed in accordance with relevant guidelines, permits, and regulations.

We extracted DNA from blood and tissue samples using the QIAGEN DNeasy Blood and Tissue Kit or a salt extraction protocol (Table 1). For blood samples extracted using the QIAGEN kit, we added Proteinase K (30 μl), RNase (4 μl), and phosphate-buffered saline (PBS; 180 μl) prior to lysing at 65°C overnight. We added ethanol, filtered the blood sample through a spin column, and rinsed the spin column with AW1 and AW2 buffers. The final DNA samples were eluted with AE buffer (150 μl) and stored at -80°C. For blood and tissue samples extracted using a salt extraction protocol, we added Proteinase K (10 μl), 20% sodium dodecyl sulfate (SDS; 20 μl), and homogenizing solution (0.4 M NaCl, 10 mM Tris–HCl, 2 mM EDTA; 200 μl) prior to lysing at 56°C overnight. We added salt solution (150 μl) to breakdown cellular components, and GlycoBlue (2 μl) to precipitate the DNA out of solution. The final DNA samples were resuspended in TE buffer (100 μl) and stored at -20°C. We carried out shallow whole-genome sequencing of all samples using an Illumina Novaseq 6000 (Novogene Corporation Inc.).

Sample	Population	Type	Extraction	Sample	Population	Type	Extraction
B-25801	DNY	Blood	Salt	*E415*	LHU	Blood	QIAGEN
B-25902	DNY	Blood	Salt	*E441-2*	JSP	Blood	QIAGEN
B-25903	DNY	Blood	Salt	*E442*	JSP	Blood	QIAGEN
B-25904	DNY	Blood	Salt	*E446*	JSP	Blood	QIAGEN
BCCH10	INY	Blood	Salt	*E447*	JSP	Blood	QIAGEN
BCCH1A	INY	Blood	Salt	*E448*	LHU	Blood	QIAGEN
BCCH1	INY	Blood	Salt	*E450*	LHU	Blood	QIAGEN
BCCH2	INY	Blood	Salt	*E451*	JSP	Blood	QIAGEN
BCCH30	INY	Blood	Salt	*E452*	JSP	Blood	QIAGEN
BCCH3	INY	Blood	Salt	*E453*	JSP	Blood	QIAGEN
BCCH4	INY	Blood	Salt	*E454-2*	JSP	Blood	QIAGEN

BCCH5	INY	Blood	Salt	*E456-3*	LHU	Blood	QIAGEN
BCCH6	INY	Blood	Salt	*E457*	JSP	Blood	QIAGEN
BCCH7	INY	Blood	Salt	*E458*	HRP	Blood	QIAGEN
BCCH8	INY	Blood	Salt	*E461*	HRP	Blood	QIAGEN
CACH32.1	ECW	Blood	Salt	*E463*	HRP	Blood	QIAGEN
CACH5.1	ECW	Blood	Salt	*E464*	HRP	Blood	QIAGEN
E012	LHU	Blood	QIAGEN	*E466*	HRP	Blood	QIAGEN
E030	LHU	Blood	QIAGEN	*E533*	LHU	Blood	QIAGEN
E031	LHU	Blood	QIAGEN	*E563*	DSU	Blood	QIAGEN
E035	LHU	Blood	QIAGEN	*E564*	DSU	Blood	QIAGEN
E039	LHU	Blood	QIAGEN	*E578*	DSU	Blood	QIAGEN
E042	LHU	Blood	QIAGEN	*E583*	DSU	Blood	QIAGEN
E054	DSU	Blood	QIAGEN	*E592*	LHU	Blood	QIAGEN
E058	DSU	Blood	QIAGEN	*E630*	DSU	Blood	QIAGEN
E060	LHU	Blood	QIAGEN	*E635*	HRP	Blood	QIAGEN
E070	LHU	Blood	QIAGEN	*E639*	HRP	Blood	QIAGEN
E076	LHU	Blood	QIAGEN	*E641*	JSP	Blood	QIAGEN
E081	LHU	Blood	QIAGEN	*E688*	JSP	Blood	QIAGEN
E082	LHU	Blood	QIAGEN	*E696*	JSP	Blood	QIAGEN
E085	LHU	Blood	QIAGEN	*E698*	JSP	Blood	QIAGEN
E095	LHU	Blood	QIAGEN	*E699*	JSP	Blood	QIAGEN
E1032	ECW	Blood	QIAGEN	*E700*	JSP	Blood	QIAGEN
E1033	ECW	Blood	QIAGEN	*E790*	LHU	Blood	QIAGEN
E1034	ECW	Blood	QIAGEN	*E809*	HRP	Blood	QIAGEN
E1035	ECW	Blood	QIAGEN	*E816*	LHU	Blood	QIAGEN
E103	LHU	Blood	QIAGEN	*E836*	LHU	Blood	QIAGEN
E104	LHU	Blood	QIAGEN	*E924*	LHU	Blood	QIAGEN
E110	LHU	Blood	QIAGEN	*E927*	JSP	Blood	QIAGEN
E111	LHU	Blood	QIAGEN	*E935*	JSP	Blood	QIAGEN
E128	LHU	Blood	QIAGEN	*E938*	JSP	Blood	QIAGEN
E132	DSU	Blood	QIAGEN	*E941*	JSP	Blood	QIAGEN
E136	LHU	Blood	QIAGEN	*E942*	JSP	Blood	QIAGEN
E137	LHU	Blood	QIAGEN	*E945*	LHU	Blood	QIAGEN
E143	DSU	Blood	QIAGEN	*E958*	LHU	Blood	QIAGEN
E162	DSU	Blood	QIAGEN	*E959*	LHU	Blood	QIAGEN
E175	LHU	Blood	QIAGEN	*E975*	JSP	Blood	QIAGEN
E232	LHU	Blood	QIAGEN	*E980*	HRP	Blood	QIAGEN
E239	LHU	Blood	QIAGEN	*E983*	HRP	Blood	QIAGEN
E240	LHU	Blood	QIAGEN	*E986*	LHU	Blood	QIAGEN

E247	LHU	Blood	QIAGEN	*E987*	LHU	Blood	QIAGEN
E271	LHU	Blood	QIAGEN	*E990*	JSP	Blood	QIAGEN
E304	LHU	Blood	QIAGEN	*E992*	JSP	Blood	QIAGEN
E328	LHU	Blood	QIAGEN	*S-77292*	LSU	Tissue	Salt
E336	DSU	Blood	QIAGEN	*S-77293*	LSU	Tissue	Salt
E346	LHU	Blood	QIAGEN	*S-77294*	LSU	Tissue	Salt
E350	LHU	Blood	QIAGEN	*S-77296*	LSU	Tissue	Salt
E392	LHU	Blood	QIAGEN	*S-77297*	LSU	Tissue	Salt
E414	LHU	Blood	QIAGEN				

Table 1: We collected blood or tissue samples from 117 individuals from three populations in the Black-capped and Carolina Chickadee hybrid zone (JSP, LHU, DSU), three allopatric Black-capped Chickadee populations (DNY, INY, HRP), and two allopatric Carolina Chickadee populations (ECW, LSU). DNA was extracted from these samples using either the QIAGEN or salt extraction protocol.

Genomic Data

We trimmed, quality-checked and aligned whole-genome sequencing reads to the Black-capped Chickadee reference genome (Accession: JAAMOC000000000) prior to calling variants. Using Trimmomatic (Bolger et al. 2014), we removed Illumina-specific adapters, trimmed low quality bases using a sliding window approach (window size: 4, required quality: 20), and discarded leading and trailing bases below a quality score of 20. Reads smaller than 90 bp post-trimming were dropped from analyses. Before and after trimming, we quality-checked reads using FastQC (Andrews 2010). We aligned trimmed reads to the Black-capped Chickadee reference genome using Burrows-Wheeler Alignment Tool (BWA; Li and Durbin 2009) and called variants using BCFtools (Li et al. 2009). We isolated biallelic SNPs from the raw variants and filtered SNPs for a minimum quality score of 80, 3x minimum coverage, minor allele frequency greater than 0.05, and a minimum of 80% individual representation (i.e., at least 80% of the individuals have data for each locus). Using the filtered SNPs, we ran ADMIXTURE (Alexander and Lange 2011; K = 2) to determine the population structure among our three

allopatric Black-capped Chickadee, two allopatric Carolina Chickadee and three sympatric populations. We compared admixture between populations using the non-parametric wilcox.test() in R v 3.6.1.

Geographic Cline Analysis

To estimate the geographic center and width of the hybrid zone, we used the quantitative trait model in the "hzar" package (Derryberry et al. 2014) in R v 3.6.1 to generate a geographic cline. To convert our genomic data into a quantitative trait for "hzar" analysis, we condensed the variation among filtered SNPs using a principal component analysis (PCA) in the "snpgdsPCA" R package (Zheng et al. 2012), and used the first principal component (PC1) as our quantitative trait. We calculated distance as the latitudinal distance (in kilometers) from the most southern, allopatric Carolina Chickadee population: Baton Rouge, Louisiana (i.e., LSU = 0 meters).

Hybrid Index and Genomic Cline Analysis

To identify patterns of introgression across the hybrid zone, we calculated hybrid index (HI) and generated genomic clines using a Bayesian Markov chain Monte Carlo (MCMC) analysis method in the "gghybrid" R package (Bailey 2020). Because patterns of introgression may be distorted by non-randomly associated loci (Geraldes et al. 2006), we filtered SNPs by linkage disequilibrium using PLINK v 2.0 (Purcell et al. 2007; window size = 50, step size = 5, r^2 threshold = 0.5) and ran "gghybrid" on the outlier loci detected using "OutFLANK" (see Outlier Detection and Gene Ontology). We compared hybrid index values between populations using the non-parametric wilcox.test() in R v 3.6.1.

Outlier Detection and Gene Ontology

To identify the biological processes associated with loci possibly involved in reproductive isolation, we identified outlier SNPs for gene ontology analysis. We identified outlier loci from SNPs filtered by linkage disequilibrium (see Hybrid Index and Genomic Cline Analysis) using the "OutFLANK" R package (Whitlock et al. 2015; minimum heterozygosity = 0.1, q-threshold = 0.05). Prior to identifying outliers, "OutFLANK" trimmed 35% of the loci from the right tail and 5% of the loci from the left tail of the *F*st distribution to generate a neutral distribution. We manually annotated *F*st outlier SNPs with BLAST (Johnson et al. 2008), a standardized map file generated in MAKER (Cantarel et al. 2008), and SnpEff (Cingolani et al. 2012) using the Black-capped Chickadee reference genome (Accession: JAAMOC000000000) for gene ontology analysis. In the PANTHER Classification System (Thomas et al. 2003), we ran a Fischer's exact test with false discovery rate (FDR) correction to identify overrepresented gene ontology categories in our annotated outlier SNP dataset. In PANTHER, we ran the statistical overrepresentation test using the closest available relative to Black-capped and Carolina Chickadees, *Gallus gallus*. To identify the genes associated with transcripts and intergenic regions (i.e., greater than 5 Kb from the gene), we manually annotated all loci, only transcripts, and only intergenic regions.

RESULTS

Filtered SNPs and Outlier Detection

Whole-genome sequencing at 5x coverage produced ~3 billion, 150-bp raw paired-end reads. After trimming, we aligned the trimmed reads to the Black-capped Chickadee reference genome to produce 73,702,591 raw variants. We isolated SNPs and filtered this dataset by quality score, coverage, minor allele frequency and percent individual representation, leaving 16,285,686 SNPs. For genomic cline analysis and outlier detection, we further filtered the dataset by linkage disequilibrium to prevent pseudoreplication by loci that are non-randomly associated, resulting in 10,794,258 SNPs. After generating a neutral *F*st distribution from ~6,476,555 loci, "OutFLANK" detected 104,016 outlier SNPs. We detected SNPs on all chromosomes before and after filtering for linkage disequilibrium. Using "OutFLANK", we identified outlier loci on all chromosomes except chromosome 16. Given the percentage of filtered SNPs detected for each chromosome, we found a significantly greater number of outlier loci on chromosomes 1, 1A, 3, 5, Z and the mitochondrial genome (Binomial Test: $P < 0.001$; Table 2).

Chromosome	SNPs Filtered by LD (Count; %)	Outlier SNPs (Count; %)	FDR Corrected P-value
1	1,100,294; 10.19	26,675; 25.65	**<0.001**
1A	691,382; 6.41	26,489; 25.47	**<0.001**
1B	22,513; 0.21	7; 0.0067	**<0.001**
2	1,425,344; 13.20	3,800; 3.65	**<0.001**
3	1,215,880; 11.26	14,469; 13.91	**<0.001**
4	761,915; 7.06	1,397; 1.34	**<0.001**
4A	276,625; 2.56	81; 0.078	**<0.001**
5	602,698; 5.58	9,319; 8.96	**<0.001**
6	227,787; 2.11	195; 0.19	**<0.001**
7	440,293; 4.08	670; 0.64	**<0.001**
8	358,075; 3.32	325; 0.31	**<0.001**
9	341,352; 3.16	212; 0.20	**<0.001**

10	278,556; 2.58	180; 0.17	**<0.001**
11	281,491; 2.61	89; 0.086	**<0.001**
12	275,928; 2.56	174; 0.17	**<0.001**
13	276,387; 2.56	217; 0.21	**<0.001**
14	256,638; 2.38	263; 0.25	**<0.001**
15	194,409; 1.80	54; 0.052	**<0.001**
16	211; 0.0020	0; 0	0.29
17	144,443; 1.34	26; 0.025	**<0.001**
18	169,252; 1.57	114; 0.11	**<0.001**
19	134,163; 1.24	228; 0.22	**<0.001**
20	216,727; 2.01	345; 0.33	**<0.001**
21	73,037; 0.68	67; 0.064	**<0.001**
22	67,596; 0.63	89; 0.086	**<0.001**
23	88,819; 0.82	19; 0.018	**<0.001**
24	103,538; 0.96	62; 0.060	**<0.001**
25	35,423; 0.33	62; 0.060	**<0.001**
26	98,359; 0.91	126; 0.12	**<0.001**
27	73,537; 0.68	130; 0.12	**<0.001**
28	73,629; 0.68	78; 0.075	**<0.001**
MT	54; 0.00050	37; 0.036	**<0.001**
Z	266,087; 2.47	17,646; 16.96	**<0.001**

Table 2: Chromosomes with a significantly higher than expected proportion of outlier loci (*Outlier SNPs*) given the percentage of loci filtered by linkage disequilibrium (*SNPs Filtered by LD*). We ran two-sided binomial tests in R using "binom.test" and adjusted p-values using a false discovery rate correction (method = "fdr" in "p.adjust"). Chromosomes with an overrepresentation of outlier loci are highlighted green.

Geographic Spread of the Hybrid Zone

We used the first principal component (PC1), which accounts for 14.32% of the variation in the filtered SNP dataset (Figure 2), to determine the change in allelic frequencies across geographic distance (Figure 3). The low estimate for the center of the geographic cline was a latitudinal distance of 1149.99 +- 0.000029 km from LSU (0 km), and the high estimate was 1156.29 +- 0.00016 km. The low estimate for the width of the hybrid zone was 23.61 +- 0.00012

km, and the high estimate was 38.30 +- 0.00041 km. These estimates place the center of the hybrid zone at a latitude around Jacobsburg State Park (1154 km), and the width of the hybrid zone between East Carolina University (580 km) and Ithaca (1337 km).

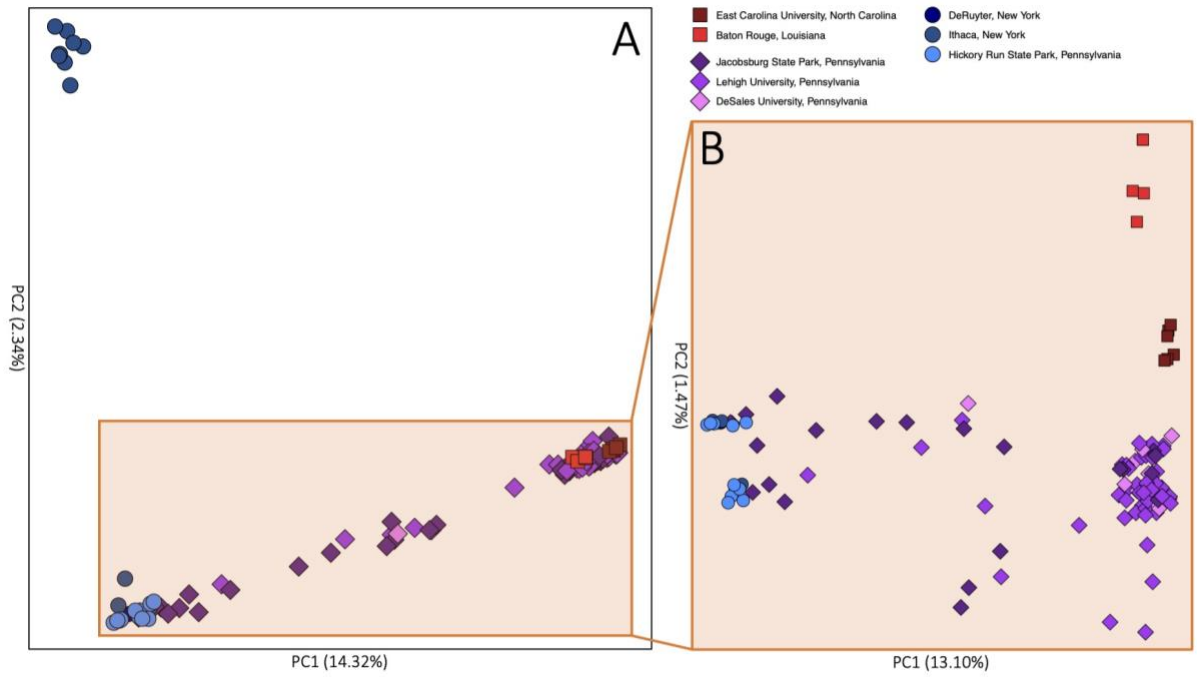

Figure 2: Principal component analysis generated from 16,285,686 filtered SNPs (**A**). To highlight the difference between allopatric Carolina Chickadee (red squares) and sympatric (purple diamonds) populations (**B**), eight individuals were dropped from DeRuyter, New York (BCCH1-8), one individual from Lehigh University (E456-3), and one individual from Jacobsburg State Park (E451). E456-3 and E451 were dropped because they became isolated from the rest of the samples after dropping BCCH1-8, suggesting E456-3 and E451 may be related.

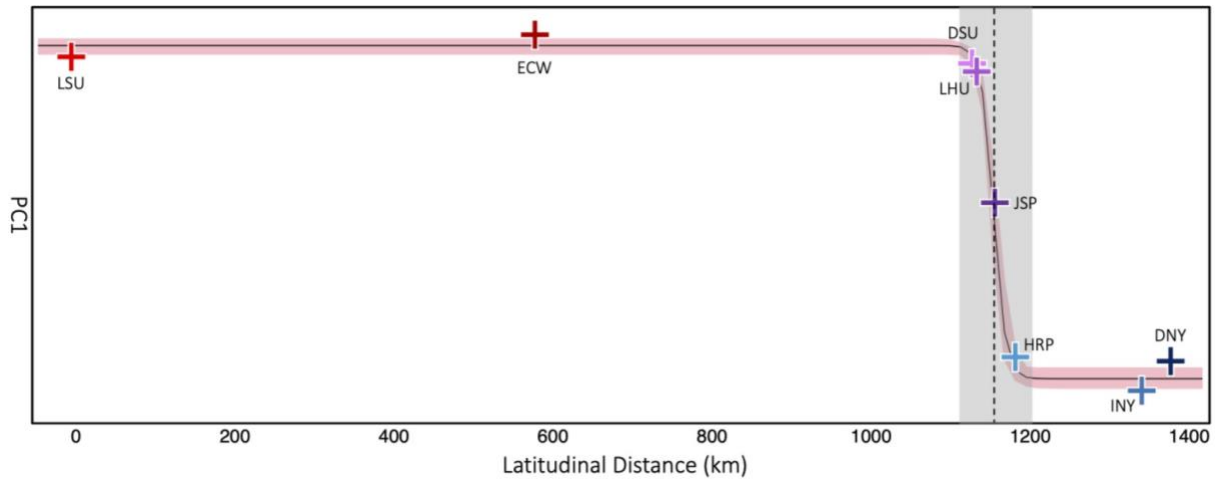

Figure 3: Geographic cline generated from principal component one (Figure 2), which represents 14.32% of the variation in the genomic data. Colored crosses represent the location of populations along the latitudinal transect (Red = allopatric Carolina Chickadee populations, Purple = sympatric populations, Blue = allopatric Black-capped Chickadee populations). The black solid line represents the maximum likelihood cline for the best "hzar" model, and the pink shading around the geographic cline is the 95% credible cline region. The dashed black line and shaded grey region represent the clinal center and width of the hybrid zone, respectively (center: 1149.99 – 1156.29 km; width: 23.61 – 38.30 km).

Hybrid Index and Population Structure

We estimated hybrid index and admixture to determine genomic differences at the population level. We calculated hybrid index in "gghybrid" using 104,016 outlier loci and admixture in ADMIXTURE using 16,285,686 filtered SNPs, where hybrid index was estimated using Bayesian inference and ADMIXTURE implemented a maximum likelihood method. For both hybrid index and admixture, individuals with a value of 1 had a Carolina Chickadee-like genome (i.e., Carolina Chickadee ancestry) while individuals with a value of 0 had a Black-capped Chickadee-like genome (i.e., Black-capped Chickadee ancestry; Figure 4). We calculated average hybrid index and admixture for each population, and determined that hybrid index and admixture were significantly different for each population (Wilcoxon Test; Table 3).

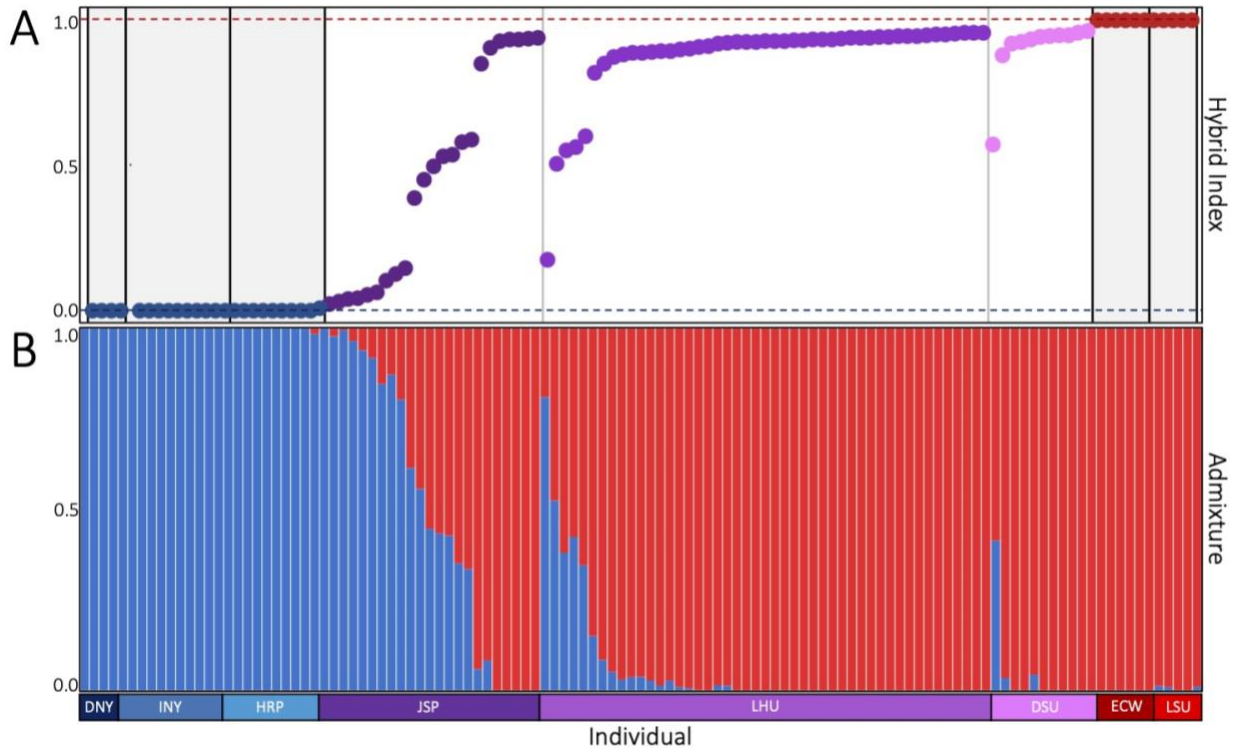

Figure 4: Hybrid index values for each individual (**A**). Populations are divided by horizontal black bars. Blue and red points inside grey bars represent allopatric Black-capped and Carolina Chickadees, respectively. Purple points inside white bars represent sympatric chickadees. ADMIXTURE plot with two populations (K), where Black-capped Chickadee ancestry is blue and Carolina Chickadee ancestry is red (**B**). Individuals are ordered by population and populations are order by latitude. Red sections along the population bar represent the allopatric Carolina Chickadee populations, purple sections the sympatric populations, and blue sections the allopatric Black-capped Chickadee populations.

Population	Individuals	Hybrid Index		Admixture		P-Value
		Average	Variance	Average	Variance	
LSU	5	1	0	0.99	0.000033	**0.0073**
ECW	6	1	0	1	0	**0.0013**
DSU	11	0.90	0.013	0.96	0.015	**<0.001**
LHU	47	0.87	0.022	0.94	0.027	**<0.001**
JSP	23	0.46	0.13	0.49	0.15	0.61
HRP	10	0.00090	0.0000080	0.0016	0.000023	**<0.001**
INY	11	0	0	0.000010	0	**<0.001**
DNY	4	0	0	0.000010	0	**0.013**

Table 3: Average and variance for hybrid index and admixture for each population. Hybrid index and admixture of 1 indicates individuals in the population have a Carolina Chickadee-like

genome, while values of 0 indicate individuals in the population have a Black-capped Chickadee-like genome. Admixture was calculated using maximum likelihood of 16,285,686 filtered SNPs, and hybrid index was calculated using Bayesian inference of 104,016 outlier SNPs. Red shading corresponds to allopatric Carolina Chickadee populations, purple shading to sympatric populations, and blue shading to allopatric Black-capped Chickadee populations.

Using a non-parametric Wilcoxon test, we found that hybrid index and admixture were significantly different between allopatric Black-capped Chickadee, allopatric Carolina Chickadee, and sympatric populations, but did not vary between populations within these categories. For allopatric Black-capped Chickadee populations, hybrid index and admixture were significantly different from allopatric Carolina Chickadee and sympatric populations ($P < 0.05$), but did not vary between allopatric Black-capped Chickadee populations (Table 4). For allopatric Carolina Chickadee populations, hybrid index was significantly different from all allopatric Black-capped Chickadee and sympatric populations ($P < 0.01$), whereas admixture was significantly different from all populations ($P < 0.05$) except DeSales University and Lehigh University (DSU | LSU: $P = 0.65$; DSU | ECW: $P = 0.24$; LHU | LSU: 1; LHU | ECW: 0.086). There were no significant differences in hybrid index or admixture between allopatric Carolina Chickadee populations. Finally, for sympatric populations, there was no significant difference in hybrid index or admixture between DeSales University and Lehigh University (HI: 0.21; admixture: 0.62). All other comparisons within the sympatric populations were significant ($P < 0.01$; Table 4). Broadly, we found that individuals in the allopatric Black-capped Chickadee populations had more Black-capped Chickadee-like genomes (Population: HI | admixture; HRP: 0.00090 | 0.0016; INY: 0 | 0.000010; DNY: 0 | 0.000010), individuals in the allopatric Carolina Chickadee populations had more Carolina Chickadee-like genomes (LSU: 1 | 0.99; ECW: 1 | 1), and sympatric populations had intermediate genomes: DeSales University and Lehigh University

were the southern sympatric populations and had more Carolina Chickadee-like genomes (DSU: 0.90 | 0.96; LHU: 0.87 | 0.94), and Jacobsburg State Park was the northern sympatric population and had more Black-capped Chickadee-like genomes (JSP: 0.46 | 0.49).

	LSU	*ECW*	*DSU*	*LHU*	*JSP*	*HRP*	*INY*	*DNY*	
LSU		*	**0.0027**	**<0.001**	**0.0011**	**<0.001**	**<0.001**	**0.0088**	Hybrid Index
ECW	0.068		**0.0014**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.0054**	
DSU	0.65	0.24		0.21	**<0.001**	**<0.001**	**<0.001**	**0.0059**	
LHU	1	0.086	0.62		**<0.001**	**<0.001**	**<0.001**	**0.0016**	
JSP	**0.037**	**0.0054**	**0.0023**	**<0.001**		**<0.001**	**<0.001**	**0.0027**	
HRP	**0.0024**	**0.0011**	**<0.001**	**<0.001**	**<0.001**		0.41	0.68	
INY	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.19		*	
DNY	**0.023**	**0.0067**	**0.0042**	**<0.001**	**0.0053**	0.49	*		
	Admixture								

Table 4: We performed a two-sided, non-parametric Wilcoxon test ("wilcox.test") to compare the means between each population for hybrid index and admixture, and corrected p-values using a false discovery rate (method = "fdr" in "p.adjust") in R. Admixture was calculated using maximum likelihood of 16,285,686 filtered SNPs, and hybrid index was calculated using Bayesian inference of 104,016 outlier SNPs. Red shading corresponds to allopatric Carolina Chickadee populations, purple shading to sympatric populations, and blue shading to allopatric Black-capped Chickadee populations.

* Due to zero variation within and between either sample, we could not compute a non-parametric Wilcoxon test. Therefore, these comparisons are considered insignificant because we could not reject the null hypothesis.

Genomic Cline Analysis

We identified 20,600 significant SNPs (i.e., $P < 0.05$) from all chromosomes of the 104,016 outlier loci. The significant genomic clines were primarily comprised of chromosomes 1 (20.63%), Z (20.04%), 1A (19.46%), 3 (17.26%), 5 (7.55%) and 2 (6.07%). The remainder of the chromosomes made up 9.00% of the significant SNPs. Among the significant genomic clines, we identified five general cline shapes that were first described in Fitzpatrick (2013): (1)

environmental selection (i.e., changes in genotype frequency are consistent with changes in environmental variables), (2) heterozygote advantage, (3) heterozygote disadvantage, (4) strong directional selection for Carolina Chickadee loci, and (5) strong directional selection for Black-capped Chickadee loci (Figure 5; Fitzpatrick 2013). Among the six chromosomes that account for ~91% of the significant genomic clines, we found patterns of introgression on all chromosomes except heterozygote disadvantage on chromosomes 2, 5 and Z (Figure 6B). Patterns of environmental variation, heterozygote advantage and heterozygote disadvantage were particularly apparent on all chromosomes, chromosome Z and chromosome 3, respectively (Figure 6A).

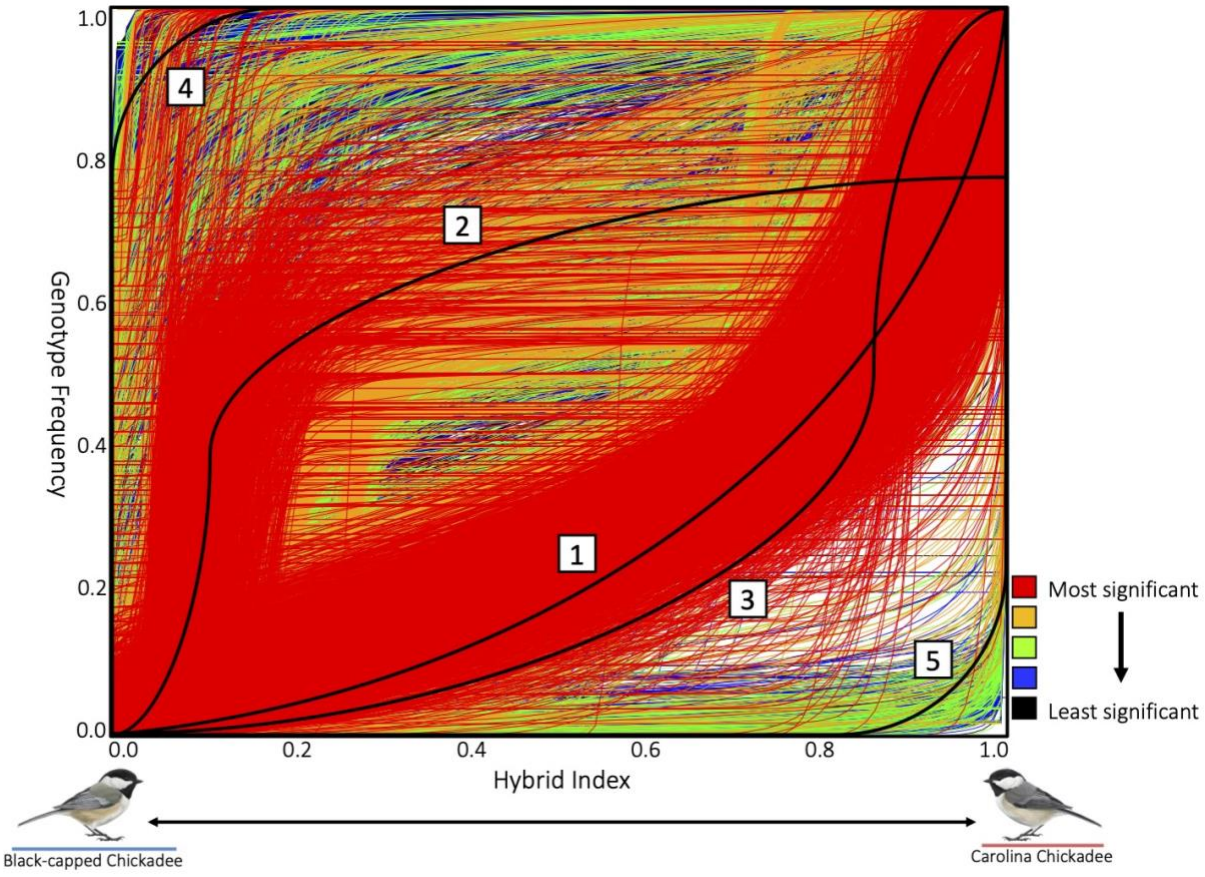

Figure 5: Of the 104,016 outlier SNPs detected by "OutFLANK", we plotted genomic clines for the 20,600 significant loci identified using "gghybrid" ($P < 0.05$). We assigned loci to equal-

sized bins by p-value to create a heatmap, where red lines represent the most significant loci. Colors are layered such that the more significant SNPs are closer to the foreground. The horizontal axis represents hybrid index where Black-capped Chickadees = 0 and Carolina Chickadees = 1. General cline shapes (i.e., patterns of introgression) reported in Fitzpatrick (2013) are depicted by solid black lines and a corresponding number: (1) environmental selection, (2) heterozygote advantage, (3) heterozygote disadvantage, (4) strong directional selection for Carolina Chickadee loci, and (5) strong directional selection for Black-capped Chickadee loci. The dashed, black line represents neutral introgression.

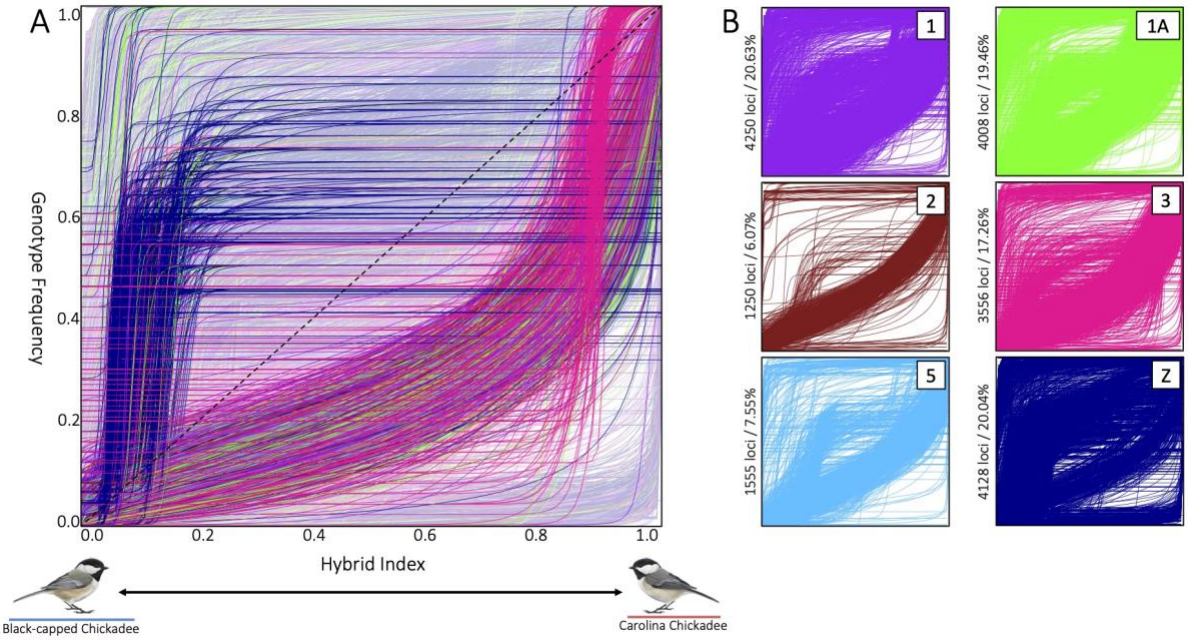

Figure 6: Genomic clines for all of the significant outlier loci (**A**). Clines in the foreground represent the 10% most significant outlier SNPs (2,060). The dashed, black line represents neutral introgression. The horizontal axis represents hybrid index where Black-capped Chickadees = 0 and Carolina Chickadees = 1. Significant outlier loci for the six chromosomes that make up ~91% of the loci (chromosome 1, 1A, 2, 3, 5 and Z; **B**). Chromosome number is indicated in the top right corner of each plot.

Gene Ontology Analysis

Of the 104,016 outlier loci, we were only able to manually annotate 34,949 SNPs (33.60%). Of these annotated loci, 13,210 were categorized as a transcript (i.e., within 5 Kb from the gene location), and 21,739 were categorized as intergenic (i.e., greater than 5 Kb from the

gene location). After manually annotating the outlier loci, we identified 2,058 unique genes for gene ontology analysis—where 1,751 genes came from transcripts, and 930 genes came from intergenic regions. Using the PANTHER Classification System, we identified 44 significantly enriched biological processes (i.e., overrepresented biological processes based on the reference list; Table 5). These biological processes largely consist of: DNA repair, cell differentiation, cellular localization, anatomical structure development, negative regulation of cellular process, transcription by RNA polymerase II, regulation of cellular macromolecule biosynthetic process, regulation of nitrogen compound metabolic process, regulation of primary metabolic process, regulation of gene expression and organelle organization. While most of the significantly enriched biological processes were associated with genes identified by intergenic loci, a number of these processes were associated with genes identified by transcripts: cell differentiation, cellular localization, organelle organization, regulation of cellular process (i.e., negative regulation of cellular process), and nitrogen compound metabolic process, primary metabolic process and cellular metabolic process (i.e., DNA repair). We did not find any underrepresented biological processes associated with either intergenic regions or transcripts.

Biological Process	Parent Biological Processes	Group ID
DNA repair (GO:0006281)	cellular response to DNA damage stimulus	GO:0006974
	response to stimulus	GO:0050896
	cellular response to stimulus	GO:0051716
	cellular process	GO:0009987
	cellular macromolecule metabolic process	GO:0044260
	cellular metabolic process	GO:0044237
	metabolic process	GO:0008152
	macromolecule metabolic process	GO:0043170
	organic substance metabolic process	GO:0071704
	nucleic acid metabolic process	GO:0090304

	nucleobase-containing compound metabolic process	GO:0006139
	heterocycle metabolic process	GO:0046483
	cellular aromatic compound metabolic process	GO:0006725
	cellular nitrogen compound metabolic process	GO:0034641
	nitrogen compound metabolic process	GO:0006807
	organic cyclic compound metabolic process	GO:1901360
	primary metabolic process	GO:0044238
cell differentiation (GO:0030154)	**cellular developmental process**	GO:0048869
	developmental process	GO:0032502
cellular localization		GO:0051641
anatomical structure development		GO:0048856
negative regulation of cellular process (GO:0048523)	**regulation of biological process**	GO:0050789
	biological regulation	GO:0065007
	regulation of cellular process	GO:0050794
transcription by RNA polymerase II (GO:0006366)	macromolecule biosynthetic process	GO:0009059
	cellular macromolecule biosynthetic process	GO:0034645
	gene expression	GO:0010467
regulation of cellular macromolecule biosynthetic process (GO:2000112)	regulation of cellular biosynthetic process	GO:0031326
	regulation of biosynthetic process	GO:0009889
	regulation of metabolic process	GO:0019222
	regulation of cellular metabolic process	GO:0031323
	regulation of macromolecule biosynthetic process	GO:0010556
	regulation of macromolecule metabolic process	GO:0060255
regulation of nitrogen compound metabolic process		GO:0051171
regulation of primary metabolic process		GO:0080090
regulation of gene expression		GO:0010468
organelle organization (GO:0006996)	**cellular component organization**	GO:0016043
	cellular component organization / biogenesis	GO:0071840

Table 5: Significantly enriched (i.e., overrepresented) biological processes identified by the PANTHER Classification System. Biological processes in bold are associated with genes identified by transcripts (i.e., loci within 5 Kb of the gene location).

DISCUSSION

We used high-resolution, whole-genome data to develop a comprehensive understanding of chickadee ancestry, advance our knowledge of hybrid zone movement, identify patterns of introgression, and determine the biological processes underlying signatures of reproductive isolation across the Black-capped and Carolina Chickadee hybrid zone. Using measures of ancestry (i.e., hybrid index and admixture) and geographic clines, we determined the center of the hybrid zone to be around Jacobsburg State Park, which is 20 km farther north than the estimated hybrid zone center in 2012. Unlike Jacobsburg State Park, the other two sympatric populations sampled (i.e., DeSales University and Lehigh University), are made up of individuals with largely Carolina Chickadee ancestry. Outlier loci demonstrated patterns of under-dominant selection in chromosome 3, over-dominant selection in chromosome Z, and environmental selection across the genome, consistent with previous avian studies (Backström and Väli 2011; Hooper et al. 2019). Finally, we identified significantly enriched biological processes that are associated with genes identified by transcripts. These biological processes are primarily associated with the regulation of cellular components (i.e., specialization and differentiation, location and organization of organelles and cellular processes) and metabolic processes (i.e., nitrogen compound, primary and cellular).

Hybrid Zone Movement and Chickadee Ancestry

Determining how allelic frequencies change along a spatial gradient provides insight into the geographic center and width of a hybrid zone. When compared across multiple generations, geographic clines can indicate the direction and speed of hybrid zone movement—valuable information for understanding why and how species distributions change over time. In two

species of Crested Newt (*Triturus anatolicus* and *T. ivanbureschi*), Wielstra et al. (2017) identified genomic footprints of the western species in the present-day range of the eastern species, suggesting eastern Crested Newt range expansion has caused the western Crested Newt to recede westward. Numerous studies have documented hybrid zone movement using geographic cline analysis, and many have attributed hybrid zone movement to climate change (Taylor et al. 2015). In Red-naped (*Sphyrapicus nuchalis*) and Red-breasted (*S. ruber*) Sapsuckers, Billerman et al. (2016) used museum specimens and species distribution models to predict the historical, present and future parental species and hybrid distributions to determine how climate change is influencing hybrid zone movement. Likewise, in the Black-capped and Carolina Chickadee hybrid zone, previous studies have documented northward movement of the hybrid zone, and attributed this movement to climate change (Taylor et al. 2014; Wagner et al. 2020). As the northern extent of the Carolina Chickadee distribution is limited by minimum winter temperatures (Taylor et al. 2014), increases in minimum winter temperatures due to climate change enables the Carolina Chickadee to advance into the Black-capped Chickadee distribution. This northward movement of Carolina Chickadees has likely caused Black-capped Chickadees to recede north because male Carolina Chickadees are dominant to male Black-capped Chickadees (Bronson et al. 2003).

Using samples collected from 2013 – 2018, we estimated the center of the Black-capped and Carolina Chickadee hybrid zone (1149.99 – 1156.29 km) to be around Jacobsburg State Park (1154 km), and the hybrid zone to be 23.61 – 38.30 km wide from north to south. In support of Taylor et al. (2014) and Wagner et al. (2020), Jacobsburg State Park is north of the 2012 clinal center estimate—which was placed between the Nolde Forest Environmental Education Center and Hawk Mountain in Pennsylvania (Figure 2 in Wagner et al. 2020)—suggesting the hybrid

zone continued to move north from 2012 – 2018. As Jacobsburg State Park is 20 km north of the 2012 estimate, hybrid zone movement increased from ~1 km per year to ~6 km per year (Wagner et al. 2020). Rather than a dramatic increase in hybrid zone movement, this discrepancy could be due to a lack of consensus on the hybrid zone's vertical width. Using 75 loci that show clinal variation, Taylor et al. (2014) determined the width of the hybrid zone to be less than 100 km. Wagner et al. (2020), however, estimated the width of the hybrid zone to be 53.8 km in 2002 and 58.4 km in 2012, within the Taylor et al. (2014) estimate but more than 20 km wider than the estimates obtained in this study.

This discrepancy in hybrid zone width could be a result of (1) our samples were collected over a longer period of time (i.e., 6 years instead of 3 years), (2) our hybrid zone transect was ~70 km east of the transect studied in Taylor et al. (2014) and Wagner et al. (2020) in what could be a narrower section of the hybrid zone, or (3) the width of the hybrid zone fluctuates over time. As the hybrid zone is continuously moving northward, sampling across many years may distort the width of the hybrid zone, especially if populations are not sampled equally across those years (e.g., LHU was primarily sampled from 2013 – 2015 and JSP was primarily sampled from 2015 – 2018). Hybrid zone width is largely determined by dispersal ability and the strength of selection on hybrid individuals (McEntee et al. 2020), indicating that geographic features potentially limiting dispersal or variations in selection might regulate the width of the hybrid zone. Therefore, geographic variation between our transect and populations studied in Wagner et al. (2020) could cause some parts of the hybrid zone to be wider than others. Finally, fluctuations in dispersal ability and selection on hybrids could lead to the width of the hybrid zone fluctuating over relatively short time periods. This, however, seems unlikely as many hybrid zones have been characterized as temporally stable (Mettler and Spellman 2009; Wang et al. 2019). Our

study, in addition to Taylor et al. (2014) and Wagner et al. (2020), provides strong support for continuous northward movement of the Black-capped and Carolina Chickadee hybrid zone, but it is unclear how hybrid zone width and speed are changing over time.

Beyond determining the spatial extent of the hybrid zone, we used estimates of chickadee ancestry (i.e., hybrid index and admixture) to determine how genomic composition varies by geographic distance in the sampled populations. Estimates of hybrid index and admixture placed the ancestral center of the hybrid zone—where individuals have relatively equal proportions of Black-capped and Carolina Chickadee ancestry—near Jacobsburg State Park, in support of geographic cline analysis. Chickadee ancestry in the other sympatric populations (i.e., DeSales University and Lehigh University), mainly consisted of individuals with a large proportion of Carolina Chickadee ancestry (>0.85 ancestry; Table 3), suggesting these populations may be near the southern limit of the hybrid zone.

Chromosomal Patterns of Introgression

To determine the regions of the genome consistent with adaptive introgression and reproductive isolation, we compared the relative proportions of each chromosome in the filtered and outlier datasets to identify what chromosomes exhibit an overrepresentation of outlier loci (Table 2). Consistent with previous avian studies, we identified an overrepresentation of outlier loci on chromosomes 1, 1A, 3, 5, Z and the mitochondrial genome (MT). In New World "mallards" (*Anas* spp.), five species recently diverged, and chromosomes 1, 2, 3, 4, 14 and Z experienced divergent selection (Lavretsky et al. 2019). Likewise, in Darwin's finches, a shift in *Geospiza scandens* allele frequencies for chromosomes 1A, 2, 3, 5 and 8 to *G. fortis* indicated gene flow between the two species (Lamichhaney et al. 2020). Interestingly, the chromosomes

with an overrepresentation of loci (except MT) are macrochromosomes (Nie et al. 2009), despite microchromosomes having twice the gene density of macrochromosomes (Smith et al. 2000) and a higher rate of nucleotide divergence (Axelsson et al. 2005). In avian genomics, d_N/d_S ratios are higher in macrochromosomes potentially explaining this discrepancy—where a higher rate of non-synonymous mutations are being fixed by positive selection in macrochromosomes (Ellegren 2007).

While we found an overrepresentation of outliers on six chromosomes, all chromosomes except chromosome 16 contained outlier loci. Interestingly, in their 76,883 SNP dataset, Wagner et al. (2020) found variants on every chromosome except 16, suggesting there may be strong selection for chromosome 16 homogeneity in the Black-capped and Carolina Chickadee hybrid zone. Likewise, in a study using Mendelian inheritance patterns to filter spurious loci, Chen et al. (2014) identified a minimum of 33 SNPs on every chromosome except 16 in Florida Scrub-Jays (*Aphelocoma coerulescens*). In birds, chromosome 16 population homogeneity may be due to the presence of the Major Histocompatibility Complex (MHC)—where nearly all of the genes on chromosome 16 have a role in immune responses, or are homologous to genes associated with the immune system in other organisms (Miller and Taylor 2016). In genes with critical function, strong selection against deleterious mutations can lead to reduced levels of genetic polymorphisms (Hudson 1995). While the present study and previous avian research (Chen et al. 2014; Wagner et al. 2020) have documented limited polymorphisms in chromosome 16, the MHC is the most polymorphic region of the genome among vertebrates (Bjorkman and Parham 1990). For example, in the Mexican Chicken (*Gallus gallus domesticus*), chromosome 16 was the only chromosome where the majority of the length (Table 2 in Gorla et al. 2017) consisted of copy number variations (i.e., number of copies for a specific gene varies among individuals in a

population). Further research should examine why chromosome 16 shows limited population variation in the Black-capped and Carolina Chickadee hybrid zone.

Using the outlier dataset, we determined the general patterns of introgression across the Black-capped and Carolina Chickadee hybrid zone, and associated those patterns of introgression with chromosomes to determine what regions of the genome show various patterns of selection. We identified five general cline shapes (i.e., patterns of introgression) that have previously been related to specific types of selection: (1) environmental selection—where environmental variables select for loci of Black-capped Chickadee ancestry, (2) heterozygote advantage—where the chance a given locus is of Black-capped or Carolina Chickadee ancestry is relatively equal, (3) heterozygote disadvantage—where a given locus is either likely to be of Black-capped (hybrid index < ~0.85) or Carolina Chickadee (hybrid index > ~0.85) ancestry, (4) strong directional selection for Carolina Chickadee loci—where the probability a given locus is of Carolina Chickadee ancestry is very high for all individuals, and (5) strong directional selection for Black-capped Chickadee loci—where the probability a given locus is of Carolina Chickadee ancestry is very low for all individuals. Beyond identifying the genomic patterns of introgression, we determined what chromosomes are associated with each type of selection. Using the six chromosomes that make up ~91% of the significant loci (Figure 6B), chromosomes 1, 1A, 2, 3 and 5 primarily showed patterns of environmental selection, chromosomes 1, 1A and Z showed strong patterns of directional selection for Carolina Chickadee loci, and chromosome Z showed a strong pattern of directional selection for the Black-capped Chickadee loci. Most notably, chromosome Z primarily showed a pattern of heterozygote advantage, and chromosome 3 primarily showed a pattern of heterozygote disadvantage (Figure 6A). In birds, numerous studies have demonstrated the importance of chromosome 3 and chromosome Z in regulating

reproductive isolation and adaptive introgression in hybrid zones (Backström and Väli 2011; Hooper et al. 2019). In regions of range overlap, *Ficedula* flycatchers exhibit a displacement in plumage characteristics (Borge et al. 2005), and plumage characteristics are linked to the Z chromosome and under selection (Sætre et al. 2003). This, and other avian studies, suggests the Z chromosome is impermeable to introgression (Rheindt and Edwards 2011)—consistent with our finding that, for many of the significant loci on the Z chromosome, there is a roughly equal probability of the loci being Black-capped or Carolina Chickadee ancestry for individuals with a hybrid index above ~0.1 (Figure 6A). Evidence of reduced introgression in the Z chromosome supports Haldane's Rule—where heterogametic hybrid offspring are expected to have reduced fitness or fertility (Rheindt and Edwards 2011). In birds, the recessive alleles that decrease hybrid fitness are more likely to be expressed in females (i.e., the heterogametic sex), leading to reduced fitness or fertility. Additionally, as mitochondrial DNA is only transmitted by females, an overrepresentation of outliers on the mitochondrial genome and patterns of reduced introgression also support Haldane's Rule (Supplementary Figure 1). Broadly, reduced introgression on the Z and mitochondrial genome reinforce the presence of postzygotic reproductive isolating barriers, particularly in female hybrids.

Enriched Biological Processes and Cognition

To determine whether cognitive breakdown in hybrid offspring is a reproductive isolating barrier in the Black-capped and Carolina Chickadee hybrid zone, we identified overrepresented biological processes by documenting the genes associated with outlier loci. Among the significantly enriched biological processes identified in this study, previous studies have shown that the biological processes we identified using transcripts are associated with cognition and

memory function. For example, epigenetic mechanisms that play a critical role in cell differentiation and development—committing an unspecialized cell to a specific fate and subsequent maturation (GO:0030154)—are also important for mediating synaptic plasticity, learning and memory (Day and Sweatt 2011). Additionally, the biochemical pathways that regulate these epigenetic mechanisms play an important role in unlearned and learned responses to food, mating and social behavior (Day and Sweatt 2011). Likewise, in humans, dystrobrevin binding protein-1 (dysbindin-1) regulates the cellular localization—the transport or maintenance of protein complexes, organelles or other substances to a certain location in the cell (GO:0051641)—of D_3 and D_2 receptors, and is associated with the development of central nervous system (CNS) disorders due to regulation of synaptic plasticity, cognition and neurotransmission (Schmieg et al. 2016). While an association between enriched biological processes and cognition provides insight into the role of cognition as a reproductive isolating barrier, it is also important to acknowledge the genes that regulate those biological processes because many genes known to regulate a certain process were not identified by outlier loci in this study.

In the klotho (KLOT) gene and the p21-activated kinase 1 (PAK1) enzyme—identified by outliers in our study—a complex relationship with cognition and survival suggests a role for cognition as a reproductive isolating barrier. In mice, a deficiency in KLOT—a gene that is highly expressed in the brain and is associated with the suppression of aging—causes cognitive impairment, suggesting this gene has a critical role in cognitive abilities (Nagai et al. 2003). Additionally, hepatoma patients with high KLOT expression had a lower survival rate compared to patients with a low KLOT expression (Chen et al. 2013). As an overexpression of KLOT leads to an overexpression of PAK1 (Chen et al. 2013), and PAK1 was associated with almost all of

the enriched biological processes (i.e., cell differentiation, organelle organization, regulation of cellular processes, nitrogen compound metabolic processes, primary metabolic processes and cellular metabolic processes), it is possible that KLOT and PAK1 have a role in causing hybrid cognitive deficiencies or reduced survival of Black-capped and Carolina Chickadee hybrids. While many of the enriched biological processes identified in this study are regulated by genes that are associated with cognition, the expression of these genes may improve cognitive abilities (Nagai et al. 2003; Schmieg et al. 2016). Genetic incompatibilities in admixed offspring can explain how these enriched biological processes, many of which are associated with genes known to improve cognitive abilities, can lead to cognitive breakdown in hybrid individuals (Orr 1996).

Conclusion

To determine whether cognition may be a postzygotic reproductive isolating barrier in Black-capped and Carolina Chickadees, we used high-resolution, whole-genome data to develop a comprehensive understanding of chickadee ancestry, hybrid zone movement, patterns of introgression, and the biological processes underlying signatures of reproductive isolation across the Black-capped and Carolina Chickadee hybrid zone. Measures of chickadee ancestry and geographic cline analysis supported previous indications of northward hybrid zone movement, probably driven by climate change, and placed the most recent center of the hybrid zone around Jacobsburg State Park, Pennsylvania. Likewise, genomic cline analysis suggested reduced introgression of the Z chromosome, in support of Haldane's Rule, and under-dominant selection on chromosome 3. Overall, signatures of reduced introgression and enriched biological processes

associated with cognition suggest that postzygotic reproductive isolation between Black-capped and Carolina Chickadees is strong, and is possibly driven by cognitive breakdown in hybrids.

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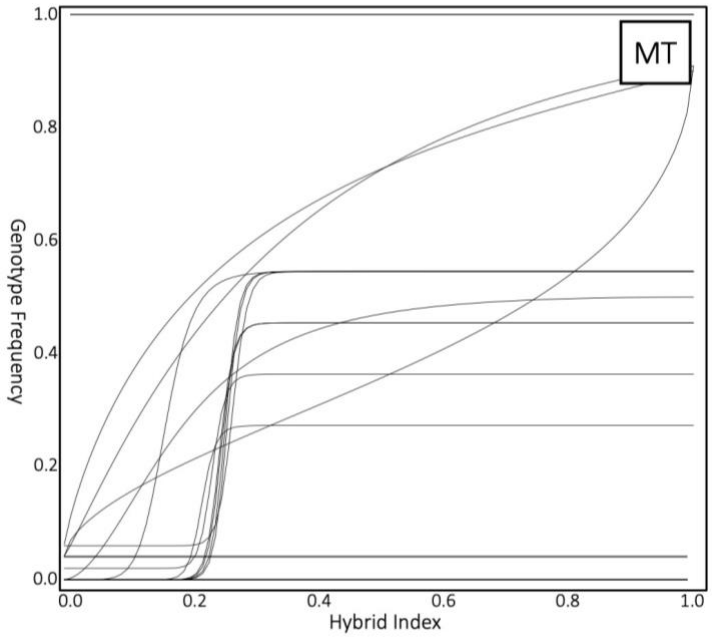
APPENDIX



Supplementary Figure 1: Genomic cline for the significant loci from the mitochondrial genome. The horizontal axis represents hybrid index where Black-capped Chickadees = 0 and Carolina Chickadees = 1.