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Population assignment reveals low migratory connectivity in a weakly structured songbird

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Abstract

Understanding migratory connectivity is essential for determining the drivers behind population dynamics and for implementing effective conservation strategies for migratory species. Genetic markers provide a means to describe migratory connectivity; however, they can be uninformative for species with weak population genetic structure, which has limited their application. Here, we demonstrated a genomic approach to describing migratory connectivity in the prothonotary warbler, *Protonotaria citrea*, a Neotropical songbird of conservation concern. Using 26,189 single nucleotide polymorphisms (SNPs), we revealed regional genetic structure between the Mississippi River Valley and the Atlantic Seaboard with overall weak genetic differentiation among populations (F_{ST} = 0.0055; 95% CI: 0.0051–0.0059). Genetic variation had a stronger association with geographic rather than environmental factors, with each explaining 14.5% and 8.2% of genetic variation, respectively. By varying the numbers of genomic markers used in population assignment models with individuals of known provenance, we identified a maximum assignment accuracy (89.7% to site, 94.3% to region) using a subset of 600 highly differentiated SNPs. We then assigned samples from nonbreeding sites to breeding region and found low migratory connectivity. Our results highlight the importance of filtering markers for informative loci in models of population assignment. Quantifying migratory connectivity for weakly structured species will be useful for expanding studies to a wider range of migratory species across taxonomic groups and may contribute to a deeper understanding of the evolution of migratory strategies.

KEYWORDS
ddRADseq, migratory connectivity, population assignment, population genetics, *Protonotaria citrea*, redundancy analysis

1 | INTRODUCTION

Migratory species are declining in abundance globally and represent a unique challenge for conservation planning (Robinson et al., 2009; Runge, Martin, Possingham, Willis, & Fuller, 2014). Understanding the spatial connections of populations throughout the full annual cycle is critical for understanding the ecology and evolutionary biology of migratory species and informing effective management decisions aimed at reversing declines (Faaborg et al., 2010; Marra, Cohen, Loss, Rutter, & Tonra, 2015). Population

dynamics may be influenced to variable extents at different stages in the annual cycle (Marra, Studds, et al., 2015; Sillet, 2000; Wilson, LaDeau, Tøttrup, & Marra, 2011). However, it is impossible to assess the influence of spatially variable stressors on any given population without an understanding of the degree of migratory connectivity (i.e., the spatial cohesiveness of populations throughout the annual cycle of their migratory movements; Webster, Marra, Haig, Bensch, & Holmes, 2002). High migratory connectivity between the breeding and nonbreeding grounds describes populations that are cohesive throughout the annual cycle, while low breeding–nonbreeding migratory connectivity describes disparate breeding populations that intermingle on the nonbreeding grounds (Finch, Butler, Franco, & Cresswell, 2017; Webster et al., 2002). Recent studies have used information on migratory connectivity to tease apart the influence of factors that could potentially explain spatial variation in observed breeding season population trends, including nonbreeding season habitat loss and climate change (Fraser et al., 2012; Kramer et al., 2018; Rushing, Ryder, Marra, & Rushing, 2016; Stanley et al., 2015; Taylor & Stutchbury, 2016). By incorporating range-wide migratory patterns into ecological models, researchers can begin to determine the drivers behind population trends and apply this knowledge to conservation and management (Hostetler, Sillett, & Marra, 2015).

To assess migratory connectivity, individuals need to be tracked across potentially large geographic distances or individuals sampled outside of the breeding grounds need to have their breeding origin determined indirectly (Webster et al., 2002). Migratory connectivity research has predominantly focused on avian species (Marra, Cohen, et al., 2015), but the methods and results are pertinent for studying migratory species across all taxa (Godley et al., 2010; Miller, Wassenaar, Hobson, & Norris, 2012; Moussy et al., 2013; Rubenstein & Hobson, 2004). While there are a variety of methods that provide data needed to quantify migratory connectivity (Hobson, 2005; McKinnon, Fraser, & Stutchbury, 2013; Webster et al., 2002), genomic markers provide a cost-effective method for estimating interseasonal connectivity of different lineages, as well as providing information about their evolutionary histories. The use of genomic markers for population assignment models requires geographically structured genetic variation on the breeding grounds. In North America, several species of Nearctic–Neotropic (hereafter: Neotropical) migratory songbirds have been shown to exhibit population structure on a broad longitudinal scale (Boulet, Gibbs, & Hobson, 2006; Irwin, Irwin, & Smith, 2011; Kelly, Ruegg, & Smith, 2005; Kimura et al., 2002; Lovette, Clegg, & Smith, 2004). However, genetic markers have generally provided weak resolving power in identifying breeding ground population structure in avian species and this has been a limitation in the use of genetic markers for studying migratory connectivity in species with more limited geographic ranges (Oyler-McCance, Oh, Langin, & Aldridge, 2016). In some avian species, a re-evaluation of population structure in studies using single nucleotide polymorphisms (SNPs) provided a finer resolution of structure (Wilson's warbler, *Cardellina pusilla*: Ruegg et al., 2014; Painted bunting, *Passerina ciris*: Battey et al., 2017) than previously reported by studies using mitochondrial DNA (*C. pusilla*: Kimura et al., 2002; *P. ciris*: Herr, Sykes, & Klicka, 2011), microsatellites (*C. pusilla*: Clegg, Kelly, Kimura, & Smith, 2003) and amplified fragment length polymorphisms (*C. pusilla*: Irwin et al., 2011).

Integrating genomic data into analyses of migratory connectivity and ecological population models for migratory birds can provide a much needed evolutionary perspective to the study of migratory connectivity (Sherry, 2018). For species spread across heterogeneous habitats, such as many migratory bird species, selective forces may vary among populations which can result in different processes affecting the spatial arrangement of genetic variation (Nosil, Egan, & Funk, 2008; Wang & Bradburd, 2014). Beyond describing population structure for use in assignment models of migratory connectivity, landscape genomics can reveal how environmental and geographic variables influence population genetic structure (Manel et al., 2010; Rellstab, Gugerli, Eckert, Hancock, & Holderegger, 2015; Sork et al., 2013). Understanding the relative influences of geographic versus environmental features is especially important in the light of climate change as this information can allow us to predict how species will respond to changes in their environment (Bay et al., 2018).

Our objective was to use genomic data to maximize resolution of spatial genetic variation and develop effective population assignment models in order to estimate migratory connectivity. Our study examined the prothonotary warbler, *Protonotaria citrea* (Parulidae), a Neotropical migratory warbler that breeds in the bottomland hardwood forests of eastern United States in disjunct populations separated by the Appalachian Mountains in the higher latitudes of its breeding range (Figure 1, Petit, 1999). Due to the clear range separation of more northern populations, we hypothesized this species would exhibit some degree of breeding population structure to allow for population assignment. Prothonotary warbler abundance is unequally distributed across the breeding range, with two regions of high concentration centred in the Lower Mississippi Valley (estimated 32% of breeding population; Partners in Flight Database, 2013) and the mid-Atlantic Coast (estimated 35% of breeding population; Partners in Flight Database, 2013). Overall, the species is estimated to be declining at a rate of 1.10%/year over the past 50 years (95% confidence intervals [CI]: −1.55%, −0.64%). However, trend estimates within the two centres of abundance vary from −2.17%/year (CI: −3.17%, −1.17%) in Louisiana to increases of 1.80%/year (CI: 0.46%, 2.12%) in North Carolina (Sauer, Niven, & Hines, 2017). The disparity between breeding population trends of prothonotary warblers makes this an ideal species for studying migratory connectivity because a depiction of migratory connectivity may provide a foundation for determining the drivers behind these differing population trends.

We first described population genetic structure on the breeding grounds, as well as the relative influence of geographic and environmental factors on observed population structure. We then validated population assignment models on known-origin individuals from the breeding range. Finally, we estimated the breeding origin

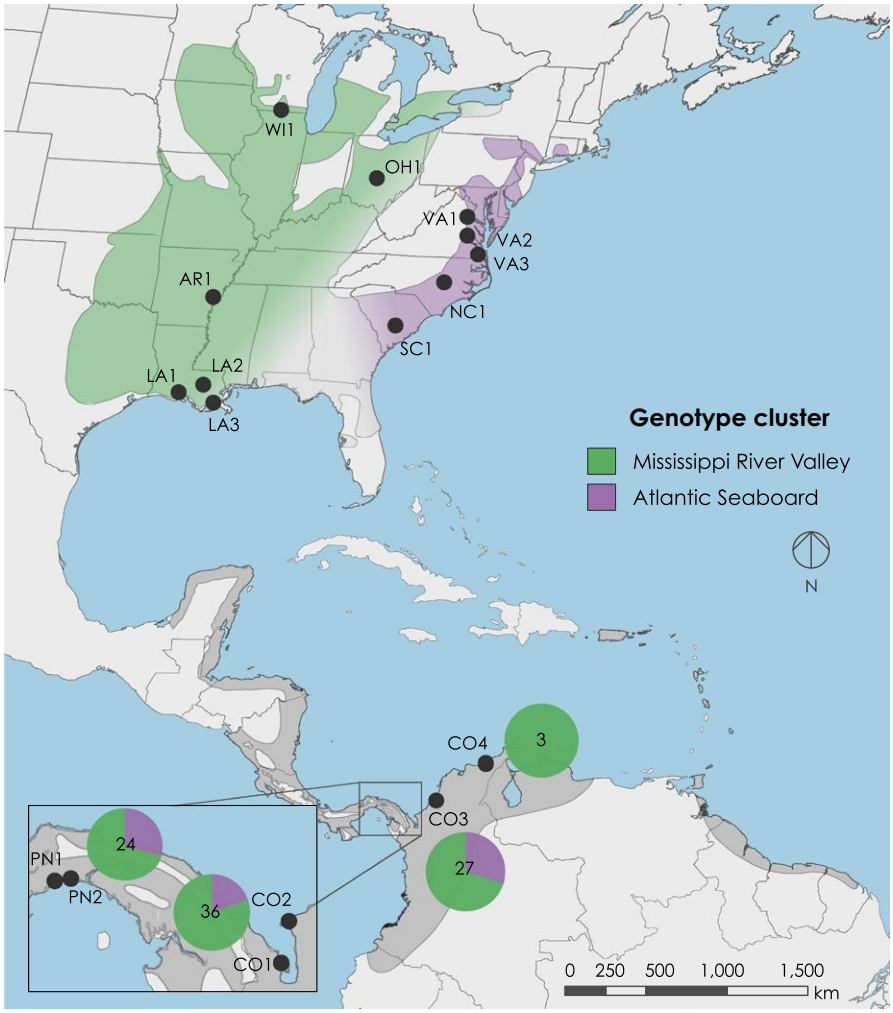

FIGURE 1 Genotype clustering and population assignment of the prothonotary warbler. Genotype clustering was determined using 26,189 SNPs in a PCA. Regional genotype clustering was overlayed on a range map of prothonotary warbler with all sampling locations shown and labelled by site ID. Site ID uses the first two letters of the state (breeding sites) or country (nonbreeding sites) followed by a number. Pie graphs depict population assignment of individuals from nonbreeding sites to regional genotype cluster. Nonbreeding sites that were geographically close were grouped together for population assignment (Table 3). The number on the pie graph depicts the total number of individuals that were assigned from the nonbreeding site grouping

of individuals sampled from the nonbreeding grounds in order to depict migratory connectivity. We discuss results in the context of conservation strategies and management focused on species with low levels of population structure and migratory connectivity.

2 | MATERIALS AND METHODS

2.1 | DNA sampling

Between 2014 and 2017, we obtained genetic samples from 265 individuals across 17 sites (range: 3–29 samples/site) distributed throughout the breeding (175 samples) and nonbreeding (90 samples) range (Table 1). The 11 breeding sites were split between the Atlantic Seaboard (Atlantic) and Mississippi River Valley (Mississippi) regions, and these will be subsequently referred to as "regions" for analyses and assignment. We collected blood samples for each individual using brachial venipuncture and preserved these samples on Whatman FTA cards (Gutierréz-Corchero et al., 2002). Collection was conducted under Virginia Commonwealth University IACUC protocol #AM10230. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following a modified lysis protocol for Whatman FTA cards. Two 1.2 mm punches of dried blood were initially incubated in 180 µl Buffer ATL for 10 min at 94°C. The sample was briefly allowed to cool and 20 µl proteinase K solution was added, and the sample was incubated at 56°C until complete lysis, approximately an hour. Following lysis, our DNA extraction procedure adhered to the manufacturer's protocol for the DNeasy Blood & Tissue Kit and we eluted samples to water.

2.2 | Polymorphism discovery and genotyping

Double digest RADseq (ddRADseq) was used to produce three multiplexed libraries for sequencing on the Illumina platform following the protocol outlined by Parchman et al. (2012). Genomic DNA was digested with two restriction endonucleases (*EcoRI* and *MseI*), and adaptor oligonucleotides, containing 10 base pair (bp) barcodes for the unique identification of individuals, were ligated to the digested fragments. The ligated fragments were amplified using PCR, and individuals with unique barcodes were pooled together in sets of 96 samples. Pooled amplified libraries were size selected for fragments in the approximate range of 300–500 bp using gel electrophoresis on 1% agarose gels and purified using QIAquick Gel Extraction Kits (Qiagen). Single-end sequencing with one multiplexed library per lane was performed by Novogene Corporation using the Illumina

TABLE 1 Sampling site locations, including site ID, name, region with genetic clustering classification for breeding sites and "nonbreeding" for Panama and Colombia sites

ID	Site	Region	Latitude	Longitude	*n*
VA1	Fort AP Hill, Virginia, US	Atlantic	38.15452	−77.32426	20
VA2	Deep Bottom, Virginia, US	Atlantic	37.407349	−77.305381	22
VA3	Great Dismal Swamp, Virginia, US	Atlantic	36.631408	−76.490525	13
NC1	Holt Lake, North Carolina, US	Atlantic	35.469092	−78.403068	9
SC1	Francis Beidler, South Carolina, US	Atlantic	33.220573	−80.354001	13
OH1	Hoover Reservoir, Ohio, US	Mississippi	40.107202	−82.88641	21
LA1	Palmetto Island, Louisiana, US	Mississippi	29.86441	−92.150251	19
LA2	Bluebonnet Swamp, Louisiana, US	Mississippi	30.367822	−91.107121	18
LA3	Barataria, Louisiana, US	Mississippi	29.783791	−90.115468	12
AR1	White River, Arkansas, US	Mississippi	34.358046	−91.090881	20
WI1	Sugar River, Wisconsin, US	Mississippi	42.530128	−89.32875	8
PN1	Panama Viejo, Panama	Nonbreeding	9.006642	−79.484717	7
PN2	Juan Diaz, Panama	Nonbreeding	9.019719	−79.44465	17
CO1	Bocas del Atrato, Colombia	Nonbreeding	8.08918	−76.836956	7
CO2	Marimonda, Colombia	Nonbreeding	8.56907	−76.81737	29
CO3	Cispata, Colombia	Nonbreeding	9.39281	−75.78397	27
CO4	Flamencos, Colombia	Nonbreeding	11.42013	−73.10123	3

Note. The last column, *n*, provides the sample size from each location.

HiSeq 4000 platform. Reads were demultiplexed and trimmed to 60 bp using the *process_radtags* program in STACKS version 2.0 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). The trimming length was based on the quality score distribution along the reads and chosen to minimize poor quality reads.

The demultiplexed FASTQ files were processed in the *dDocent* bioinformatics pipeline (Puritz, Hollenbeck, & Gold, 2014) with minimal filtering thresholds of 3× coverage per locus and presence of loci in at least 10 individuals, which yielded 145,260 putative SNPs. For the resulting variant call format (VCF) files, we used *VCFtools* (Danecek et al., 2011) to select for biallelic SNPs with less than 50% missing data and remove any indels, which retained 114,632 SNPs. We selected for SNPs that were between −0.5 and 0.5 F_{IS}, greater than 0.01 minor allele frequency, and had a PHRED quality score greater than 20, resulting in 41,328 SNPs. We further reduced the SNP data set to one SNP per RAD tag (random SNP) to avoid issues with linkage disequilibrium, and this resulted in a set of 26,189 SNPs that were used as the initial data set for subsequent analyses.

2.3 | Population structure

We used multiple complementary methods to quantify and describe the extent of genetic population structure across the breeding range, as this will provide valuable insights into the statistical power for assignment of individuals sampled on the nonbreeding grounds. First, we conducted principal component analysis (PCA) of multilocus genotypes to visualize potential groupings of the 175 sampled breeding individuals (Patterson, Price, & Reich, 2006). Outlier individuals, defined as multilocus genotypes whose PCA coordinates exceeded six standard deviations from the centroids of the first ten principal components (PCs), were removed from the data set. In order to assess the effect of variation in amounts of missing data across sites on observed patterns in PC space, we performed an analysis of variance (ANOVA) on the amount of missing data per individual among breeding sites. In the approach to PCA described by Patterson et al. (2006), missing data are imputed as the centred and standardized mean. If missing data are not random across breeding sites, then observed patterns of population structure could be artefactual. We excluded sites with high levels of missing data from a subsequent PCA and compared its patterns to those observed using the full set of data.

To complement PCA, we identified putative genetic clusters by implementing discriminant analysis of principal components (DAPC; Jombart, Devillard, & Balloux, 2010) in the package ADEGENET (Jombart, 2008) in R (version 3.4.4, R Core Development Team, 2018). DAPC is not based on a specific population genetics model assumption and performs *K*-means clustering on genetic distances in order to identify genetic clusters. We selected the number of principal components to use in the discriminant analysis based on a cross-validation of 1,000 permutations. *K*-means clustering solutions were compared using the Bayesian information criterion (BIC). DAPC was performed using all 26,189 SNPs and also using a subset of SNPs based on results from the population assignment. Additionally, we employed the program STRUCTURE (Pritchard, Stephens, & Donnelly, 2000), based on population genetic assumptions of an admixture

model with correlated allele frequencies and no prior information on sampling locations, to elucidate broad-scale compartmentalization in the spatial genetic structure of samples from the breeding grounds. We ran STRUCTURE with 50,000 burn-in iterations followed by another 100,000 Markov chain Monte Carlo steps for values of *K* 1 to 11. We conducted five replications for each *K* value. STRUCTURE was run using a subset of SNPs defined by assignment accuracy results, and the optimal *K* value from these results was then used to inform a subsequent STRUCTURE run, using all the SNPs, for the identified *K* value. We produced bar plots in CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015). We inspected the bar plots and followed guidelines of Evanno, Regnaut, and Goudet (2005) to identify the most likely number of clusters (*K*).

In addition to characterizing overall clustering, population structure was described using multilocus *F*-statistics (Weir & Cockerham, 1984) estimated globally and pairwise among all sampling sites using the R package HIERFSTAT (Goudet, 2005). Confidence intervals (95%) were generated for each statistic by bootstrapping ($\alpha = 0.05$, $n = 1{,}000$) across loci. We conducted a hierarchical analysis of molecular variance (AMOVA) in which we tested for differences in genetic variance among sampling sites (nested within regions) and differences between regions (Excoffier, Smouse, & Quattro, 1992). Significance testing was performed by permuting ($\alpha = 0.05$, $n = 1{,}000$) individuals among the hierarchical levels. Independent of the magnitude of population structure, we also examined the extent to which population differentiation was spatially arranged under a model of isolation by distance ($F_{ST}/(1 - F_{ST}) \sim \log_{10}$(Euclidean distance)) using a standard Mantel test (1,000 permutations) in the R package ADEGENET (Jombart, 2008).

2.4 | Redundancy analyses

Beyond describing the magnitude of genetic structure on the breeding ground, we wanted to determine the degree that genetic variation was associated with features of the local environment. We assessed association between breeding population genetic structure and environmental parameters through redundancy analysis (RDA), a form of constrained ordination that uses predictor matrices to explain multivariate response data (Legendre & Gallagher, 2001). Our full RDA consisted of individual genotype PC scores (see below) as the response variables and matrices of environmental and geographic factors as the predictor variables. We performed RDA at the individual level because we did not have a sufficient number of sampling locations for a robust site-level analysis. When assessing the effect of environmental factors on genetic variation, it is essential to account for the spatial distribution of sampling locations as this can confound the interpretation of environmental variation (Excoffier & Ray, 2008). Therefore, in conjunction with the full RDA, we used partial RDAs that were conditioned separately on environmental and geographic factors. The full set of environmental variables consisted of 27 bioclimatic variables for the 1961–1990 period that were gathered for each site (Table S3) using ClimateNA (Wang, Hamann, Spittlehouse, & Carroll, 2016). Latitude, longitude and elevation, as well as quadratic and cross-product combinations of latitude and longitude, were combined into a matrix of geographic variables (Table S3) following Legendre and Legendre (2012). To reduce dimensionality of predictor variables, a PCA was performed separately for the environmental and geographic matrices. We retained the PCs that cumulatively explained at least 90% of the variation in each case for use in the RDAs. PCA was performed on the individual genotypes of breeding range samples, and significant PCs were retained as the response variables for the RDA. Significance ($\alpha = 0.05$) of the genotype PCs was determined by comparing standardized eigenvalues to a Tracy–Widom distribution (Patterson et al., 2006). To determine the optimal combination of predictor variables, we performed stepwise model selection using permutation tests. The global significance of the RDA was assessed with an analysis of variance (ANOVA; $\alpha = 0.05$, 1,000 permutations), and a conditional RDA was performed to partition variance between the geographic and environmental predictors (Borcard, Legendre, & Drapeau, 1992). The joint effect of geography and environment on genetic variance was defined as the remaining proportion of variance explained by the full RDA after subtracting the proportion of variance explained by the partial RDAs conditioned on geographic and environmental variables. All steps of the RDA were conducted in R using the VEGAN package (Oksanen, Blanchet, & Kindt, 2013).

2.5 | Population assignment of known-origin breeding samples

We attempted to assign individuals from a hold-out sample to their known origin at two different scales—breeding site and breeding region based on maximum likelihood of multilocus genotype probabilities (Paetkau, Calvert, Stirling, & Strobeck, 1995). In order to maximize assignment accuracy and minimize bias, we used a two-part analysis involving a training-set/hold-out protocol and leave-one-out cross-validation following the methods described by Anderson (2010). The first part of the analysis determined the ideal number of markers to use for assignment accuracy, and the second part of the analysis determined the level of assignment accuracy achieved. By using separate sets of individuals for population allele frequencies and assignment in the hold-out set, we avoided the inherent upward bias in predicted accuracy that occurs when combining classifier and training data (Anderson, 2010). In the leave-one-out cross-validation, population allele frequencies are calculated with the removal of one individual from the data set and then the individual is assigned to a population based on the calculated allele frequencies. Assignment tests were performed using twenty bootstrapped data sets, each containing a 2:1 ratio of training and hold-out individuals randomly sampled from each breeding site. Only SNPs with alleles present in every breeding sampling site were used in order to circumvent the issue of calculating genotype frequencies of zero. To test the effect of number of markers on assignment accuracy, we calculated expected genotype frequencies in a training set with subsets of SNPs (200, 400, 600, 800, 1,000, 1,200, 1,500, 2,000 and 3,000 SNPs). We ranked loci by

single-locus F_{ST} estimates (from HIERFSTAT) and ordered the loci by decreasing values of F_{ST}. The subset of SNPs with the highest assignment accuracy was used in a leave-one-out cross-validation model (see Anderson, 2010) to describe the variation in assignment success across sites and regions. We examined the effect of sample size of sites on the population assignment accuracy using a nonlinear regression. All assignment tests were conducted with custom scripts in R which are available through GitHub (https://github.com/mgdesaix/populationAssignment).

2.6 | Migratory connectivity

In order to determine the strength of migratory connectivity between nonbreeding and breeding sites, we first assigned individuals sampled on the nonbreeding grounds to their breeding origin in one of the two regions (Mississippi vs. Atlantic). We then compared the proportion of individuals assigned to each region with estimates of relative breeding abundance of those regions. If, at each nonbreeding sampling location, the proportion of individuals assigned to each breeding region is similar to the estimates of abundance in those regions, this would suggest that individuals from both regions are mixing among the nonbreeding sites (i.e., low migratory connectivity). However, if a nonbreeding sampling location contained a higher proportion of individuals assigned to a breeding region than would be expected by estimates of breeding abundance, then that would show individuals from different breeding regions remained separate on the nonbreeding ground (i.e., high migratory connectivity). As in the assignment methods above, we limited the total number of SNPs to loci that had two alleles in both of the regions and ranked loci by single-locus F_{ST} estimates. We used the optimal number of loci as determined above to calculate allele frequency by region, and assignment was determined by the maximum product of expected genotype frequencies across loci. To estimate the degree of mixing of breeding individuals on the nonbreeding sites, we used a chi-square test ($\alpha = 0.05$) to compare the proportion of individuals assigned to region from each nonbreeding site to an estimate of relative abundance (Partners in Flight Database, 2013) between the regions on the breeding ground.

3 | RESULTS

3.1 | Population structure

In the PCA using all 26,189 SNPs, the first two PCs explained 0.89% and 0.85% of the genetic variation, respectively, and most individuals were clustered together except for several outlier individuals (Figure S1). After the iterative removal of 13 outliers, the PCA explained 0.88% (PC1) and 0.82% (PC2) of the genetic variation and showed distinct genetic clustering of individuals into the Atlantic and Mississippi regions (Figure 2a). The results of an ANOVA on the distribution of individual-level missing genetic data among breeding sites suggested that the SC1 and OH1 sites had higher levels of missing data than the other breeding sites (Figure S2). After removing those sites and reperforming PCA, the genetic clustering patterns remained the same. Therefore, we concluded that the clustering patterns were not an artefact of missing data and we retained all individuals and sites for subsequent analyses.

Overall F_{ST} across sites was 0.0055 (95% CI: 0.0051–0.0059) when using all 26,189 SNPs. Pairwise genetic differentiation measured by F_{ST} ranged from 0 to 0.0107 among the 11 sampling locations (Table S1). No significant genetic differentiation ($\alpha = 0.05$) was found among the following pairs of sampling locations: VA1 & VA3, VA3 & NC1 and NC1 & SC1. Based on an AMOVA, genetic structure across all hierarchical levels was similarly low yet significant, with less genetic differentiation among regions ($\Phi_{RT} = 0.0082$, $p < 0.05$; Table 2) than among sites nested within regions ($\Phi_{SR} = 0.0183$, $p < 0.002$). Variance decomposition revealed the vast majority of genetic variation being contained within sites (97.4%), with a small amount of genetic variation contained among sites nested within regions (1.8%) and among regions (0.8%). The results of the Mantel test for all breeding sites showed there was a significant positive correlation between genetic and geographic distances ($r^2 = 0.40$, $p = 0.001$, Figure S3). When examined within the regions separately, the Atlantic sites had no association between genetic and geographic distances ($r^2 = 0.44$, $p = 0.26$), while the Mississippi sites had a strong positive association between genetic and geographic distances ($r^2 = 0.61$, $p = 0.004$).

Performing PCA with 600 SNPs retained the same regional clustering of individuals as with the full set but also separated the breeding sites LA2 and VA2 apart from other sites (Figure S1). Additionally, when PCA was performed for the regions separately, clustering resolution was increased with more breeding sites being distinguishable from each other (Figure S1). The proportion of variance explained in the first two PCs increased from 0.89% and 0.85%, respectively, to 1.77% and 1.67% when using the subset of SNPs. In the DAPC, using 26,189 SNPs identified $K = 1$ with the lowest BIC and using the subset of 600 SNPs identified $K = 2$ as having the lowest BIC (Figure S4). Using $K = 2$ in the discriminant analysis with 600 SNPs resulted in the clustering of breeding sites predominantly between the Atlantic and Mississippi regions (Table S2). With a subset of 600 SNPs, $K = 2$ clusters had the highest delta K and clustering was evident between sites in the Atlantic and Mississippi regions with high levels of admixture (Figure S5). There was a smaller peak of delta K for $K = 4$ clusters, and this revealed OH1 and VA2 sites each as their own cluster, in addition to the regional clustering. Higher levels of K further split breeding sites into their own groups. Using all 26,189 SNPs for $K = 2$, STRUCTURE produced bar plots that, upon visual inspection, did not reveal separation of individuals between 2 clusters (Figure S5). For the purposes of assignment, we retained K of 2 clusters (Atlantic and Mississippi regions; Figure 1).

3.2 | Redundancy analyses

A PCA of the 23 annual bioclimatic variables obtained from ClimateNA (Wang et al., 2016) revealed environmental heterogeneity across breeding sites (Figure S6). PC axes 1 and 2 of the environmental variables (from here on, ENV_PC1 and ENV_PC2) accounted for the vast majority of the variation among breeding sites (92.3%) and were retained

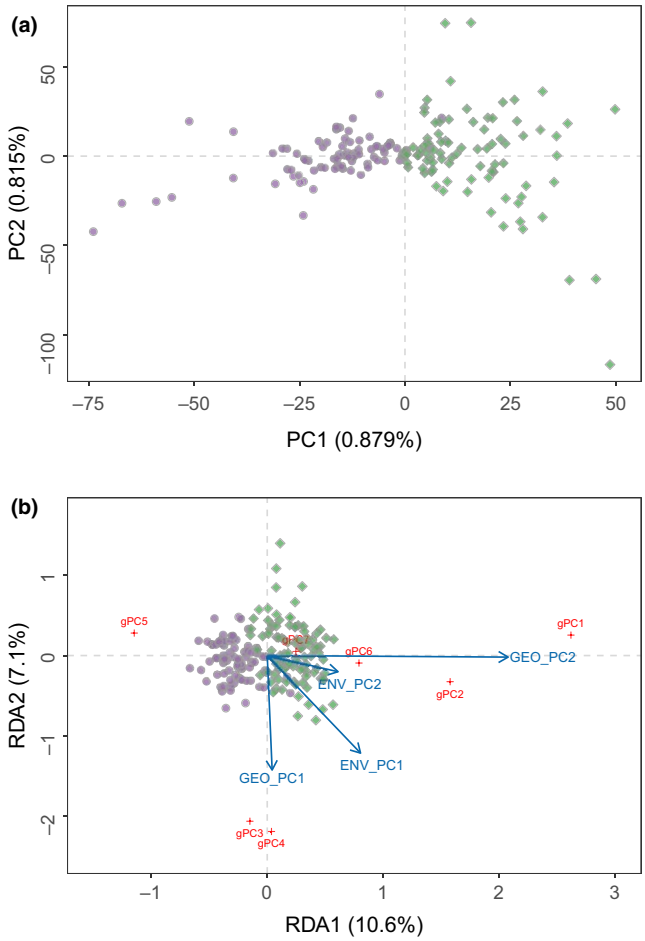

FIGURE 2 Principal component analysis and RDA. (a) PCA on 26,189 SNPs and individual outliers removed that had principal component values of more than six standard deviations on the first ten principal components. (b) Triplot of RDA, using a subset of 600 SNPs, with the *x*-axis and *y*-axis representing scaled RDA values for sampled individuals. Statistically supported environmental and geographic factors in the RDA models are represented as arrows, and the length of the arrows corresponds to magnitude of effect for geographic (GEO_PC) and environmental (ENV_PC) principal components. Genotype PC (gPC) scores are represented by red crosses and labelled. The reported per cent variance explained is of the constrained variance. In both plots, individuals from the Atlantic region are represented by purple circles and individuals from the Mississippi region are represented by green diamonds

TABLE 2 AMOVA table for the differences at the hierarchical levels of region and site (nested within region)

Source	*df*	Φ	Variation (%)	*p*
Region	1	0.0082	0.82	<0.05
Site	9	0.0181	1.80	<0.002
Error	164	0.0262	97.38	<0.001

Note. In the linear model, the error term is derived from the individuals within sites. The designation of region was based on the genetic clusters shown in the PCA plot, while sites correspond to all of the breeding sites. Significance was tested by permutation (n = 1,000) across the hierarchical levels.

for RDA. ENV_PC1 had relatively uniform loadings (range: −0.23 to 0.23) across all variables and explained 79.8% of the variation, while ENV_PC2 had wider ranging loading values (range: −0.47 to 0.28) and explained 12.5% of the variation. Generally, ENV_PC1 had higher loadings of variables directly associated with temperature, while ENV_PC2 had higher loadings of several variables measuring moisture, with the addition of a measurement of historical extreme temperature (Table S3). In the PCA on geographic variables, PC1 and PC2 (from here on, GEO_PC1 and GEO_PC2) explained 62.0% and 35.2%, respectively, of the variation in the data and were retained for the RDA. GEO_PC1 had a strong positive correlation with variables representing the latitude of the breeding sites (r^2 = 0.84, p < 0.001, $F_{1,9}$ = 48.13). Accordingly, GEO_PC2 was highly correlated with the longitudinal gradient of the breeding sites (r^2 = 0.99, p < 0.001, $F_{1,9}$ = 1,973).

For the response matrix, we used the subset of the 600 highest differentiated SNPs. A comparison of standardized eigenvalues to a Tracy–Widom distribution identified the first seven PCs as significant and these PCs cumulatively accounted for 10.6% of the genetic variation (individually ranging from 1.3% to 1.8%). Stepwise model selection retained all predictor variables (two environmental PCs and two geographic PCs) in the optimal model. Across all breeding individuals, the RDA was significant (ANOVA; F = 13.303, p < 0.001) and RDA axes 1 and 2 accounted for 10.6% and 7.1% of the constrained variation, respectively (Figure 2b). In the partial RDA conditioned on geographic predictors, the environmental variables explained 8.2% of the total genetic variance (constrained and unconstrained) and in the partial RDA conditioned on environmental variables, 14.5% of the genetic variance was associated with geography, while 1.2% of the genetic variance was explained by the joint effect of geography and environment. The genetic clusters revealed through PCA were predominantly partitioned longitudinally, and this barrier was evident in the strong association between GEO_PC2 and PC1 of the RDA genotype response matrix (r^2 = 0.43, p < 0.001, $F_{1,173}$ = 130.9).

3.3 | Validation of assignment models

We considered assignment accuracy at the spatial scales of site and region. In the hold-out validation test, assignment accuracy of individuals to sampling site varied with the number of markers used for assignment (Figure 3). We identified 600 SNPs as the optimal number of markers when ranked in descending order of single-locus F_{ST}. Using the least number of markers tested, 200 SNPs, the median assignment accuracy of individuals to site across training sets was 76.4% (range: 65.5%–85.5%). Assignment accuracy peaked at 83.6% (range: 76.4%–87.3%) with the use of 600 SNPs and declined to 50.0% (range: 40.0%–63.6%) with the maximum number of markers tested, 3,000 SNPs. The maximum threshold of 3,000 SNPs was determined from the 20 training data sets which had a range of 3,513 to 3,826 available SNPs, based on the criteria of having two alleles present in every population. Assignment of individuals to region followed a similar pattern and had the highest assignment accuracy of 94.6% using 600 SNPs (range: 90.9%–98.2%). We performed the leave-one-out cross-validation

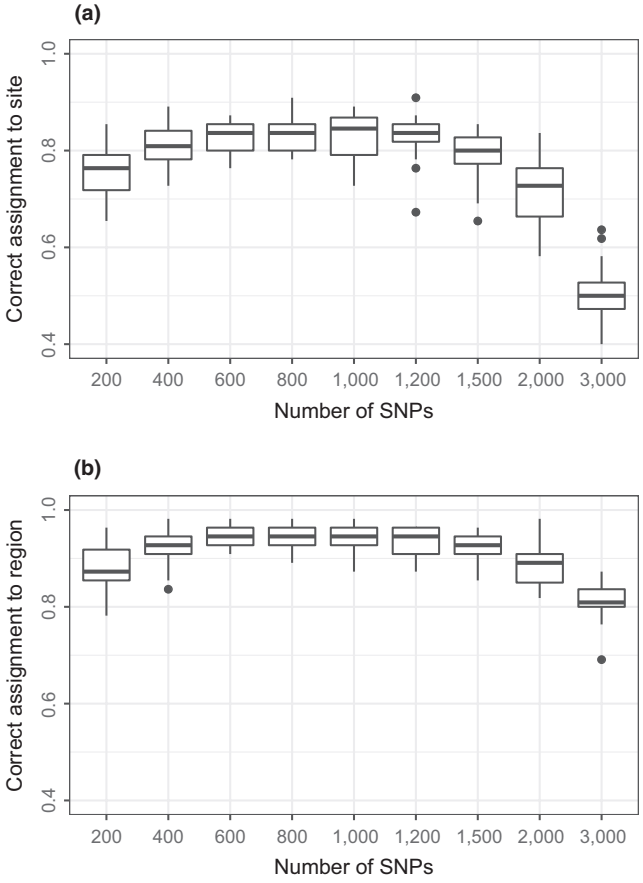

FIGURE 3 Accuracy of assignment models at site and regional scales. In both plots, the *y*-axis corresponds to the proportion of individuals that were correctly assigned to known-origin site (a) or region (b) across 20 bootstrapped training sets in the hold-out validation model. The *x*-axis is categorically divided by the number of SNPs used to assign hold-out individuals to sites and regions. The trajectory of assignment success followed the same pattern in both plots, with assignment accuracy peaking achieving a maximum from 600 to 1,200 SNPs. For subsequent analyses with a subset of the data, 600 SNPs were retained since they provided the highest accuracy with the least amount of data

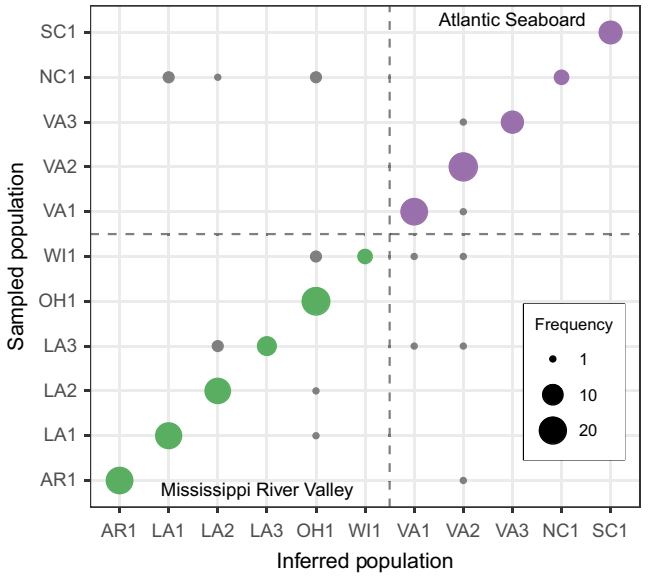

FIGURE 4 Results from the leave-one-out cross-validation model. The size of the plotted circles is scaled by the number of assigned individuals from the sampled population (*y*-axis) to the inferred population (*x*-axis; range 1–22). The circles on the diagonal correspond to individuals that were correctly assigned by site, with sites from the Mississippi region represented by green and sites from the Atlantic region represented by purple. The grey circles off the diagonal represent individuals that were incorrectly assigned to site. Compared to the hold-out validation tests, the leave-one-out cross-validation assignment resulted in a modest increase in assignment accuracy by site, 89.7%, and an equal value of regional assignment accuracy, 94.3%

assignment with 600 SNPs and the F_{ST} of these SNPs ranged from 0.072 to 0.421. With 600 SNPs, the overall F_{ST} across breeding sites was 0.0471 (95% CI: 0.0454–0.0488). Compared to the hold-out validation tests, the leave-one-out cross-validation assignment resulted in a modest increase in assignment accuracy by site, 89.7%, and an equal value of regional assignment accuracy, 94.3% (Figure 4). Assignment accuracy by site was strongly correlated with the number of individuals sampled at each site ($r^2 = 0.84$, $p = 0.0007$, $F_{2,8} = 20.84$), and assignment accuracy plateaued when sites had at least 13 individuals (Figure S7). The assignment likelihoods are provided in the Supporting Information (Table S4).

3.4 | Migratory connectivity

For the assignment of nonbreeding individuals to an origin on the breeding grounds, we assigned individuals to a general region rather than a specific site for two reasons: (a) given the large breeding range of the species, it is improbable that any individual sampled from the nonbreeding ground actually originated from any one of our sampled sites, and (b) we noted that assignment accuracy by site was affected by sample size (Figure S7), and because of this we had low assignment accuracy to some of the sites (Figure 4). Due to close geographic proximity, we combined two of the sites in Panama together as well as two of the sites in Colombia when we conducted the assignment of nonbreeding individuals. Based on these groupings, sample size by site ranged from 3 to 36. There were 19,046 loci that had two alleles in individuals from both of the regions (i.e., not fixed for all individuals in one region), and when reduced to 600 loci with the highest F_{ST}, they ranged in F_{ST} from 0.044 to 0.250. Across all nonbreeding sites, 22 samples (24.4%) were assigned to the Atlantic region and 68 samples (75.6%) were assigned to the Mississippi region. At every site, more individuals were assigned to the Mississippi region than the Atlantic (Table 3). Using relative abundance data from the Partners in Flight Database, 2013), we determined our defined regions of Atlantic and Mississippi to account for 35% and 65%, respectively, of the breeding abundance of prothonotary warblers. Specifically, the Atlantic region contained Partners in Flight Database (2013) estimates from the Atlantic Cost Joint Venture, while the Mississippi region contained estimates from

the Lower Mississippi Valley, East Gulf Coastal Plain, Gulf Coast and Central Hardwoods Joint Ventures. A chi-square test of nonbreeding assignment proportions across the nonbreeding site groupings failed to reject the null hypothesis of deviation from the relative breeding abundance proportions ($\chi^2 = 4.18$, $df = 4$, $p = 0.38$). Thus, the individuals from the two breeding regions were distributed throughout the nonbreeding sites at a comparable proportion to the relative breeding abundance estimates of those two regions.

4 | DISCUSSION

In this study, we used genomic markers to reveal weak multilocus genetic structure and describe migratory connectivity in a Neotropical migratory songbird. In doing so, we have highlighted the importance of selecting informative genomic markers for population assignment models. A subset of 600 SNPs selected by F_{ST} amplified the weak signal of population structure across all methods examined (PCA, DAPC and STRUCTURE). Based on the consensus of methods identifying two lineages between the Atlantic and Mississippi breeding regions, we assigned nonbreeding individuals to breeding region with high assignment accuracy (>90%). In addition, we found a higher proportion of genetic variation on the breeding ground was associated with geography than environment. This result suggests that the genetic divergence in prothonotary warblers is predominantly driven by neutral processes. We demonstrated a high level of accuracy in assigning individuals to known origin, despite weak structure, and identified low breeding–nonbreeding migratory connectivity. Our study provides an effective approach to incorporating genomic data into studies of migratory connectivity in weakly structured species and is broadly applicable to migratory species across taxonomic groups.

4.1 | Patterns of population structure across the breeding grounds

Our results provide evidence of regional breeding structure of prothonotary warbler populations between the Mississippi and Atlantic regions. The weak genetic differentiation within different hierarchical levels (site and region) indicates substantial genetic connectivity among sites within these two regions and/or a recent divergence of large and possibly structured ancestral populations (Marko & Hart, 2011). We demonstrated that the observed genetic structure in prothonotary warblers, although small in magnitude, is in part explained by a model of isolation by distance. We observed this pattern across the entire breeding range and within the Mississippi region, though there is no evidence of isolation by distance within the Atlantic region. The demonstration of isolation by distance within the Mississippi region provides support that the overall model of isolation by distance across the breeding range was not solely driven by the separation of genetic clusters.

We used thousands of SNPs to provide a measure of weak regional genetic structure in the prothonotary warbler. These results are in line with other studies demonstrating that warblers of the eastern United States do not have strong genetic structure among populations, if any structure at all (Avise & Ball, 1992; Deane, McCoy, Robertson, Birt, & Friesen, 2013; Klein & Brown, 1994; Winker, Graves, & Braun, 2000). In the northern part of the prothonotary warbler breeding range, the genetic clusters follow the geographical separation by the Appalachian Mountains; however, we did not have sufficient sampling coverage in the contiguous southern portion of the range to determine boundaries of the clusters. In a comparative phylogeography study of eastern North America, no avian species (of 16 examined) with genetic structure had a genetic discontinuity along the Appalachian Mountains (Soltis, Morris, McLachlan, Manos, & Soltis, 2006). However, our finding of population structure split by the Appalachian Mountains in the prothonotary warbler is not surprising in the light of Soltis et al. (2006) concluding that phylogeography of the eastern United States lacks consistent spatial patterning due to the large number of barriers uniquely affecting each individual species. For prothonotary warblers, the Appalachian Mountains and surrounding foothills constitute a region of little suitable breeding habitat (e.g., bottomland hardwood forest).

In addition to describing population structure, we found that geography explained more genetic variation (14.5%) than environment (8.2%) in a 600 SNP response matrix. The majority of genetic variability was not explained by the constrained axes (76.2%), which was to be expected given the overall weak population structure. The RDA results, along with evidence of isolation by distance from the Mantel test, suggest that genetic drift has a strong role in genetic differentiation among prothonotary warbler breeding sites. Additionally, the observed genetic variation associated with environment may be indicative of local adaptation, given that breeding sites varied in environmental conditions. The amount of total variance explained, as well as the individual contributions of geography and environment, was generally higher than reported in other avian studies using RDA (Friis et al., 2018; Szulkin, Gagnaire, Bierne, & Charmantier, 2016). This may be due to our use of a much smaller subset of 600 SNPs data filtered by F_{ST} than random sets of more than 10,000 SNPs.

Environmental association analyses of avian species have found ecological factors such as temperature (Andrew, Jensen, Hagen, Lundregan, & Griffith, 2018; Friis et al., 2018; Seeholzer & Brumfield, 2017; Termignoni-Garcia et al., 2017; Zhen et al., 2017), precipitation

TABLE 3 Values are given for the number of individuals assigned from each nonbreeding site to the corresponding region, and percentages of assigned individuals to region are listed in parentheses

Nonbreeding site	Mississippi	Atlantic
PN1	6 (85.7%)	1 (14.3%)
PN2	11 (64.7%)	6 (35.3%)
CO1	6 (85.7%)	1 (14.3%)
CO2	23 (79.3%)	6 (20.7%)
CO3	19 (70.4%)	8 (29.6%)
CO4	3 (100.0%)	0 (0.0%)

(Bay et al., 2018; Friis et al., 2018; Zhen et al., 2017) and habitat (Szulkin et al., 2016) to be associated with spatial patterns of genomic variation. In our study, the relation of ENV_PC1 with temperature may be indicative of local adaptation associated with temperature differences, including seasonality, among the breeding sites. For example, aquatic prey are an important component of prothonotary warbler nestling diet in a population in the Atlantic Seaboard (Dodson, Moy, & Bulluck, 2016), and the distribution and emergence of these aquatic insects are strongly affected by temperature (Ward & Stanford, 1982). Varying abundance and distribution of terrestrial and aquatic prey across the range of prothonotary warblers could lead to local adaptation based on food availability, as shown in other species (Card et al., 2018; Chaves et al., 2016). It is important to note that only a subset of the genome will be associated with ecological factors and that our study incorporated only 26,189 SNPs, thus we may not have sampled important markers nor considered other relevant predictors of adaptive variation in the RDA (Meirmans, 2015). Although out of the scope of this study, a comprehensive investigation of bioclimatic variables closely tied to more proximate factors for prothonotary warblers (e.g., seasonal temperature windows that correspond with aquatic insect growth efficiency and emergence success) could help us better understand ecological influences on genetic variation.

Although we solely examined environmental variables from the breeding grounds, migratory species may have a higher proportion of genetic variation associated with migratory pathways than with breeding sites (as is the case in anadromous salmon; Micheletti, Matala, Matala, & Narum, 2018). A recent light-level geolocator study described two distinct migratory pathways for prothonotary warblers with more western breeding birds crossing the Gulf of Mexico to the Yucatán peninsula and eastern breeders migrating south through Florida (Tonra2019, i2019). The breeding individuals with different migratory routes generally aligned with our genotype clustering results. One exception was that individuals from a site in Ohio (considered in our study to be part of the western genotype cluster) followed the eastern migratory route down to Florida (Tonra et al., i2019). This difference in migratory routes within a genotype cluster provides a potentially useful system for future research to disentangle the proportion of genetic variation associated with migratory pathways versus breeding sites.

4.2 | Marker selection for assignment accuracy

Despite minimal genetic structure, we assigned individuals sampled on their breeding grounds to their sampling sites in the hold-out validation model with high accuracy. Our results provide evidence that weakly differentiated markers are uninformative in population assignment and add noise to the prescribed models, consequently decreasing the overall performance of the model. Similar decreases in performance of population assignment models have been documented by Sylvester et al. (2017) in a broad analysis of methods for filtering SNPs in population assignment models. Importantly, we also found that filtering SNPs had a profound effect on the ability of different methods to detect a weak signal of population structure. Using the subset of 600 highly differentiated SNPs in STRUCTURE, DAPC and PCA, we identified two genetic lineages, separated between the Atlantic and Mississippi breeding regions, as appropriate for population assignment. Using all 26,189 SNPs, we identified the same two genetic lineages in PCA as with the subset of SNPs, while STRUCTURE and DAPC did not provide support for two lineages. These results highlight the importance of consulting different methods for describing population structure when conducting genetic population assignment. While we used F_{ST} to select SNPs, random forest is another highly effective method for marker selection that can lead to higher assignment accuracy than selecting by F_{ST} (Sylvester et al., 2017).

Our assignment accuracy in the leave-one-out cross-validation model (89.7% to site and 94.3% to region) was comparable to another migratory connectivity study using molecular markers (Ruegg et al., 2014), which reported 80%–100% accuracy for assigning individuals to six groups following the same assignment method (Anderson, 2010). Ruegg et al. (2014) used 96 highly divergent SNPs for their analyses and reported a global F_{ST} of 0.179. Our study used 600 SNPs, and the global F_{ST} with these markers was 0.047 (F_{ST} = 0.0055 with 26,189 SNPs). In order to more accurately compare to Ruegg et al. (2014), when we reduce our SNP data set to the same ratio used in their study (96 SNPs out of 19,707 SNPs, 0.48%), then the global genetic differentiation with 0.48% of our data set is F_{ST} = 0.079 (127 SNPs). These results may suggest a need for increasing numbers of markers for population assignment as population differentiation decreases. Further corroborating this pattern are the results from population assignment of American lobsters (*Homarus americanus*; Benestan et al., 2015), in which they achieved maximum assignment accuracy (>80%) using 3,000 SNPs with considerably weaker genetic differentiation (F_{ST} = 0.0019 reported across 8,144 SNPs).

Our assignment accuracy was strongly driven by the sample size of the site being assigned to and our results indicate a need of at least 13 samples per site for assignment accuracy greater than 90% (Figure S7). Although the magnitude of genetic differentiation among our sites does not preclude accurate assignment, lower genetic differentiation may result in the need for a larger sample size to achieve similar results. Our results are similar to other studies using ddRADseq that have shown at least 34 samples per site may be needed to reach maximum assignment accuracy in populations with weaker genetic differentiation (F_{ST} = 0.0019, Benestan et al., 2015). While there is no prescriptive formula for the minimum sample size needed for a study, these results may serve as guidelines for sampling design when considered with general estimates of genetic differentiation, power analyses and the resolution needed for the specific study. An important aspect to note when filtering highly differentiated markers for population assignment is that parallel genetic signatures of adaptive variation among geographically disparate sites could confound assignment. While this scenario may be unlikely, a simulation of the effects on assignment from this type of scenario could provide guidance for population assignment models. In our study, the results of the RDA showed the vast majority of genetic variability was not

associated with environment, which suggests that genetic signatures of adaptive variation are not confounding the results of population assignment.

4.3 | Conservation implications of low migratory connectivity

We provide strong evidence of low migratory connectivity in the core nonbreeding range of the prothonotary warbler. Altogether, the nonbreeding individuals across each sampling site were predominantly assigned to the Mississippi breeding region (76%). The proportions of assigned individuals to region are comparable to the relative abundance of prothonotary warblers in these two breeding regions, signifying the mixing of individuals on the nonbreeding ground (i.e., low migratory connectivity). If, as we suggest, individuals from various breeding locations are intermingling across the nonbreeding range, the species may be relatively resilient to nonbreeding habitat loss in any one location (Gilroy, Gill, Butchart, Jones, & Franco, 2016). However, the habitat specificity of the prothonotary warbler makes the species particularly vulnerable to habitat loss at sites throughout the annual cycle (Petit, 1999), as does the fact that the majority of individuals appear to overwinter in one region (northern Colombia; Tonra et al., i2019) On the breeding grounds, despite a reduction in the rate of land conversion (e.g., bottomland forest converted to agricultural land in the Mississippi Alluvial Valley) and wetland draining over the past 50 years, forested wetlands in the eastern United States are still in decline (Sucik & Marks, 2015). And on the wintering grounds, mangrove forests, their preferred nonbreeding habitat (Petit, 1999), may be completely lost to sea level rise in the next 100 years (Duke et al., 2007).

One caveat to our results is that our nonbreeding sites were concentrated within the centre of the nonbreeding range where we might expect more mixing to occur than on the periphery of the range (Finch et al., 2017). However, this may not be an issue given that for prothonotary warblers, Tonra et al. (2019) showed that 91% of 33 prothonotary warbler individuals sampled with geolocators from across the breeding range migrated to northern Colombia for the nonbreeding season, with the remaining birds wintering in neighbouring Panama and Venezuela. This suggests that northern Colombia may constitute a core nonbreeding area for prothonotary warblers despite being approximately one-fifth the size of the entire nonbreeding range. Thus, our lack of sampling along the periphery of the nonbreeding range likely only excluded a very small proportion of the nonbreeding population. A management benefit of having a concentrated area of prothonotary warbler abundance on the nonbreeding grounds is that conservation resources can be focused on a smaller nonbreeding area that would benefit numerous breeding populations.

5 | CONCLUSIONS

In this study, we demonstrated the utility of filtering high-resolution SNP markers for achieving high accuracy in population assignment models. Despite weak genetic differentiation among sites, individuals can be accurately assigned to sites using relatively few samples (<20 individuals) and a moderate number of genomic markers (<1,000 SNPs). Using this technique, genetic migratory connectivity studies can describe sufficiently fine-scale population structure for the use of population assignment models for an increasing number of species. For migratory connectivity studies, these methods are especially beneficial for species and taxa that are too small or otherwise unable to have physical tracking devices attached to their bodies. Our identification of low migratory connectivity in prothonotary warblers allows us to work towards a full annual cycle model for this species, a crucial step towards the conservation and management of migratory species (Marra, Cohen, et al., 2015). Future studies should continue to expand our knowledge of the full annual cycle of migratory songbirds (e.g., migratory route variability) and incorporate genomic data to explore the evolutionary processes that underlie interspecific variation in migratory connectivity.

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AUTHOR CONTRIBUTIONS

M.G.D., L.P.B., C.B.V., R.J.D., T.J.B. and C.M.T. designed the study. M.G.D., L.P.B., C.B.V., T.J.B., C.M.T. and J.A.R. organized field

sampling and procured funding. M.G.D. generated the genomic data and performed the data analysis with contributions from A.J.E. and R.J.D. The majority of the manuscript was written by M.G.D. and all authors edited the manuscript.

DATA ACCESSIBILITY

The scripts for population assignment are available on GitHub (https://github.com/mgdesaix/populationAssignment). Genomic data (SNP calling in 012 format) and the climate input data are available on Dryad: https://doi.org/10.5061/dryad.hb1g263.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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