doi:10.1111/evo.12510

Spatiotemporally consistent genomic signatures of reproductive isolation in a moving hybrid zone

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Received November 1, 2013
Accepted July 28, 2014

Studies of hybrid zone dynamics often investigate a single sampling period and draw conclusions from that temporal snapshot. Stochasticity can, however, result in loci with spurious outlier patterns, which is exacerbated by limited temporal or geographic sampling. Comparing admixed populations from different geographic regions is one way to detect repeatedly divergent genomic regions potentially involved in reproductive isolation. Temporal comparisons also allow us to control partially for the role of stochasticity, but the power of temporal sampling has not yet been adequately explored. In North America, black-capped (*Poecile atricapillus*) and Carolina (*P. carolinensis*) chickadees hybridize in a contact zone extending from New Jersey to Kansas. The hybrid zone is likely maintained by strong intrinsic selection against hybrids, and it is moving north. We used a reduced representation genomic approach and temporally spaced sampling—two samples of ~80 individuals separated by a decade—to determine the pattern and consistency of selection and genomic introgression in the chickadee hybrid zone. We report consistently low introgression for highly divergent loci between *P. atricapillus* and *P. carolinensis* in this moving hybrid zone. This is strong evidence that these loci may be linked to genomic regions involved in reproductive isolation between chickadees.

KEY WORDS: **Black-capped chickadee, Carolina chickadee, genomic cline, genotyping by sequencing (GBS), introgression.**

New species can arise when genetic differentiation leads to reproductive isolation between diverging lineages. Determining the genetic architecture of divergence and reproductive isolation is key to understanding the speciation process and we focus on genetic divergence in this study. Recent studies in natural populations have found substantial support for the genic or semipermeable genome view of speciation, which posits that divergence and isolation are often properties of individual genetic loci—and potentially small regions surrounding them—rather than large blocks of the genome (Key 1968; Bazykin 1969; Barton and Hewitt 1981; Rand and Harrison 1989; Harrison and Rand 1989; Harrison 1990; Wu 2001; Nosil and Feder 2012). When approached with powerful new sequencing technologies and methods of data analyses, the investigation of naturally hybridizing populations is providing novel insights into the genetic architecture of adaptive divergence and reproductive isolation (Payseur et al. 2004; Teeter et al. 2010; Gompert and Buerkle 2011a,b; Gompert et al. 2012a,b; Janousek et al. 2012; Kingston et al. 2012; Luttikhuizen et al. 2012; Carneiro et al. 2013; Larson et al. 2013, 2014; Parchman et al. 2013).

Hybrid zones—geographic regions where genetically distinct groups of individuals interact and produce offspring of mixed

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

ancestry—have long been recognized as tractable windows on the evolutionary process (Hewitt 1988; Barton and Hewitt 1989; Harrison 1990, 1993; Virdee and Hewitt 1994; Hewitt 2001; Grant et al. 2005; Teeter et al. 2010; Larson et al. 2013, 2014). The interaction of differentiated genomes in hybrid individuals in nature, and the subsequent endogenous and/or exogenous selection on new genomic combinations, provides an opportunity to explore the evolution of reproductive barriers and, ultimately, speciation in natural settings (Endler 1977; Hewitt 1988; Barton and Hewitt 1989; Harrison 1993; Buerkle and Lexer 2008; Gompert et al. 2012b). Genetic regions of exceptional differentiation between hybridizing species, and those that exhibit reduced introgression, may contain loci that contribute directly to reproductive isolation (Endler 1977; Hewitt 1988; Barton and Hewitt 1989; Harrison 1990). Quantifying admixture and introgression in hybrid zones can lead to the identification of genomic regions involved in hybrid fitness and assortative mating (Szymura and Barton 1986; Gompert et al. 2012a,b), and studies of hybridizing species have found that these regions are frequently located on sex chromosomes (Carling et al. 2010; Teeter et al. 2010; Ellegren et al. 2012; Taylor et al. 2012). Here, introgression is defined as the movement of alleles between gene pools via admixture.

In all hybrid zones, recombination in admixed individuals creates novel combinations of parental genotypes, breaking down genomic parental ancestry blocks (Barton and Hewitt 1985, 1989; Buerkle and Lexer 2008). The behavior of divergent genetic regions during hybridization—the extent of introgression—relies upon the effect of the genetic region on hybrid fitness and the linkage of that region with other regions. If allelic variants within a genetic region cause reduced viability or fertility in hybrids, or if they contribute to assortative mating, then they should experience limited introgression (Barton and Hewitt 1989; Harrison 1993; Buerkle and Lexer 2008). Genetic regions with allelic variants that increase fitness should introgress rapidly, and neutral genetic regions (assuming they are unlinked to regions under selection) should introgress proportionally to the distance of dispersal (gene flow) out of the hybrid zone and the duration of the period of interbreeding (hybrid zone age; Barton and Hewitt 1989).

Introgression can be quantified by examining the distribution and movement of alleles in geographic space (geographic introgression; e.g., Szymura and Barton 1986; Carling et al. 2010; Teeter et al. 2010; Taylor et al. 2012) or by examining the movement of blocks of parental ancestry into different genomic backgrounds (genomic introgression; e.g., Szymura and Barton 1986; Gompert et al. 2012b; Fitzpatrick 2013; Parchman et al. 2013). Here, we focus on genomic introgression. Loci involved in reproductive isolation—those that decrease the fitness of hybrids—should exhibit reduced genomic introgression, which can manifest as loci exhibiting patterns of underdominant selection (i.e., the fitness of the heterozygous genotype is lower than either homozygous genotype). This process ultimately results in persistent correlations between parental alleles and parental genetic backgrounds for loci involved in reproductive isolation in admixed populations, but not necessarily for loci that are not involved in reproductive isolation, which may be free to introgress (Barton and Hewitt 1985, 1989; Szymura and Barton 1986; Gompert and Buerkle 2011b; Gompert et al. 2012b).

Generally, the insights we gain on the evolutionary process from the study of hybrid zones are snapshots, often at unknown positions, along the time course of speciation. An underlying assumption is that these snapshots are representative of longer term patterns of selection and introgression between the interacting species. Further, loci identified as exhibiting patterns of differentiation and introgression that differ from neutral expectations are often assumed to be involved in reproductive isolation or adaptive divergence. Stochastic processes (biological or from sampling) can, however, generate variation in genetic patterns among loci that can mimic various forms of selection, including reproductive isolation and adaptive divergence. This can confound outlier approaches (Gompert and Buerkle 2011b; Gompert et al. 2012b). Comparing locus-specific patterns of differentiation and introgression in different geographic or temporal samples (comprised different sets of admixed individuals) is one way to determine if locus-specific nonneutral patterns are the product of selection or stochastic variation. It is important to note, however, that the samples will not necessarily be evolutionarily unique and that shared population history will play a role in generating locus-specific patterns.

Only in a few cases have investigators been able to sample hybrid zones in a way that allows comparisons of their genomic dynamics across multiple time periods, and few have done so using multilocus or "genome-wide" datasets (Szymura and Barton 1986; Rand and Harrison 1989; Butlin and Ritchie 1991; Szymura and Barton 1991; Virdee and Hewitt 1994; Bridle et al. 2001; Buggs 2007; Smith et al. 2013). Recent studies of tension zones (hybrid zones primarily maintained by a balance of dispersal into the hybrid zone and intrinsic selection against hybrids; Barton and Hewitt 1985; Harrison 1993) have illustrated that selection and genomic introgression between hybridizing species can vary in space, potentially in response to environmental variation, genetic drift, and/or metapopulation dynamics (Teeter et al. 2010; Gompert and Buerkle 2011b). These factors, and potentially selection, can also vary temporally, but no comparison of selection and introgression has been conducted across temporally separated periods in the same hybrid zone. Therefore, we know little about the general consistency of genomic signatures of selection and introgression in hybrid zones over time.

Black-capped (*Poecile atricapillus*) and Carolina (*P. carolinensis*) chickadees are closely related (Gill et al. 2005; Harris

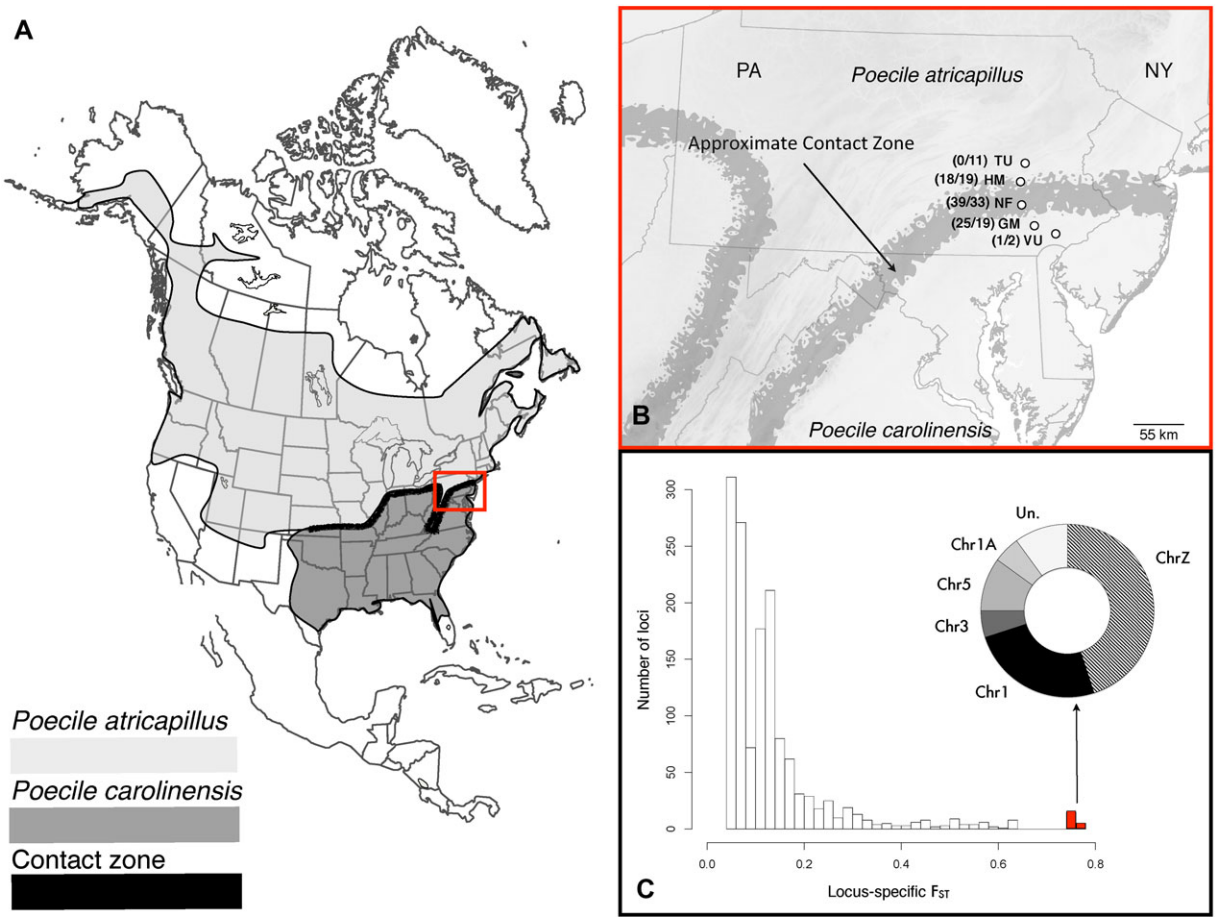

Figure 1. **Context of the study. (A) Approximate *Poecile atricapillus* and *P. carolinensis* breeding distributions in North America. Black textured line denotes approximate location of the contact zone. (B) Distribution of parental and admixed chickadee research populations in southeastern Pennsylvania. Number of samples from each location in parentheses beside site abbreviation (historical, 2001–2002; contemporary, 2011–2012). TU, Tuscarora State Park (40.80°N; −76.03°W); HM, Hawk Mountain Sanctuary (40.65°N; −76.00°W); NF, Nolde Forest Environmental Education Center (40.27°N; −75.95°W); GM, Great Marsh (40.14°N; −75.73°W); VU, Villanova University campus (40.04°N; −75.34°W). (C) Histogram of locus-specific genetic differentiation between allopatric *P. atricapillus* and *P. carolinensis* populations. Interspecific outlier loci indicated by red shading. Putative chromosomal distribution of interspecific outlier loci based on alignment of GBS tags to the *T. guttata* genome indicated in donut graph.**

et al. 2013), forest-dwelling North American passerines that hybridize in a narrow but long zone of contact that stretches from New Jersey to Kansas (Fig. 1A). Both species are important subjects for behavioral ecology studies and much is known about their basic ecology (e.g., Otter et al. 1998; Christie et al. 2004; Curry et al. 2007; Harvey and Freeberg 2007; Olson et al. 2010). Hybridization between these species has been the focus of numerous studies in various locations along their hybrid zone (Bronson 2002; Bronson et al. 2003a,b, 2005; Reudink et al. 2006, 2007; Davidson et al. 2013), and historical introgression during periods of range expansion and contraction have likely played a role in shaping genomic divergence between the species. Hybrid chickadees experience strong intrinsic selection (i.e., lower hatching success of hybrid clutches) leading some authors to suggest that the chickadee hybrid zone is a tension zone (Bronson et al. 2003b, 2005). Additionally, the hybrid zone in southeastern Pennsylvania has moved north ∼11 km over the past decade in response to climate change (Fig. 2A, B; Reudink et al 2006, 2007; Taylor et al. 2014). Hybrid zone movement in Ohio has occurred at a similar rate (Bronson et al. 2003a,b, 2005). This rate of movement closely matches the distance of natal dispersal over one year (R. Curry, unpubl. ms.).

Here, using two samples of ∼80 individuals separated by a decade, we compare patterns of genomic introgression at 1425 loci within this moving avian tension zone to gain a better understanding of the spatiotemporal consistency of selection and genomic introgression between hybridizing species. To do this we employ recently developed Bayesian genomic cline analyses, implemented in the program bgc, that determine the probability of locus-specific ancestry as a function of genome-wide admixture

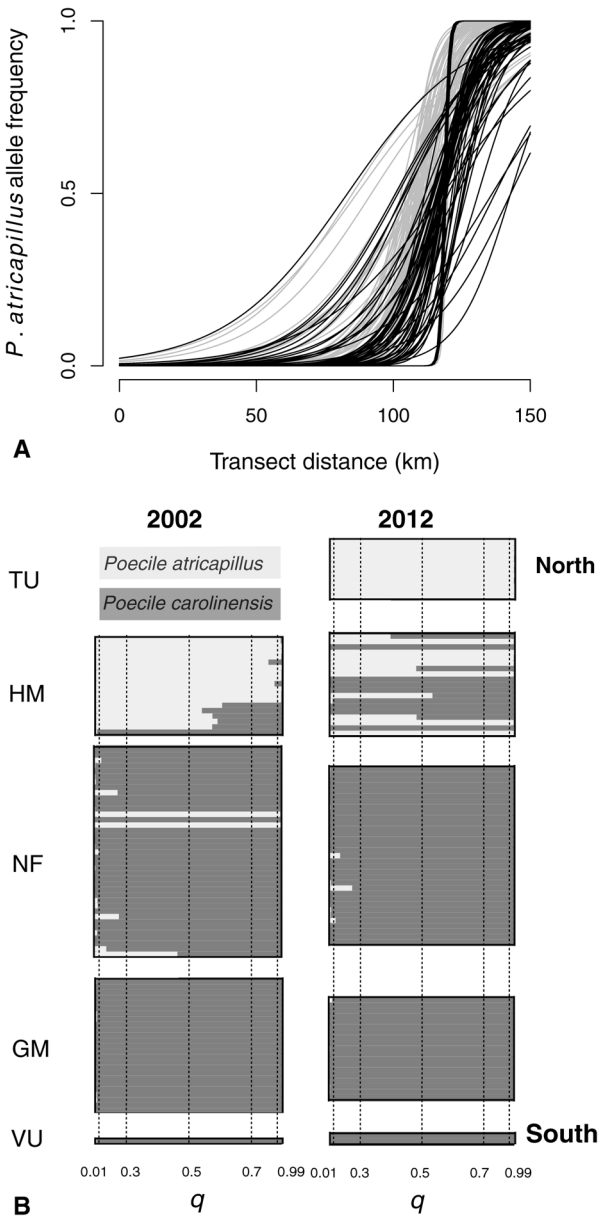

Figure 2. Genomic evidence for hybrid zone movement. Figure modified with permission from Figure 1 in Taylor et al. (2014). (A) Locus-specific geographic clines depicting *P. atricapillus* allele frequencies for 75 clinal loci. Historical samples (2000–2002) in gray, contemporary samples (2010–2012) in black. (B) Bayesian assignment probabilities from STRUCTURE for *P. atricapillus* (light gray) and *P. carolinensis* (dark gray) at $K = 2$. Each horizontal line represents one individual. q = the probability of assignment to each genetic population. Dashed lines indicate threshold q values used to categorize individuals (see text). Population acronyms as in Figure 1B.

in putatively admixed individuals (detailed in methods; Gompert and Buerkle 2011a,b; Gompert et al. 2012a,b). Recent simulations from these analyses under a variety of demographic conditions show that underdominant selection (i.e., against heterozygotes) and directional selection (i.e., favoring an advantageous allele) have detectable and predictable effects on locus-specific genomic cline parameters, but that some caution should be used in the interpretations of patterns given the influence of demography on parameter estimates (Gompert and Buerkle 2011a,b; Gompert et al. 2012a,b). The locus-specific cline parameters modeled in bgc are α and β. The α parameter describes locus-specific increases or decreases in the probability of ancestry of a designated parental population from the base probability, which is predicted by the hybrid index of that individual. The β parameter describes locus-specific excesses or reductions in ancestry-based linkage disequilibrium from the base probability, again predicted by the hybrid index of that individual and in relation to a designated parental population (see Methods for more detailed information; Gompert and Buerkle 2011a,b; Gompert et al. 2012a,b). Positive nonzero estimates of the genomic cline parameter β are expected with underdominant selection when selection against admixed individuals is strong and gene flow is high, with assortative mating or selection that prevents the formation of admixed individuals, or when population structure exists within the hybrid zone; however, when selection is weak and gene flow is low, underdominant selection is more likely to affect the genomic cline parameter α (Gompert et al. 2012a). Significantly positive or negative estimates of the genomic cline parameter α are expected with directional selection under a variety of demographic scenarios, and are indicative of adaptive divergence when correlated with elevated estimates of F_{ST} (Gompert et al. 2011b). Simulations using bgc have shown that stochastic drift is more likely to affect the genomic cline parameter α than β, indicating that drift is more likely to mimic adaptive introgression than reproductive isolation (Gompert and Buerkle 2011b).

Here, we test the hypothesis that the genomic signature of reproductive isolation between hybridizing chickadees will show that divergent loci (those potentially involved in reproductive isolation) are affected by spatiotemporally consistent underdominant selection. Results from the aforementioned simulation studies allow us to make specific predictions about locus-specific patterns of differentiation (F_{ST}) and introgression (bgc parameters) in the chickadee hybrid zone under the hypothesized scenario. Intrinsic selection against chickadee hybrids is strong (Bronson et al. 2005a) and dispersal into the contact zone is high (involving northward expansion of *P. carolinensis*; Taylor et al. 2014). Knowing this, we predict that loci involved in reproductive isolation (i.e., affected by underdominant selection) should exhibit both high levels of differentiation (F_{ST}) between the species and positive nonzero estimates of the genomic cline parameter β (from bgc). This pattern should be spatiotemporally consistent if it is not the result of stochastic variation. Alternatively, if reproductive isolation between hybridizing chickadees were weak, we would predict that highly divergent loci (from F_{ST}) would exhibit estimates of the genomic cline parameter β that are not significantly

different from zero (from bgc), but may be associated with significantly values of the genomic cline parameter α.

Methods

SAMPLE COLLECTION AND PREPARATION

We collected blood samples from locally resident breeding chickadees as described in Reudink et al. (2006) as part of a long-term study of chickadee hybridization in southeastern Pennsylvania. Samples included in this study were selected from two time periods that were 10 years apart (2000–2002 and 2010–2012) and are the same samples analyzed in Taylor et al. (2014). We chose individuals with the goal of having as even sampling as possible across available sampling locations in each time period, but otherwise arbitrarily. For the earlier time period (2000–2002), samples were available from four locations (Fig. 1B). An additional location (Tuscarora State Park; hereafter, TU; Fig. 1B) was added to the sampling regime in 2006 to ensure that one of the sampling sites remained ahead of the moving hybrid zone (Curry 2012a,b). Samples from TU, which is in Barnesville (Rush and Ryan Townships), Schuylkill County, Pennsylvania, came from 50 artificial nest snags that we installed beginning in 2006. All resident breeding chickadees from this site that we have tested to date have the most common *P. atricapillus* mtDNA haplotype for cytochrome *b* (using methods described in Reudink et al. 2007), and results from STRUCTURE indicate that this site is composed of "genetically pure" *P. atricapillus* (Fig. 2B). This site makes up the northern limit of the 2010–2012 transect, which has five geographic sampling points that span the hybrid zone (Fig. 1B).

We extracted DNA from 190 samples using Qiagen DNeasy extraction kits (Qiagen, Valencia, CA) and standard blood extraction protocols, eluted the DNA in water, and concentrated it using a vacuum centrifuge. Original blood samples are archived at Villanova University (Villanova, PA). DNA extractions are archived at the Cornell Lab of Ornithology (Ithaca, NY).

GENOMIC DATA GENERATION

The genotyping-by-sequencing (GBS) libraries were prepared at the Cornell Institute for Genomic Diversity (IGD), following the protocols of Elshire et al. (2011), using the enzyme PstI for digestion. In short, plated DNA was digested with PstI following which two adaptors were added to each well: one barcode adaptor (containing a unique barcode, Illumina sequencing primer 1 [P1], and a sticky end) and one common adaptor (containing Illumina sequencing primer 2 [P2] and a sticky end), resulting in a library of fragments for each individual, barcoded with one of 96 unique barcodes. Following adaptor ligation, samples (individually barcoded libraries) within a single plate were pooled to create two GBS libraries, each containing 95 uniquely barcoded individuals plus one negative control. These GBS libraries were cleaned and then subjected to polymerase chain reaction with two primers, one containing Illumina P1 and one containing P2. Both primers also contained a sequence complementary to the oligonucleotides that coat the Illumina flow cell. Each GBS library was then sequenced on one lane of an Illumina HiSeq 2000 (100 base pair [bp], single-end) at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY).

GBS data were processed following White et al. (2013) and as described in Taylor et al. (2014). Illumina data files were filtered to individual genotypes using the Universal Network Enabled Analysis Kit (UNEAK) pipeline (Lu et al. 2013), which is available as part of TASSEL 3.0 (Bradbury et al. 2007). The UNEAK pipeline retains reads with a barcode, a restriction enzyme cut site, and no ambiguous bases (Ns) in the 64 bp of the sequence following the individual barcode, and trims all acceptable reads to 64 bp after the barcode. The pipeline then clusters reads into tags (groups of identical reads) and stores counts of the tags present in each barcoded individual. All unique tags are then merged, and their counts in the whole sample of individuals are stored. The pipeline then performs a pairwise alignment of tags. Tag pairs with 1 bp mismatches are considered as candidate single nucleotide polymorphisms (SNPs). Reciprocal pairs of tags are retained as SNPs according to standard protocols of the Cornell IGD with a user-specified error tolerance rate (0.03 here). After SNP identification, counts of each tag (or allele) are output for each locus and each individual. Following UNEAK filtering, we recalled individual genotypes using a global sequencing error rate of 0.03 and following the method detailed in Lynch (2009; GitHub: https://github.com/mgharvey/GBS_process_Tom_White). Genotype likelihood was calculated using a binomial sampling distribution. A genotype was called if its Akaike information criterion value (Burnham and Anderson 2002) was at least 4 lower than the next best genotype. If this condition was not met, the genotype was coded as "missing." We discarded loci with a mean observed heterozygosity greater than 0.75 as a way to filter out potential paralogs. Alternative trials discarding loci with heterozygosity values between 0.5 and 1 did not notably change our results (data not shown). The final dataset consisted of 1425 loci that could be called confidently in at least 80% of 167 individuals (23 of the original 190 sequenced individuals were discarded due to generally low coverage), which were evenly distributed across historical ($n = 83$) and contemporary ($n = 84$) datasets (Fig. 1B). The loci were primarily intergenic (determined from BLAST and Bowtie analyses detailed below) and were distributed on chromosomes 1–28 (except chromosome 16) and the Z chromosome (assuming synteny with zebra finch, *Taeniopygia guttata*; see Results).

Table 1. Interspecific F_{ST} outlier loci with putative chromosomal position and locus-specific F_{ST} estimates calculated from allopatric *P. atricapillus* (TU 2010–2012) and *P. carolinensis* (GM 2000–2002).

Locus ID	Position	Female	Male	Combined
2402	Z	0.69	0.77	0.75
6773	Z	0.70	0.78	0.77
26109	Un	0.70	0.75	0.75
40606	Z	0.68	0.79	0.76
41934	Z	0.68	0.78	0.75
52178	3	0.55	0.77	0.76
60421	1	0.68	0.75	0.74
64664	Z	0.76	0.78	0.76
81898	Z	0.76	0.78	0.76
82180	Z	0.76	0.77	0.76
82750	5	0.77	0.78	0.76
87715	1	0.76	0.78	0.76
98617	Z	0.76	0.78	0.76
115173	1A	0.68	0.75	0.75
141683	1	0.76	0.77	0.76
143902	Z	0.76	0.78	0.76
143986	1	0.76	0.77	0.76
147414	5	0.76	0.77	0.76
151111	Un	0.75	0.77	0.75
154344	Z	0.76	0.77	0.76

Average interspecific F_{ST} = 0.11. Outlier loci designated as such if the F_{ST} estimate for a locus was greater than the 0.95 quantile (combined and male datasets) or 0.90 quantile (females) of the genome-wide average F_{ST}. All of the F_{ST} outlier loci in the combined, male, and female analyses exhibit patterns of genomic introgression from bgc that differ from neutral expectations.

GENOME-WIDE DIFFERENTIATION

We quantified genome-wide differentiation between chickadee species using the Bayesian implementation of the F-model described and implemented in Gompert et al. (2012b). Samples from 2000 to 2002 from GM and VU made up the pure *P. carolinensis* sample and allopatric samples from 2010 to 2012 from TU constituted the pure *P. atricapillus* group. Previous analyses with STRUCTURE indicated that these samples consisted of genetically pure *P. carolinensis* and *P. atricapillus*, respectively (Fig. 2B). As in Parchman et al. (2013), we treat this locus-specific measure of differentiation as equivalent to F_{ST} (Balding and Nichols 1995; Nicholson et al. 2002). Posterior probabilities of F_{ST} for each locus, as well as genome-wide average F_{ST}, were calculated using 30,000 Markov chain Monte Carlo (MCMC) steps, discarding the first 3000 steps as burn-in. We assessed mixing by examining the MCMC output and calculated locus-specific F_{ST} in genotype uncertainty mode (option-m) and using the logit-transformed F_{ST} model (option-l). Outlier loci were designated as such if the F_{ST} estimate for a locus was greater than the 0.95 quantile of the genome-wide average F_{ST}. This hard cutoff approach does not account for the role of demographic processes in creating spurious outliers; however, more rigorous outlier detection is beyond the scope of this article. Additionally, spurious outliers would not be expected to show spatiotemporal consistency in independently calculated genomic cline parameters from bgc, which we report (see Results). We also quantified genome-wide differentiation using sex-specific datasets. The results were congruent with the combined dataset and are presented in Table 1. See Supplemental Data for command line.

GENOMIC CLINE ANALYSES

To examine the behavior (introgression or lack thereof) of each locus relative to genomic background in putatively admixed individuals, we used the Bayesian genomic cline (bgc) model with genotype uncertainty (Gompert and Buerkle 2011b; Gompert et al. 2012b). This model relates the hybrid index of an individual (here probability of *P. carolinensis* ancestry) to the probability that a locus exhibits ancestry from parental population 1 (here parental population 1 is *P. carolinensis*; Gompert and Buerkle 2011b; Gompert et al. 2012b). The function is described by two locus-specific genomic cline parameters α (genomic cline center) and β (genomic cline rate; Gompert and Buerkle 2011b; Gompert et al. 2012b). Under the null model, the probability of a locus displaying ancestry of parental population 1 is directly predicted by the hybrid index of an individual, such that increasing hybrid indices should be reflected in increased probability of a locus displaying ancestry of parental population 1 (Gompert and

Buerkle 2011b; Gompert et al. 2012b). If the probability that a locus displays ancestry from parental population 1 is significantly different from the probability predicted by hybrid index, then the α and/or β parameters for that locus-specific genomic cline will differ significantly from the null expectation, and the confidence intervals for these genomic cline parameters will not include zero (Gompert and Buerkle 2011b; Gompert et al. 2012b). Such loci are inferred to display patterns of introgression that diverge from neutral expectations (Gompert and Buerkle 2011b; Gompert et al. 2012b).

For the purposes of this study, *P. carolinensis* represents parental population 1 and the probability of *P. atricapillus* ancestry is 1 – (probability of *P. carolinensis* ancestry). Increases or decreases in the probability of *P. carolinensis* ancestry from the base probability (predicted by hybrid index) for a given locus are specified by the genomic cline center parameter α, for which positive and negative values indicate an increase or a decrease in the probability of *P. carolinensis* ancestry, respectively. Excesses or reductions in ancestry-based linkage disequilibrium are denoted by β: positive values indicate excess ancestry-based linkage disequilibrium (i.e., *P. carolinensis* locus-specific ancestry confined to *P. carolinensis* genomic background and *P. atricapillus* locus-specific ancestry confined to *P. atricapillus* genomic background), whereas negative values indicate reduced ancestry-based linkage disequilibrium (e.g., locus-specific ancestry is less strongly associated with genomic background; Gompert et al. 2012b).

Modeling of various forms of selection (e.g., directional selection, underdominant selection, epistasis) has shown that specific patterns of α and β can be related to some, but not all, forms of selection and that their patterns are influenced by the demography of the specific hybrid zone and by the overall strength of selection (see Introduction; Gompert and Buerkle 2011a,b; Gompert et al. 2012a,b). Pure *P. carolinensis* and *P. atricapillus* allele frequencies were calculated from the same allopatric samples we used to calculate genome-wide differentiation (see above). For each time period, individuals sampled from Nolde Forest Environmental Education Center and Hawk Mountain Sanctuary were pooled and treated as the potentially admixed population. MCMC was used to estimate marginal posterior probability distributions for the hybrid index of each individual in each time period and the genomic cline parameters α and β were calculated for each locus in each time period. We ran the program for 2,000,000 generations, discarding the first 3000 as burn-in with a thinning parameter (−t) of 100, and assessed convergence by inspecting the MCMC output. We also analyzed the data separated by sex. The results were congruent with the combined dataset and we do not present them here. See Supplemental Data for bgc command line. We assessed consistency of our results by running three separate analyses.

BLAST AND BOWTIE ANALYSES

To ascertain (1) if any SNPs occurred in genic regions (particularly SNPs that exhibited clinal variation across the hybrid zone), and (2) the putative chromosomal position of loci, we used BLAST to compare GBS tags to expressed sequence tag (EST) and protein sequence databases (SwissProt), and aligned the GBS tags to the *T. guttata* genome using Bowtie (Warren et al. 2010). Given the high level of avian genomic synteny, aligning GBS tags to the *T. guttata* genome can provide putative chromosomal positions of loci when genomic resources are not available for nonmodel avian species (Ellegren et al. 2012). GBS tags were compared to the EST database using blastn (Altschul et al. 1997) with parameters: word_size = 11; gapopen = 5; gapextend = 2; penalty = −3; and reward = 2. Sequences were compared to the SwissProt database using blastx with default parameters. Loci were identified as putatively genic if they had an expectation value $e < 1 \times 10^{-5}$ in matches against the EST database or $e < 1 \times 10^{-3}$ in matches against SwissProt database. blastx was used to determine if the genic SNPs were synonymous or nonsynonymous. GBS tags were aligned to the *T. guttata* genome (taeGut1 assembly) using Bowtie 2 (Langmead and Salzberg 2012) with the option "–very-sensitive-local." A Perl script was used to determine the exact position of the SNP within an alignment. ANNOVAR (Wang et al. 2010) was then used with the taeGut1 refGene database to assign each SNP that aligned to the *T. guttata* genome to one of the following classes: exonic, splicing, ncRNA, UTR5, UTR3, intronic, upstream, downstream, or intergenic.

Results

GENOTYPING BY SEQUENCING

Illumina sequencing of 190 individuals on two lanes resulted in 496,872,131 reads. This dataset was trimmed to 400,000,000 reads by the criteria that each contained a unique barcode, a cut site remnant, and no ambiguous sites. Prior to filtering, the data consisted of 103,641 SNP loci with mean coverage of 2× (minimum coverage per individual 0.08×, maximum coverage per individual 302×). Of 190 individuals, 167 passed initial filtering. Sequencing failures appeared to occur primarily in samples with DNA concentrations below 10 ng/μl. The UNEAK pipeline was run on the data from the remaining 167 individuals ($n_{\text{historical}} = 83$, $n_{\text{contemporary}} = 84$), identifying 20,363 biallelic SNP loci, many of which had low coverage or were present in only a handful of individuals. When loci with more than 20% missing data and with observed heterozygosity >0.75 were excluded, 1425 loci were retained, with a mean coverage of 22× (minimum coverage per individual 12×, maximum 239×). The SNP data have been uploaded to Dryad doi: 10.5061/dryad.7gg47.

GENOME-WIDE DIFFERENTIATION

Locus-specific differentiation varied widely across the marker set, but was generally low (mean $F_{ST} = 0.11$; Fig. 1C). The maximum locus-specific estimate of F_{ST} was 0.77 and 20 loci fell above the 0.95 quantile of the genome-wide average F_{ST}, representing outlier loci (Fig. 1C; Table 1). F_{ST} outlier results were consistent when sexes were analyzed separately, but estimates from females were slightly reduced. For the female-only dataset, all of the outlier loci identified in the combined and male-only datasets fell within the 0.90 quantile, and no loci fell within the 0.95 quantile (Table 1). As noted in the methods, the populations used to calculate interspecific F_{ST} were composed of genetically pure individuals of each species (Fig. 2B). Type 1 errors (false positives) are a concern with genomic data, which generally involves estimation of parameters from datasets containing many more loci than individuals. We would not expect F_{ST} outlier loci to exhibit consistent spatiotemporal correlations with parameter estimates from genomic cline analyses under a neutral model. Estimates of the β parameter from our bgc analyses are spatiotemporally consistent for the F_{ST} outlier loci identified here in temporally separated samples of admixed individuals (see below); however, shared population history is likely playing a role in generating this pattern. Although we do not include a classical statistical correction for multiple tests, our spatiotemporal comparison gives us confidence that the loci we have identified as interspecific F_{ST} outliers are not the product of stochastic variation. Additionally, parameter estimates (cline center and width) from a geographic cline approach are coincident and narrow in both time periods for all loci identified here as F_{ST} outliers, which we would not expect if these F_{ST} outliers were the product of stochastic drift (Fig. 2A; Table S1 in Taylor et al. 2014).

GENOMIC CLINE ANALYSES

Locus-specific genomic introgression varied across the genome in both time periods; however, there was spatiotemporal consistency in the dominant signatures of both genomic cline parameters, and in the distribution of hybrid indices (Figs. 3–6; Tables S1 and S2). This remained true when the sexes were analyzed separately (Table S3). Both genomic cline parameters were significantly correlated between time periods ($R^2_{\alpha} = 0.27$, $P < 0.001$; $R^2_{\beta} = 0.49$, $P < 0.001$; Fig. 6A, B), indicating that the reported patterns are at least in part the result of consistent forces of selection. Shared population history most likely plays a role in these consistent patterns as well. Regardless, the skewed pattern in Figure 6B would not be expected under a neutral model. The scatter associated with both plots is likely the result of stochastic variation and/or sampling error between time periods (see Discussion).

We considered a locus to exhibit excess ancestry if the 95% credibility interval (CI) did not include zero (Gompert et al. 2012b). Similar proportions of loci exhibited excess *P. carolinensis* ancestry (lower bound of 95% CI > 0) in each time period. However, loci with excess *P. atricapillus* ancestry (lower bound of 95% CI < 0) were slightly more common in the historical dataset than the contemporary dataset (Figs. 3A, 4; Table S1). This difference is likely the result of stochastic variation related to demography between time periods. Only seven loci exhibited similar patterns of ancestry with respect to their α values between time periods (Table S1).

Seventy-three loci exhibited excess ancestry-based linkage disequilibrium (i.e., reduced introgression) in the historical dataset and 50 loci exhibited the same pattern in the contemporary dataset (Figs. 3B, 4; Table S2). Forty-eight of these loci exhibit the same patterns in each time period, and locus-specific β values are correlated between the time periods ($R^2_{\beta} = 0.49$, $P < 0.001$; Fig. 6B). This correlation remains significant when the effect of temporally correlated F_{ST} estimates is removed: the residuals from the regressions between β values and F_{ST} (see below) between time periods are correlated ($R^2_{\beta\text{-residuals}} = 0.27$, $P < 0.001$; Fig. S1). Additionally, β values within each time period are positively correlated with their residuals (Fig. S2A, B) and plots of F_{ST} by residuals (Fig. S2C, D) are clustered around zero, as would be expected for an appropriately fitted regression.

GENETIC DIFFERENTIATION AND INTROGRESSION

No estimates of α for the 20 interspecific F_{ST} outlier loci were significantly greater than zero in either time period (Figs. 3A, 5B; Table S1); however, all F_{ST} outlier loci had significantly positive estimates of β in the historical time period and 19 of 20 outlier loci had significantly positive estimates of β in the contemporary time period (Figs. 3B, 5C; Table S2). Genetic differentiation between *P. atricapillus* and *P. carolinensis* was significantly correlated with positive values of the genomic cline rate parameter β in both time periods ($R^2_{\text{historical}} = 0.39$, $P < 0.0001$, $R^2_{\text{contemporary}} = 0.38$, $P < 0.0001$; Fig. 5C; Table S2). Because of the slight nonlinearity in the relationships between β and F_{ST} (Fig. 5C), we used generalized additive models (mgcv library in R with $k = 2$ for the smoothing term of the model in order for the spline to describe a very smooth nonlinear relationship; Wood 2006) to fit regression lines and calculate residuals. The correlation remained when loci with F_{ST} estimates below 0.1 were excluded (642 loci), indicating that the pattern is not being driven by loci with low F_{ST} estimates ($R^2_{\text{historical}} = 0.39$, $P < 0.0001$, $R^2_{\text{contemporary}} = 0.42$, $P < 0.0001$), which has been shown generate spurious correlations in bgc data through simulations (Gompert and Buerkle 2011b). The probability that the same 19 loci would exhibit significantly positive estimates of β in both time periods is low. The 19 loci showing excess ancestry-based linkage disequilibrium (β values with 95% confidence limits not overlapping zero) each has a probability of 0.05 by chance alone of being an outlier in either time period. This assumption is an oversimplification given that the two sampling

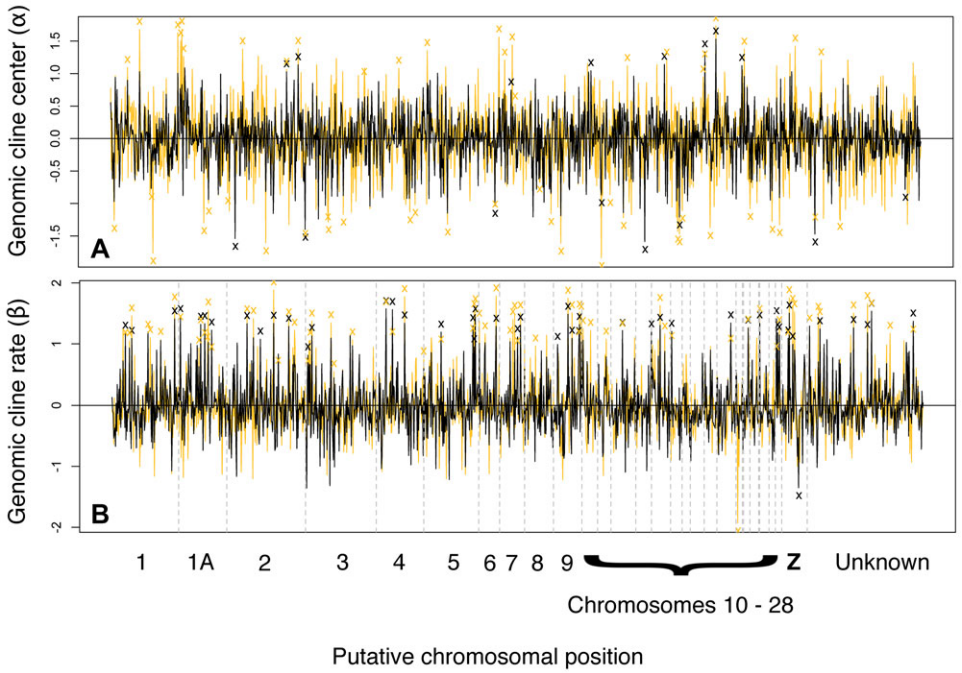

Figure 3. **Locus-specific Bayesian genomic cline parameters. (A) Bayesian genomic cline center (α) and (B) Bayesian genomic cline rate (β). Loci ordered by putative chromosomal position as determined by alignment of GBS tags to the *T. guttata* genome. Loci exhibiting excess ancestry (loci for which confidence intervals did not include zero) indicated by ×. Historical data in yellow, contemporary data in black.**

periods have shared population history; however, given this simplified assumption, the fact that these same loci were outliers in both the first and second time period is unlikely. We acknowledge that this is an oversimplification and ignores the fact that the two temporal samples are not independent given their shared population history. Although population history is likely influencing these patterns (i.e., the samples are not completely independent given the relatively short timescale of sampling and shared history of the populations) this remains robust evidence that these loci are likely involved, or are linked to regions involved, in reproductive isolation between *P. atricapillus* and *P. carolinensis*. There was no association between elevated F_{ST} and positive or negative values of α in either time period (Fig. 5B; Table S1).

PUTATIVE CHROMOSOMAL POSITION, DIFFERENTIATION, AND INTROGRESSION

Based on alignments with the *T. guttata* genome, the 1425 loci were distributed across the chickadee genome on chromosomes 1 through 28 (excluding chromosome 16), and the Z chromosome (Fig. 3). The sex-linked Z chromosome was significantly more likely than autosomes to have F_{ST} outlier loci: nine of the 20 interspecific outlier loci were putatively on the Z chromosome (Fig. 1C; $\chi^2 = 14.8$, $P = 0.01$). The majority of F_{ST} outlier loci also had significantly positive β values in both time periods (Fig. 5C; Table S2). One hundred and forty-three SNPs were genic (i.e., within genes) based on comparisons to the SwissProt and EST_OTHERS databases (SwissProt = 22, EST_OTHERS = 121). Loci with high F_{ST} estimates were not enriched for genic regions. Alignment of the data to the *T. guttata* genome did not reveal many genic regions: the majority of matches, including all of the outlier loci, were intergenic (Table S4). Although intergenic, several outlier loci were located near genes involved in oxidative phosphorylation, gene expression, and the endocrine system (Table S5). Alignment of GBS tags to the *T. guttata* genome illustrates that the 1425 locus dataset utilized here is putatively distributed across most chromosomes in the avian genome; however, we do not evaluate linkage between markers here because of their low density within the genome.

Discussion

The most notable result from our analysis of the chickadee hybrid zone is the identification of a subset of loci linked to genomic regions exhibiting spatiotemporally consistent patterns of elevated divergence and reduced introgression. Introgression patterns for loci showing elevated divergence (F_{ST} outliers) were consistent in samples of admixed individuals separated by a decade in this moving hybrid zone, across multiple generations of admixture. These loci are potentially linked to regions under selection and may be involved in reproductive isolation

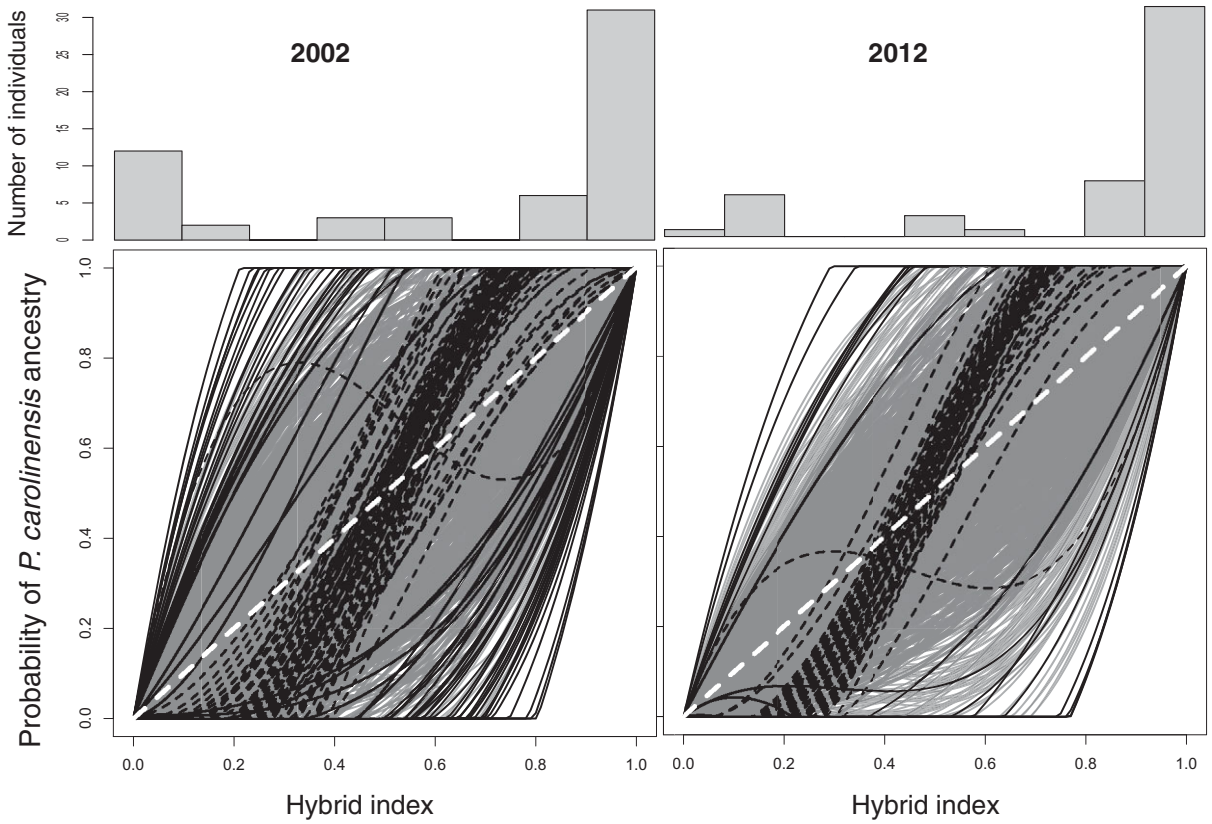

Figure 4. **Estimated genomic clines for all 1425 loci in each time period. Each line represents the genomic cline for a single locus. Solid black lines indicate loci for which the 95% CI of α did not include zero, dashed black lines indicate loci for which the 95% CI of β did not include zero, and gray lines indicate loci for which the 95% CIs of both α and β included zero. Dashed horizontal white line gives ϕ = h. Marginal histogram shows hybrid indices for the putatively admixed individuals in each time period (hybrid index of pure *P. atricapillus* = 0.0 and pure *P. carolinensis* = 1.0).**

between chickadees (i.e., they are likely not the product of stochastic drift; Barton and Hewitt 1985,1989; Szymura and Barton 1986; Harrison 1990; Gompert and Buerkle 2011b; Gompert et al. 2012b; Carneiro et al. 2013; Parchman et al. 2013). As hypothesized, the most divergent loci between *P. atricapillus* and *P. carolinensis* exhibit nonneutral patterns of introgression consistent with underdominant selection. It is important to note, however, that population structure can also produce the pattern recorded here, and that significantly positive estimates of genomic cline rate parameter β in the manakin (*Manacus* spp.) hybrid zone in Central America were recently attributed to population structure (Parchman et al. 2013). Given that there is no evidence of population structure below the level of species differentiation within the chickadee hybrid zone, and that dispersal between populations is likely high, underdominant selection is more likely than population structure to produce the patterns of reduced introgression we report (Fig. 2B).

For the majority of loci, locus-specific patterns of introgression did not differ from null expectations in either time period (i.e., genomic cline parameters α and β did not differ significantly from zero). However, in addition to the loci identified as F_{ST} outliers, a subset of loci exhibited patterns of introgression that differed from null expectations (Tables S1 and S2). We did not recover any evidence for directional selection: no estimates of the genomic cline center parameter α were associated with F_{ST} outlier loci (Gompert et al. 2012b). Sampling error and stochastic variation are likely influencing these results; however, the temporally consistent nature of the patterns of introgression could indicate a role for selection in their distribution (Fig. 6A, B). Given the short timescale of the study these patterns may also be consistent due to shared population history. Unfortunately, we currently lack a model of how neutral genomic introgression should change over time making the interpretation of the patterns we report difficult. Population bottlenecks or drift in small populations could produce patterns from stochastic processes that may persist over a 10-year period, but we have no evidence that either chickadee population has experienced a population bottleneck in recent history.

A large proportion of the F_{ST} outlier loci experiencing reduced introgression was putatively located on the Z chromosome, and a subset may be associated with oxidative phosphorylation

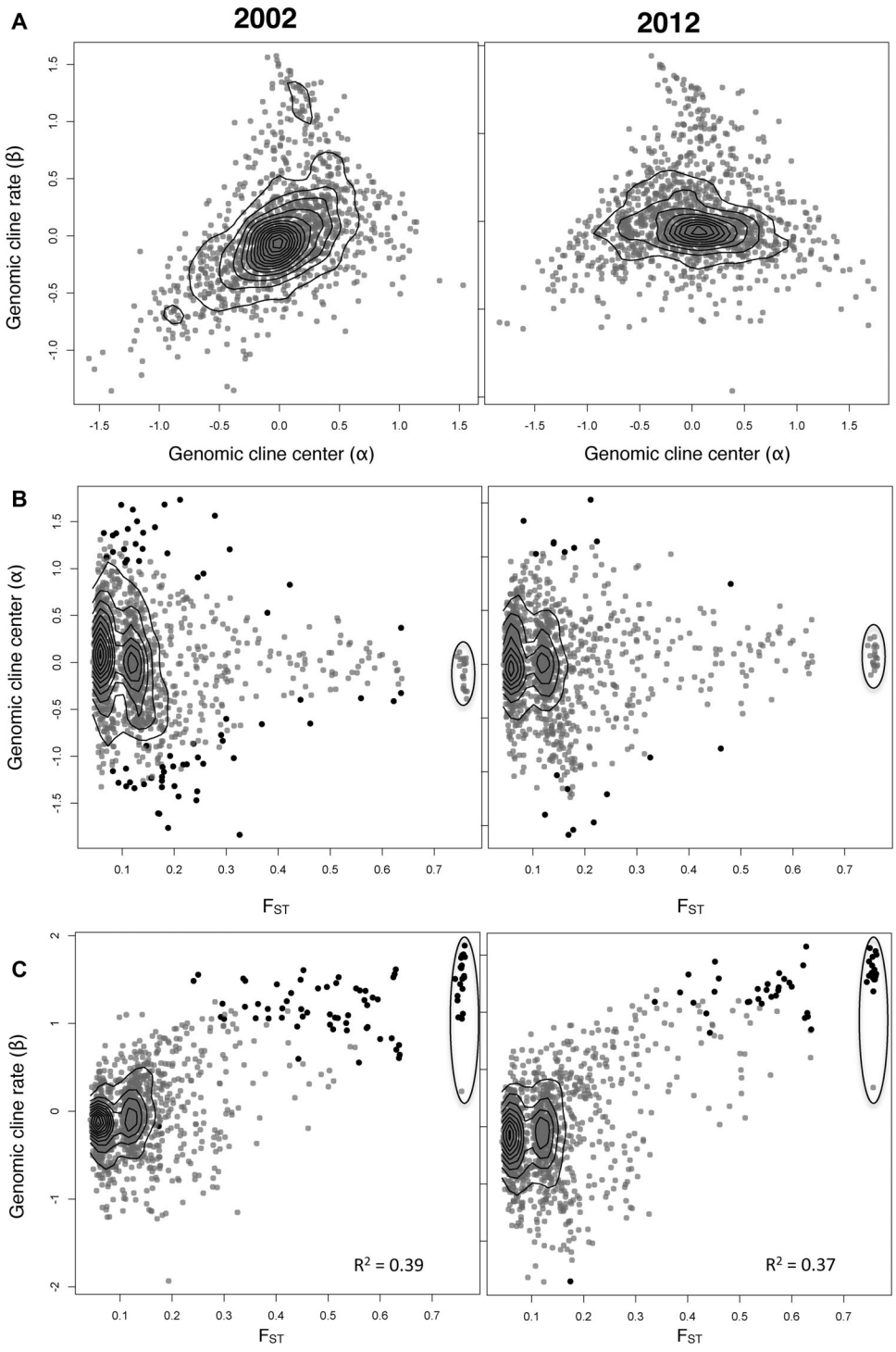

Figure 5. Temporal comparisons of selection and introgression. (A) Scatterplot showing relationships between locus-specific Bayesian genomic cline center (α) and rate (β) parameters illustrating variable genomic introgression in admixed populations. Each point represents one of 1425 loci. Contour lines depict the joint density of estimated cline parameters. (B) Scatterplot showing relationship between locus-specific genomic cline center α and interspecific F_{ST}. Each point represents one of 1425 loci. Contour lines depict the bivariate density. Black points are loci with 95% CI for α that did not include zero. Positive values of α indicate elevated *P. carolinensis* ancestry; negative values indicate elevated *P. atricapillus* ancestry. F_{ST} outlier loci indicated by black ellipse. (C) Scatterplot showing relationship between locus-specific genomic cline rate β and interspecific F_{ST}. Each point represents one of 1425 loci. Contour lines depict the bivariate density. Black points denote loci with 95% CI for β that did not include zero. Positive values of β indicate excess ancestry-based linkage disequilibrium (i.e., *P. carolinensis* locus-specific ancestry confined to *P. carolinensis* background). F_{ST} outlier loci indicated by black ellipse.

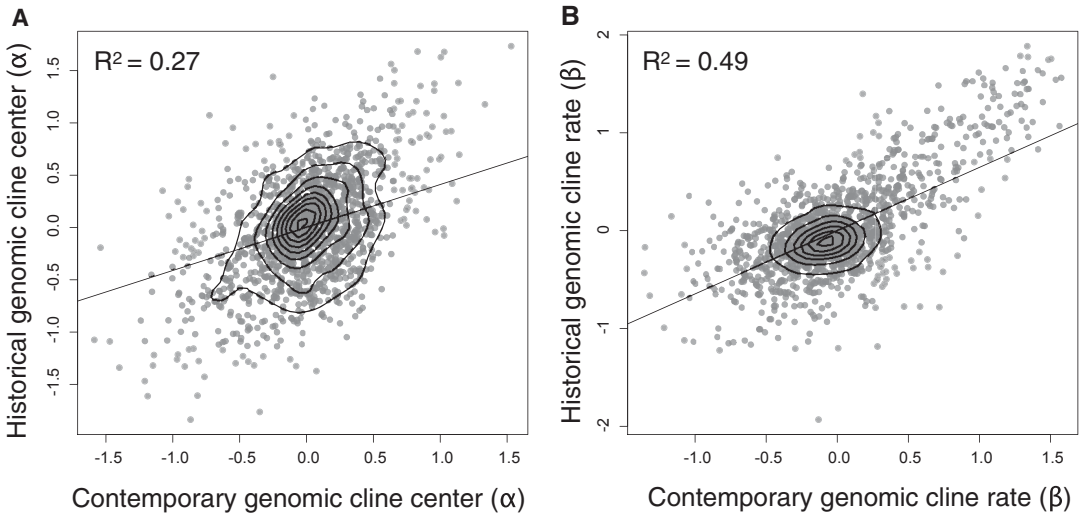

Figure 6. Temporal comparisons of Bayesian genomic cline parameters. Scatterplots illustrating significant correlations between historical and contemporary locus-specific Bayesian genomic cline parameters for (A) cline center (α) ($R^2 = 0.39$, $P < 0.0001$) and (B) cline rate (β) ($R^2 = 0.38$, $P < 0.0001$). Each point represents one of 1425 loci. Line represents linear regression.

and microRNA genes (Fig. 1C; Table S4). The loci associated with oxidative phosphorylation and microRNA genes are potentially linked to causal variants, but are almost certainly not the causal variants themselves. Although these are intriguing patterns, constraints related to current levels of genomic annotation make these genetic regions more appropriate targets for future investigations that can explore the functional genetics of these loci in greater detail.

COMPLEXITY OF DIVERGENCE AND REPRODUCTIVE ISOLATION

Overall, genetic differentiation and introgression vary across the chickadee genome at least at the scale of the chromosome. This result is consistent with other studies of hybridization and speciation that report heterogeneous genomic differentiation (Nosil et al. 2008; Hohenlohe et al. 2010; Gompert et al. 2012a,b; Janousek et al. 2012). Loci with nonneutral patterns of introgression were distributed across multiple chromosomes in the chickadee genome, with some localized on the Z chromosome, but we cannot evaluate their fine-scale distribution within chromosomes with the current dataset. It appears from these data that a relatively small proportion of loci contribute to reproductive isolation between chickadee species (20 of 1425 loci); however, analyses of a substantially greater number of loci are needed to evaluate robustly this aspect of reproductive isolation. In general, however, our results parallel those of others that have identified narrow regions of divergence and introgression widely distributed across the genome of hybridizing species, but elevated differentiation of the sex chromosomes (Ellegren et al. 2012; Carneiro et al. 2013; Parchman et al. 2013). Linkage mapping and/or whole genome sequencing will help clarify this pattern on a finer genomic scale.

Along with sampling error and Type I error rates, which we have addressed earlier (see Results), stochastic processes or selection on standing variation not directly related to reproductive isolation may have influenced the patterns of differentiation and introgression we report (Mani and Clarke 1990; Orr and Turelli 2001). Indeed, reproductive isolation can evolve without divergent selection via mechanisms including genetic drift and biased gene conversion (Gavrilets 1997; Gavrilets et al. 1998; Fierst and Hansen 2010). Broadly, our results are consistent with other studies that suggest that selection associated with divergence and reproductive isolation—species barriers—is complex. However, our spatiotemporal sampling scheme has allowed us to detect rigorously a series of loci that are linked to genomic regions likely involved in reproductive isolation between chickadees.

IMPORTANCE OF THE Z CHROMOSOME IN AVIAN SPECIATION

Numerous studies of hybrid zone dynamics have recorded reduced geographic introgression and higher differentiation of sex-linked loci (Carling and Brumfield 2008; Storchová et al. 2010; Teeter et al. 2010; Gompert and Buerkle 2011b; Trier et al. 2014). This pattern could be the result of a smaller effective population size for sex-linked loci, but often appears to be a signature of increased divergence of sex chromosomes relative to autosomes facilitated by lower recombination rates, a higher proportion of infertility alleles on the sex chromosomes, meiotic drive, or Z-maternal interactions (Charlesworth et al. 1987; Frank 1991; Badyaev et al. 2003; Ellegren 2011). For example, between European rabbit subspecies the steepest recorded geographic clines were for X and Y chromosome markers (Carneiro et al. 2013), and

patterns of reduced geographic and genomic introgression of sex-linked loci have been recorded across the mouse hybrid zone and within *Passera* hybrid complex in Europe (Macholán et al. 2007; Trier et al. 2014).

Birds have a ZZ/ZW sex determination system in which females are the heterogametic sex. The most comprehensive genomic examination of avian hybridization to date, between collared (*Ficedula albicollis*) and pied (*F. hypoleuca*) flycatchers, found significantly greater differentiation between the species on the Z chromosome than on the autosomes, and suggested that the sex chromosomes were farther along the speciation continuum than the autosomes (Ellegren et al. 2012). Additionally, there is evidence that female preferences and male traits under sexual selection are linked on the Z chromosome in *Ficedula* flycatchers (Ellegren et al. 2012). In birds, linkage between preference and trait could substantially accelerate the process of speciation on the Z chromosome compared to the autosomes (Albert and Otto 2005). Other studies of avian hybridization have also recorded a pattern of reduced introgression and higher differentiation of the Z chromosome compared to autosomes (Carling and Brumfield 2008; Carling et al. 2010; Storchová et al. 2010; Taylor et al. 2012, 2013).

As in the *Ficedula* flycatchers, the Z chromosome appears farther along the speciation continuum than the autosomes of *P. atricapillus* and *P. carolinensis* (Ellegren et al. 2012). Female chickadees evaluate male quality in part by assessing dominance interactions, but tight linkage between male dominance traits and female preferences seems less likely (see below; Otter et al. 1998; Curry et al. 2007). Male *P. carolinensis* tend to be dominant in captive interactions with male *P. atricapillus* (Bronson et al. 2003a), but whether male *P. carolinensis* are dominant over *P. atricapillus* in field situations remains unknown (Curry 2005; Curry et al. 2007). Field evidence shows that female *P. atricapillus* will choose male *carolinensis*-like males as extra-pair sires (Reudink et al. 2006), which could suggest that dominance traits and preferences are not linked on the Z chromosome in chickadees. Whether dominant behavior and preference for dominance could be linked on the Z chromosome is uncertain, as male dominance is likely to be an emergent behavior that depends on many other behavioral and physiological traits.

SPATIOTEMPORAL CONSISTENCY AND TIME SINCE DIVERGENCE

The spatiotemporally consistent signatures of reproductive isolation in the chickadee hybrid zone are likely the result of strong intrinsic selection against hybrids throughout the population history of these two species, which should be stronger for hybridizing species with older divergence times (Coyne and Orr 1989, 1997; Tubaro and Lijtmaer 2002). Earlier phylogenetic reconstructions based on mitochondrial DNA markers indicated that *P. atricapillus* and *P. carolinensis* likely diverged from their common ancestor more than 2.5 million years ago, are not sister species, and may represent a relatively divergent hybridizing species pair (Gill et al. 2005). However, a recent multilocus phylogenetic analysis of the new world chickadees that included sequence data from 40 nuclear genes (Harris et al. 2013) recovered a highly supported sister species relationship between *P. atricapillus* and *P. carolinensis*; the incongruent sister relationships in mitochondrial versus nuclear gene trees may be the result of ancient mitochondrial introgression from an additional species, the mountain chickadee (*P. gambeli*). A comprehensive analysis of the genomic architecture of reproductive isolation should aim to evaluate selection and introgression in hybridizing species pairs representing a range of "times since divergence" (e.g., Singhal and Moritz 2013). Spatiotemporal comparisons of selection and introgression in admixed populations between less-divergent species or subspecies may provide additional insight into the consistency of the genomic architecture of reproductive isolation through time and across geography.

GENERAL CONCLUSIONS

Our spatiotemporal comparisons have allowed the rigorous detection of genomic regions likely involved in reproductive isolation between hybridizing chickadees. Although the genomic architecture of divergence and reproductive isolation between *P. atricapillus* and *P. carolinensis* is complex, a higher than random proportion of divergent loci reside on the Z chromosome, as in other avian systems. This represents the first spatiotemporal analysis of a hybrid zone with a genome-spanning dataset. These results will facilitate comparisons to other moving hybrid zones and we encourage others to explore geographically and temporally replicated samples from admixed populations when evaluating the genomic architecture of reproductive isolation between hybridizing organisms.

ACKNOWLEDGMENTS

Thanks to J. Good, D. Irwin, two anonymous reviewers, E. L. Larson, and R. G. Harrison for their helpful comments on the manuscript. This research was supported through the Fuller Lab of Evolutionary Biology at the Cornell Lab of Ornithology and by Villanova University. Discussion with Z. Gompert, W. Hochachka, L. Campagna, N. A. Mason, G. Bradburd, and M. G. Weber aided analyses. A. Talaba, L. Stenzler, S. Mitchell, and C. Acharya contributed to laboratory analyses. We thank M. W. Reudink, S. L. Guers, and many other students in the Curry laboratory for contributions to field data collection. We thank Villanova University, the Nature Conservancy-Pennsylvania, Hawk Mountain Sanctuary Association, and Pennsylvania DCNR for permission to conduct research at the field sites.

DATA ARCHIVING

The doi for our data is 10.5061/dryad.7gg47.

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Associate Editor: J. Good

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Residuals from the relationship between the β value of each locus and F_{ST} with residuals calculated separately for the historical and contemporary time periods.

Figure S2. Scatterplots of goodness-of-fit information from the generalized additive models from which residuals were calculated for Figure S1.

Table S1. Bayesian genomic cline results of α parameter (genomic cline center) for loci showing significant excess of *P. carolinensis* (+) or *P. atricapillus* (−) ancestry in either time period ordered by descending interspecific F_{ST} with 95% confidence intervals (CI; LB = lower bound, UB = upper bound).

Table S2. Bayesian genomic cline results of β parameter (genomic cline rate) for loci showing significant presence (+) or absence (−) of locus-specific ancestry (95% confidence intervals did not include zero) in either time period ordered by descending interspecific F_{ST} with 95% confidence intervals (CI; LB = lower bound, UB = upper bound).

Table S3. Comparison of Bayesian genomic cline results of β parameter (genomic cline rate) for loci showing significant presence or absence of locus-specific ancestry (95% confidence intervals did not include zero) in either time period ordered by descending interspecific F_{ST} between total dataset (T) and male only (M) and female only (F) datasets.

Table S4. Results from alignment of GBS tags to Zebra Finch genome.

Table S5. Interspecific F_{ST} outlier loci (all are intergenic) and their closest putative genes, and annotations based on alignment to the *T. guttata* genome.