Received: 24 March 2023 | Revised: 23 August 2023 | Accepted: 30 August 2023

DOI: 10.1111/mec.17129

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Whole genome assessment of a declining game bird reveals cryptic genetic structure and insights for population management

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Handling Editor: Paul A. Hohenlohe

Abstract

Population genomics applied to game species conservation can help delineate management units, ensure appropriate harvest levels and identify populations needing genetic rescue to safeguard their adaptive potential. The ruffed grouse (*Bonasa umbellus*) is rapidly declining in much of the eastern USA due to a combination of forest maturation and habitat fragmentation. More recently, mortality from West Nile Virus may have affected connectivity of local populations; however, genetic approaches have never explicitly investigated this issue. In this study, we sequenced 54 individual low-coverage (~5X) grouse genomes to characterize population structure, assess migration rates across the landscape to detect potential barriers to gene flow and identify genomic regions with high differentiation. We identified two genomic clusters with no clear geographic correlation, with large blocks of genomic differentiation associated with chromosomes 4 and 20, likely due to chromosomal inversions. After excluding these putative inversions from the data set, we found weak but nonsignificant signals of population subdivision. Estimated gene flow revealed reduced rates of migration in areas with extensive habitat fragmentation and increased genetic connectivity in areas with less habitat fragmentation. Our findings provide a benchmark for wildlife managers to compare and scale the genetic diversity and structure of ruffed grouse populations in Pennsylvania and across the eastern USA, and we also reveal structural variation in the grouse genome that requires further study to understand its possible effects on individual fitness and population distribution.

KEYWORDS
balancing selection, *Bonasa umbellus*, chromosome inversion, population genomics, spatial genetic variation, wildlife management

David P. L. Toews and Julian D. Avery contributed equally to this work.

1 | INTRODUCTION

Managing wildlife populations is a complex task that requires knowledge of multiple factors, including spatial genetic variation. In recent years, advances in high-quality genome sequencing have made it possible to generate a substantial amount of data on genetic variation within and between populations (Brandies et al., 2019; Paez et al., 2022). Along with the increasing availability of genomic data comes an exciting potential to transform the field of wildlife management (Hohenlohe et al., 2021; Toews et al., 2018; Theissinger et al., 2023). For example, assessing genetic structure and levels of gene flow within a population is essential for management actions that aim to reduce the underlying risks of genetic variability loss associated with rapid population declines and fragmentation (Hohenlohe et al., 2021; Luna et al., 2022). By leveraging genome-wide information, wildlife managers can gain a deeper understanding of the needs of declining populations, ultimately leading to more effective conservation efforts.

Many wildlife species in North America are in rapid decline (Brown et al., 2019; Pimm & Askins, 1995; Sauer & Link, 2011), and genomic data can help identify which target species or populations are most at risk and need targeted conservation efforts. This is especially important for species that experience harvest or have socio-economic importance (Allendorf et al., 2008). For instance, genomic data have been crucial for salmonid conservation efforts in the North American Pacific Northwest, allowing for adaptive harvest models in real time (Garner et al., 2016), and for the identification of non introgressed populations of the heavily managed red-legged partridge (*Alectoris rufa*) in Europe, highlighting populations that warrant protection (Forcina et al., 2021). There is a growing push to develop genomic resources for game and threatened species conservation as environmental and anthropogenic stressors increasingly impact populations (Hogg et al., 2022), particularly when increased resolution is needed over traditional molecular approaches (Garner et al., 2016).

Species simultaneously declining in abundance and experiencing harvest are particularly vulnerable, and resource managers need detailed information that helps them spatially prioritize populations. The ruffed grouse (*Bonasa umbellus*) is an iconic North American game bird with considerable socio-economic importance (Knoche & Lupi, 2013). Ruffed grouse, which are dependent upon early successional habitats, have experienced steep population declines in recent decades. This downward trend is likely due to a combination of factors, including habitat loss and fragmentation, predation pressure, climate change, and disease (Dessecker & McAuley, 2001; Stauffer et al., 2018). Pennsylvania is one of the states hardest hit by this decline, with estimates suggesting that the number of ruffed grouse has decreased by as much as 70% since the early 1960s (Fink et al., 2022; Sauer et al., 2014). Reduced occupancy due to forest fragmentation is one of the most significant threats to ruffed grouse populations in Pennsylvania, especially in the southernmost areas of the commonwealth (Stauffer et al., 2018). Habitat fragmentation alters essential ecological processes and imposes serious genetic risks to small, isolated populations (Cheptou et al., 2017; Debinski & Holt, 2000; Haddad et al., 2015). As a result, the gene pool of each fragment can become increasingly isolated, leading to genetic divergence, and loss of genetic diversity through drift and inbreeding. These processes can be further accelerated by selection, as different fragments may be subject to varied selection pressures (Cheptou et al., 2017).

More recently, mortality caused by West Nile Virus (WNV) has been a major driver of ruffed grouse declines, potentially affecting the connectivity of local populations (Nemeth et al., 2021; Stauffer et al., 2018). West Nile Virus is a mosquito-borne virus that was first detected in North America in 1999 and has since influenced the decline of several bird populations across North America (LaDeau et al., 2007). Ruffed grouse are particularly susceptible to WNV infection, and mortality rates can be as high as 30% with negative population effects likely to continue (Nemeth et al., 2017, 2021; Stauffer et al., 2018). This can result in a loss of genetic connectivity between populations, as well as lower population density and reduced reproductive success (Charlesworth, 2003; Charpentier et al., 2005; Frankham, 1996). To mitigate the impact of low connectivity and WNV on ruffed grouse populations, conservation measures should focus on creating and maintaining large tracts of suitable habitat (Nemeth et al., 2021; Stauffer et al., 2018). However, as human development continues to fragment and degrade forest habitats, ruffed grouse populations will become more isolated from each other, increasing the risk of losing genetic diversity and adaptive potential, making them more vulnerable to WNV outbreaks and Allee effects (Berec et al., 2007).

Previous ruffed grouse population genetic studies have indicated that significant population divisions can be attributed to the combined effect of macrogeographic barriers (e.g. the Rocky Mountains in the western US) and unsuitable habitat (Honeycutt et al., 2019; Jensen et al., 2019; Perktaş, 2021). However, these studies have been conducted on a large scale, encompassing most of the species' distribution, and have only examined a limited number of genetic markers. To date, no research has thoroughly investigated the effects of landscape-level variation and recent abundance declines on the species' population connectivity using comprehensive genomic data.

The use of population genomics to test hypotheses of genetic diversity and structure in fragmented habitats has revolutionized our understanding of wild population interactions (Hohenlohe et al., 2021). Genomics is bringing important insights into variation in neutral and adaptive loci, illustrating how environmental and landscape-level factors shape populations in different ways (Barbosa et al., 2021), which is critical to understand at smaller scales when conducting translocations and reintroductions. Genomics is also shedding light on cryptic population structure, with important implications for captive breeding and the aforementioned efforts to move individuals and augment populations (Pedersen et al., 2018; Silva et al., 2018).

Whole-genome resequencing, particularly when employing highly contiguous, chromosome level reference genomes, has played a vital role in identifying genetic structural variations, such as inversions, duplications and deletions (Mérot et al., 2020; Wold et al., 2021). These structural variants can significantly influence gene function, thereby controlling polymorphisms in phenotypic traits of ecological and

evolutionary importance (Wellenreuther & Bernatchez, 2018). Consequently, they can also have profound effects on individual and population fitness (Berdan et al., 2021; Hager et al., 2022). Recognizing these structural variants and their potential relationships with adaptive traits can bring important insights into conservation biology and species management, especially for economically important species. By managing individuals with compatible genetic traits, negative impacts on fitness can be avoided, and the preservation of genome-wide diversity can be promoted (Wold et al., 2021), especially in the context of reduced connectivity due to habitat fragmentation.

In the case of ruffed grouse in Pennsylvania, sequencing the complete genomes of individuals from different populations can help to identify patterned genetic variation on an effectively manageable scale. This information can then be used to delineate management units, ensure appropriate harvest levels, prioritize populations of conservation importance and identify populations that need genetic rescue (Allendorf et al., 2008; Funk et al., 2012; Hohenlohe et al., 2021). Beyond that, understanding the spatial genetic variation of different ruffed grouse populations can also provide a benchmark where wildlife managers can compare and scale the genetic diversity and structure of ruffed grouse, not only in Pennsylvania but also across the eastern USA.

We use the ruffed grouse as a model for genomic game species population characterization because of the multiple synergistic threats from disease, habitat loss and fragmentation, and the need to inform effective game species management efforts. We sequenced and assembled the first high-quality, chromosome-level, reference genome for ruffed grouse, along with 54 individual low-coverage genomes to assess fine-scale genetic diversity and structure across Pennsylvania. As a null hypothesis, ruffed grouse populations show

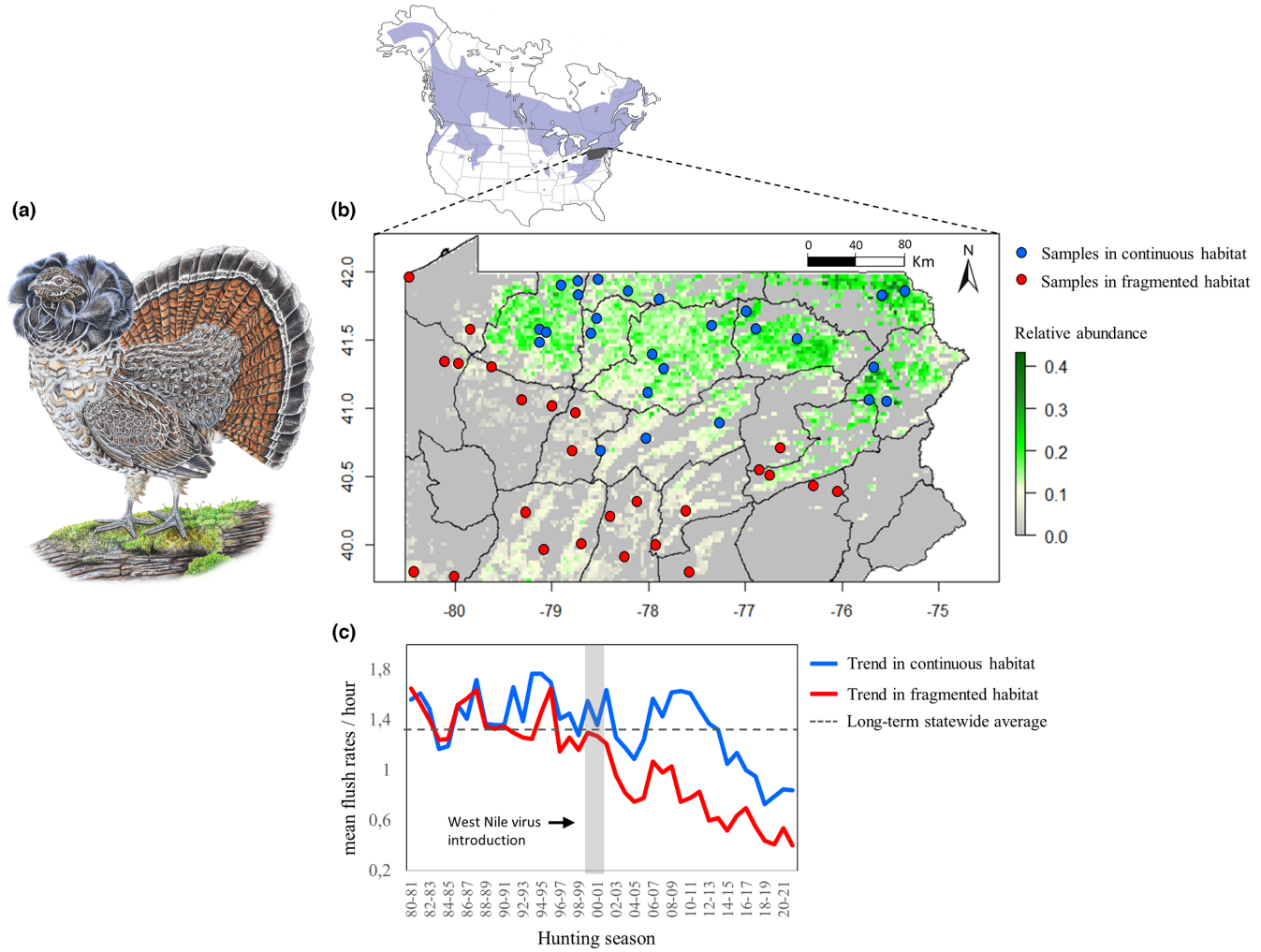

FIGURE 1 Map of ruffed grouse sample distribution and relative abundance in Pennsylvania, USA, divided into Wildlife Management Units (WMU). (a) The ruffed grouse (*Bonasa umbellus*), illustrated by Andreza Silva. (b) On the map, blue circles refer to individuals collected in WMU with continuous habitats (high local connectedness), and red circles are individuals collected in fragmented habitats (low local connectedness). The relative annual abundance of the ruffed grouse is depicted in grey (no record) and gradients of light yellow (low abundance) and light to dark green (high abundance). (c) Mean ruffed grouse flushes per hour, divided into trends in regions with a higher proportion of continuous (blue line) and fragmented (red line) forest habitat referent to the long-term statewide average (dashed line), as reported by the Pennsylvania Game Commission during hunting season surveys from 1980 to 2021.

no significant signs of genetic differentiation due to the natural maintenance of gene flow by dispersal, regardless of habitat conditions or past population reductions. Alternatively, we hypothesize that ruffed grouse are experiencing reduced connectivity (i.e. low gene flow) due to a combination of extensive historical habitat fragmentation and population decline caused by WNV mortality, especially in the southernmost distributions of the commonwealth (Figure 1). Our genetic predictions are that isolated localities with a history of severe demographic decline and patchy habitat show signs of genetic differentiation (i.e. substructure or reduced gene flow) when compared to individuals sampled from continuous habitats and historically more stable populations. To test this hypothesis, we (1) determined the population genetic diversity and structure of ruffed grouse across Pennsylvania, (2) assessed migration rate variation across the landscape to identify potential barriers to gene flow, (3) quantified the impact of habitat fragmentation on genetic connectivity, relative to other geographic features, (4) identified genomic regions with high differentiation and (5) estimated and compared genetic diversity and signals of selection both genome-wide and between potential genomic clusters. Access to this genomic data is essential to understand the relationship between functional connectivity and environment in the recent history of this prized game bird, thus producing relevant spatial information to improve management efforts.

2 | METHODS

2.1 | Reference genome sequencing, assembly and mapping

The sample of ruffed grouse was obtained by salvage on 27 February 2021 where blood was obtained, while the bird was alive, put into BD microtubes, and sent immediately on wet ice to DoveTail genomics. The genome sequencing was completed by DoveTail (see details in Appendix S1). The initial assembly was produced by generating continuous long reads run on a PacBio Sequel II to a depth of 136X coverage. The individual sequenced for the reference genome was deposited at the Cornell University Museum of Vertebrates in Ithaca New York, with catalog no. CUMV-59724.

To quantify the completeness of the genome assembly, we used BUSCO 4.0.5 (Manni et al., 2021) with the eukaryota_odb10 loci. For the annotation of the genome, repeat families found in the genome assemblies were identified de novo and classified using the software package RepeatModeler 2.0.1 (Flynn et al., 2020). Coding sequences from *Coturnix japonica*, *Gallus gallus* and *Taeniopygia guttata* were used to train the initial ab initio model for *Bonasa umbellus* using the AUGUSTUS 2.5.5 (Stanke et al., 2008). To help assess the quality of the gene prediction, AED scores were generated for each of the predicted genes as part of the MAKER pipeline (Cantarel et al., 2008). Genes were further characterized for their putative function by performing a BLAST search (Boratyn et al., 2013) of the peptide sequences against the UniProt database. tRNA was predicted using the software tRNAscan-SE 2.05 (Chan et al., 2021). For more details on the genomic library preparation pipeline, completeness quantification, and genome annotation procedures, see Appendix S1.

2.2 | Sampling design

To represent spatial genetic variability of ruffed grouse across the Pennsylvania commonwealth, we selected 54 samples within two categories of habitat connectivity, that is continuous and fragmented (Figure 1). Categorical classification of the samples into continuous and fragmented habitats was based on the distribution pattern of ruffed grouse relative annual abundance using eBird data (Fink et al., 2022) and local connectivity levels per Wildlife Management Units (WMUs, sensu Pennsylvania Game Commission https://www.pgc.pa.gov/Wildlife/HabitatManagement/WildlifeManagementUnits/Pages/default.aspx) calculated using The Nature Conservancy's Resilient Land Mapping Tool (https://maps.tnc.org/resilientland/). Local connectedness was calculated by measuring the amount and configuration of barriers such as main roads, urbanized areas, farmland and forestry land (Figure S1). Furthermore, to identify population-specific management units based on the genetic data, the sampling design aimed to represent all current WMUs where ruffed grouse populations are not presumed extirpated. All samples were from hunter-harvested grouse tissues donated to PGC between 2014 and 2020 (Table S1). We used different tissues such as feathers, toe pads, muscle and dried skin to obtain adequate yields of DNA (Table S1).

2.3 | DNA extraction and genomic library preparation

We extracted total DNA from different tissue types (Table S1) using the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's protocol. All different tissue types yielded adequate DNA yield (>23 ng/μL) for downstream applications. All samples were equalized to ~2 ng/μL and we used bead-linked transposomes (BLT) to shred and tagment DNA with adapter sequences. Finally, we prepared sequencing libraries using the Illumina DNA Prep protocol and sent them to the Pennsylvania State University Genomics Core Facility for sequencing (150 nt, paired-end) in a single NextSeq Hight Output Lane, targeting genomic coverage of ~5X per sample.

2.4 | Bioinformatics and population genomic structure

We removed the sequence adapters and quality trimmed reads using AdapterRemoval 2.1.7 (Schubert et al., 2016), following the '-collapse-trimns -minlingth 20 -qualitybase 33' options. Alignment of reads to the new *B. umbellus* reference genome was performed with BowTie2 (Langmead & Salzberg, 2012), while

Polymerase chain reaction duplicates were marked with Picard (Broad Institute, 2021). Evaluation of genomic data quality statistics and coverage was performed using qualimap 2.2.1 (Okonechnikov et al., 2016).

We used multivariate and Bayesian approaches to evaluate ruffed grouse population structure. First, we performed principal component analysis (PCA) to find genomic clustering signals using PCAngsd (Meisner & Albrechtsen, 2018), which uses genotype likelihoods from variable sites as inputs to compose a covariance matrix. Genotype likelihoods were estimated at ANGSD 0.938 (Korneliussen et al., 2014) using the GATK model (McKenna et al., 2010) option -GL 2, with the removal of low-quality reads (–remove_bads -minMapQ 20 -minQ 20), reads with unmapped pairs (–only_proper_pairs) and restricted to sites with significant evidence for the presence of SNPs (-SNP_pval 1e-6). Then, we tested the number of ancestral populations (*K*) using sNMF (Frichot et al., 2014). Since sNMF requires individual differences in called SNPs, we output genotype calls (-doGene 4 -postCutoff 0.99 -minMaf 0.06 -SNP_val 1e-6) from ANGSD. We tested models with 100 replicates, 100 iterations and an alpha regularization parameter of 1000 for each value of *K* (from 1 to 6).

We assessed the spatial pattern of gene flow across the landscape using the Estimated Effective Migration Surface (EEMS, Petkova et al., 2015) method. This approach measures the decay of genetic similarity of individuals from geographically indexed data, highlighting areas that potentially deviate from the null expectation of isolation by distance (IBD), thus identifying potential barriers to gene flow. To capture the spatial heterogeneity of gene structure across the distribution of our samples, we selected a deme size of 300 and applied a run with MCMC length of 20×10^6 and burn-in 2×10^6. We then used the program eems.plots in R (R Core Team, 2022) to evaluate convergence and plot the results. We also tested whether the mean values of genetic similarity residues (decorrelated with geographic distance), inbreeding coefficient (*F*-statistic), and nucleotide diversity are significantly different between individuals sampled in continuous vs. fragmented forest habitats using non-parametric *t*-tests. Estimates of per-individual inbreeding coefficient accounting for population structure were performed in the ngsF program (Vieira et al., 2016) and nucleotide diversity was estimated using ANGSD.

2.5 | Isolation by resistance

To assess the impact of habitat fragmentation, geographic distance, and terrain elevation on the genetic connectivity of grouse populations, we employed two isolation-by-resistance (IBR) approaches using Mantel tests and Maximum Likelihood Mixed Models of Population Effects (MLPE; Clarke et al., 2002). First, to estimate the proportion of spatial genomic variation explained only by geographic distance and habitat resistance, we conducted Euclidean distance (ED), least-cost path (LCP) and resistance distance (RD) analyses using the R package gdistance 1.6.4 (van Etten, 2017). Since grouse abundance and occupancy are positively related to the availability of forested habitat (Stauffer et al., 2018), we derived habitat resistance from Global Tree Cover maps (Hansen et al., 2013) at 1 arc second resolution (~30 km^2 per pixel) and converted to conductance values using the transition function of the gdistance package (Figure S2). LCPs and RDs were calculated between each pair of individuals using the functions costDistance and commuteDistance, respectively (van Etten, 2017). To determine the proportion of genetic distance variance explained by the predictors (ED, LCP, and RD), we conducted Mantel tests with 10,000 permutations using vegan 2.6.4 package (Oksanen et al., 2019).

Next, to account for the nonindependence of genetic distances between pairs, we used mixed effects least squares regression and penalty models with correlation structure (Clarke et al., 2002) by employing the 'lme' function in the nlme 3.1–152 (Pinheiro et al., 2020) and the corMLPE 0.0.3 R packages (https://github.com/nspope/corMLPE). In this approach, the construction of the habitat resistance matrix involved identifying areas with low forest cover presence (height <1 m), which are presumed to hinder grouse dispersal (i.e. gene flow) and therefore exhibit higher resistance values (Dessecker & McAuley, 2001; Yoder et al., 2004). In this case, we consider that forested areas promote gene flow (resistance = 0.1), while nonforested areas prevent it (resistance = 0.9). Terrain elevation variables were accessed using raster maps with a resolution of 2.5 m per pixel of the SRTM elevation data from WorldClim 2.1 (https://www.worldclim.org/).

To ensure the avoidance of highly correlated predictors, we applied the dredge function from the MuMIn 1.43.17 R package (https://github.com/rojaff/dredge_mc), eliminating models with a correlation coefficient (r^2) greater than .6. The best models for investigating the IBR were identified using delta Akaike's information criterion (ΔAIC <2; Harrison et al., 2018), with confidence intervals for association coefficients estimated through the restricted maximum likelihood (REML) method (Silk et al., 2020). Likelihood ratio tests were performed to determine the best model among the nested models using the anova.lme function in the nlme R package, and the significance of predictors were evaluated through chi-square contingency table tests using the drop1 function in the stats R package. To quantify the variance explained by the model, we calculated the conditional coefficients of determination (conditional R^2) using the MuMIn R package (Nakagawa & Schielzeth, 2013). The relative importance of each predictor in explaining genetic connectivity was determined by summing the AIC weights across all models with ΔAIC <2 using the get.models and importance functions in the MuMIn package.

2.6 | Genome-wide differentiation, diversity, and neutrality test

To assess differentiation between genomic clusters discovered in our population structure analyses, we estimated the fixation index (F_{ST}) using ANGSD. For this approach, we generated the site frequency spectrum (SFS) of SNPs between each genomic cluster using the realSFS tool implemented in ANGSD. We then generated

windowed estimates of F_{ST} at 10 kb across the genome to identify divergent regions when comparing different genetic clusters. F_{ST} results across the genome were visualized through a manhattan plot using the R package qqman 0.1.8 (Turner, 2018). We determined which annotated genes are present within the highly differentiated genomic regions using the ruffed grouse reference genome annotation. Finally, we estimated admixture proportions between individuals from different gene clusters using NGSadmix (Skotte et al., 2013).

We estimated nucleotide diversity (π) and tested the expectation of neutral genetic evolution (i.e. drift-mutation equilibrium), and calculated Tajima's *D* (Tajima, 1989) across the genome and within genomic clusters using ANGSD. We calculated both π and *D* for each site in non-overlapping 10 kb windows across the genome by estimating SFS in the realSFS tool. These summary statistics can reveal signs of selection (e.g. selective sweep and balancing selection) in regions across the genome, so we tested whether the values of π and *D* are significantly different between genomic clusters relative to background genomic variation. To test the significance of the comparisons, we performed a nonparametric Kruskal–Wallis Test in R.

3 | RESULTS

3.1 | Genomic statistics and population genomic structure

The resulting genome assembly was 1.004 Gbp, with a scaffold N50 of 69.1 Mbp, and 97.25% complete and single-copy BUSCOs. It is available via NCBI at PRJNA1008140 accession no. We resequenced 54 ruffed grouse specimens across the state of Pennsylvania, USA, yielding an average per individual genome-wide coverage of ~5.3X (ranging from 2.3 to 9.8X).

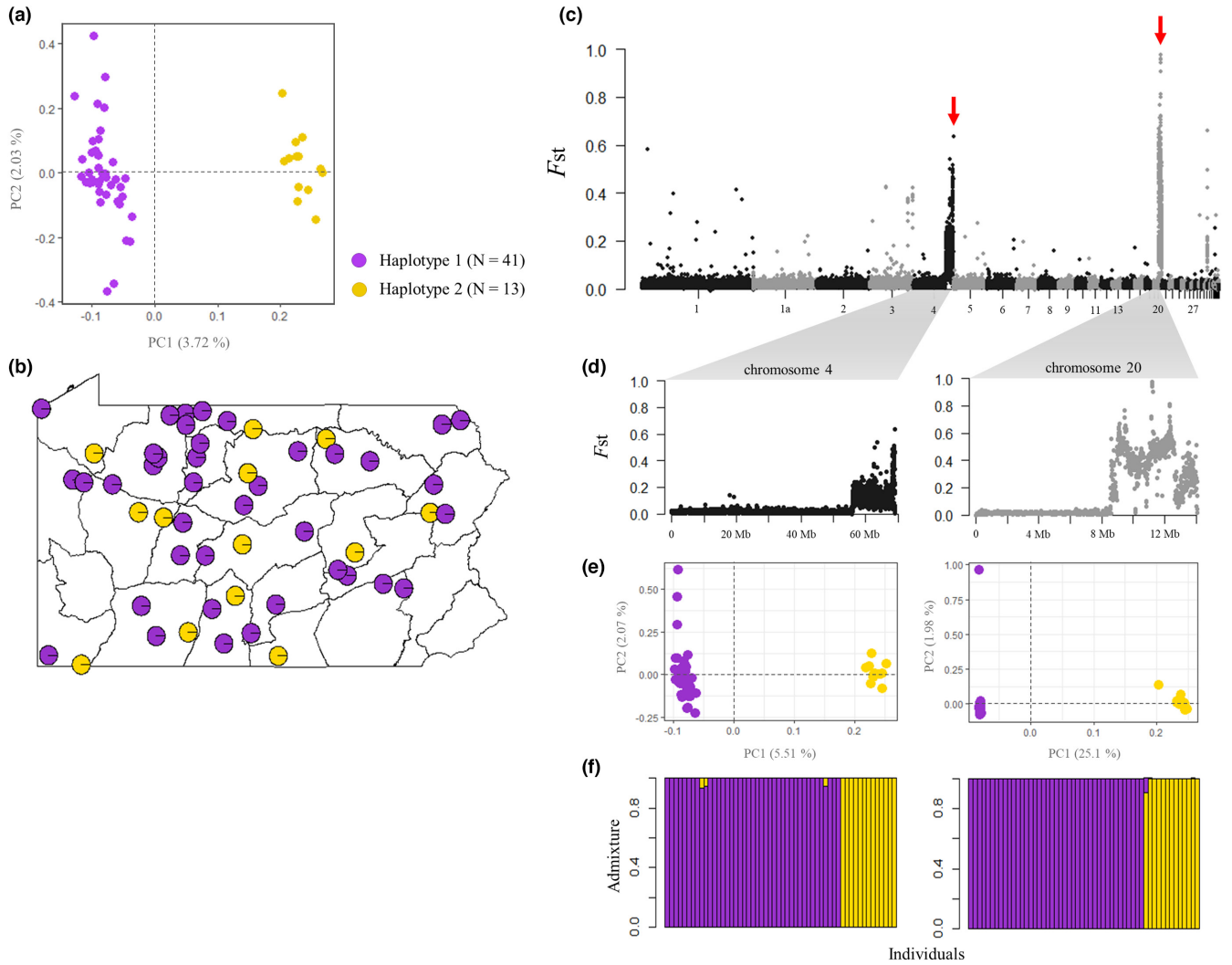

FIGURE 2 Ruffed grouse genome-wide differentiation. (a) Principal Component Analysis (PCA) showing two genomic clusters (or haplotypes), where purple circles represent haplotype 1, and yellow circles haplotype 2. (b) Distribution of haplotypes within the ruffed grouse population in Pennsylvania, USA. (c) Windowed F_{ST} estimates (10 kb) comparing the two genomic clusters, (d) within chromosomes 4 and 20, which show large blocks of differentiation. (e) Results of PCA and (f) Admixture of the two divergence regions identified on chromosomes 4 and 20.

PCA results for whole-genome SNPs showed two genomic clusters (Figure 2a), which we describe as haplotypes 1 and 2. Haplotype 1 comprises 41 (75.9%), and haplotype 2 contains only 13 (24.1%) samples. However, these clusters have no obvious geographical correlation (Figure 2b). Comparisons of genomic differentiation between the two genomic clusters indicated two large blocks of differentiation (F_{ST}) associated with chromosomes 4 (mean F_{ST} = .385) and 20 (mean F_{ST} = .126) (Figure 2c). These differentiation blocks span 14 Mbp on chromosome 4 and 6 Mbp on chromosome 20 (Figure 2d), representing ~2% of the genome. The levels of differentiation on these chromosomes were 7- to 22-fold greater than the global genome-wide differentiation values F_{ST} = .017. PCA and NGSadmix of the SNPs found on these chromosomes corroborate the two haplotype groups, indicating no intermediates, where individuals that possess a haplotype for chromosome 4 possess the same haplotype on chromosome 20 (Figure 2e,f, and Table S2).

When we excluded SNPs within the two highly differentiated blocks, our PCA was unable to recover clear genomic clusters corresponding to the two haplotypes (Figure 3a). These same reduced data sets also did not recover strong geographic genetic substructure in the sNMF analysis, suggesting that genetic variation within the Pennsylvania ruffed grouse population is the best fit for the $K = 1$ model (Figure S3). Visualizations of K values >1 also failed to recover any signs of shallow geographic substructure (Figure S4). On the contrary, migration surface estimates showed some degree of cryptic population structure, with lower gene flow rates (m) in inferred areas with extensive habitat fragmentation in the southwestern and southeastern regions. These are areas with a long history of urbanization in the state of Pennsylvania (Figure 3b), compared to the Allegheny Plateau highlands in the north-central part of the state. The highlands have relatively contiguous forested habitats and exhibited higher than average migration rates (Figure 3b). Differences between mean genomic similarity residuals (when controlling for the effect of geographic distance) were significant ($p < .001$) between individuals sampled in continuous habitats compared with fragmented forest habitats (Figure 3c). Despite showing a slight variation difference, the mean per-individual inbreeding coefficient (F) between the two treatments were not significantly different (Figure 3d; $p = .38$). Nucleotide diversity did not exhibit notable variance among individuals from continuous vs. fragmented habitats. Individuals residing in continuous habitats displayed an average π value of 0.00518 (SD = 0.00216), while those in fragmented habitats exhibited an average π value of 0.00502 (SD = 0.00207).

3.2 | Isolation by resistance

The Mantel test results indicated a strong and statistically significant correlation between genetic distance and habitat resistance distance ($r^2 = .487$, $p < .001$). The correlation between genetic distance and Euclidean distance was also significant, albeit weaker ($r^2 = .341$, $p = .001$). Least-cost path exhibited the lowest correlation with genetic distance ($r^2 = .285$, $p < .001$). These findings support the idea that habitat resistance serves as the most reliable predictor. Notably, the close correlation values suggest that the Euclidean distance did not deviate significantly from the least-cost path distance.

In the MLPE analysis, the best-fit models (characterized by ΔAIC <2) recovered the three variables—geographic distance, elevation, and habitat (i.e. forest cover)—as significant predictors of genetic connectivity (Table 1). However, only the models incorporating habitat and geographic distance as predictors demonstrated significant chi-squared values among all the models examined (Table 1). The most robust statistical model for IBR was the one emphasizing habitat as a resistance factor to gene flow (Figure 4a and Table 1), accounting for approximately 59.1% of the observed genetic variation (Conditional R^2). The model based on geographic distance ranked second, explaining 21.7% of the data's variation (Conditional R^2; Table 1). Habitat and geographic distance exhibited the highest relative importance values among the tested models, while elevation did not exhibit a noticeable impact on grouse population genetic connectivity (Figure 4b).

3.3 | Genetic diversity and selection

The mean nucleotide diversity (π) for the whole genome (WG) was $\pi = 0.0051$ (Figure S5a). This value was close to the diversity of haplotype 1 but considerably lower than the values observed for haplotype 2 on chromosomes 4 and 20 (Figure S5a). Tajima's D metric for testing the neutral evolution of SNPs showed contrasting selection patterns between genomic clusters (Figure S5b). Although the WG showed broad Tajima's D values, the mean value was negative (mean $D = -0.54$), suggesting excess rare and low-frequency alleles (Figure S5b). The same pattern can be observed for haplotype 1, on both chromosomes 4 (mean $D = -0.32$) and 20 (mean $D = -0.25$). In contrast, haplotype 2 on chromosome 4 ($D = 0.42$) and 20 (mean $D = 1.79$), showed positive mean values, indicating the low presence of rare alleles.

Within the two high differentiation blocks, several annotated genes were identified, of which 212 annotated genes and 408 genes with unknown functions were identified within chromosome 4, while chromosome 20 had 132 annotated genes and 64 with unknown functions. Among the annotated genes with high differentiation were diacylglycerol kinase-theta (*DQGK*) and *TDRD7* on chromosome 4 and *FSIP2*, *IFT52*, and *MAFβ* on chromosome 20 (Figure 5a). Except for *FSIP2*, most of these genes showed distinct signs of selection associated with a chromosomal haplotype and low levels of nucleotide diversity (Figure 5b).

4 | DISCUSSION

This study represents a contribution to the field of game species population genomics by investigating the genetic structure of the declining ruffed grouse population in Pennsylvania, USA. Despite

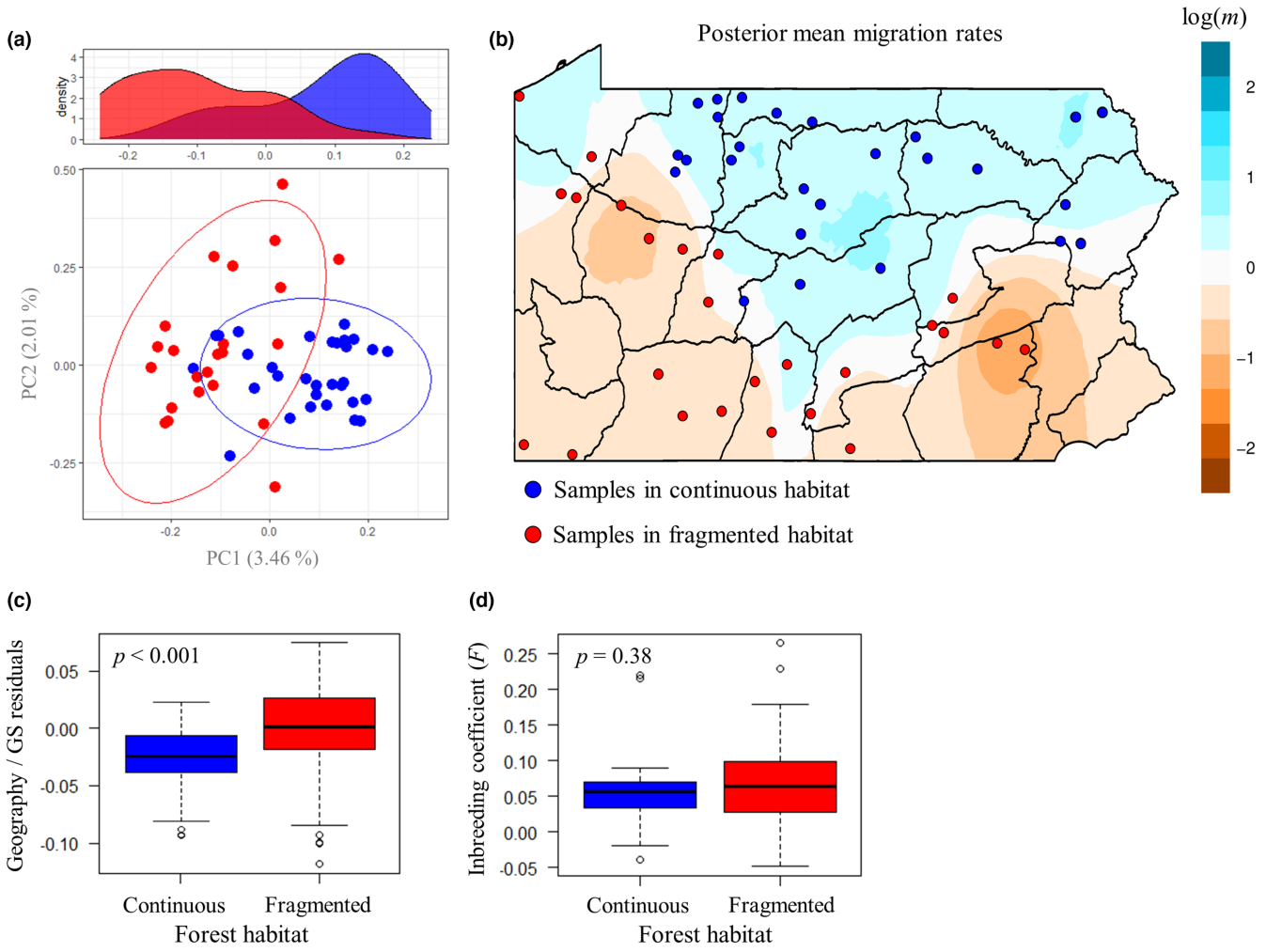

FIGURE 3 Genetic structure of the ruffed grouse population in Pennsylvania, USA, after removing SNPs within the chromosomal inversions. (a) Principal component analysis and PC1 density distribution (3.46% variation) showing the genomic relationship between individuals sampled in fragmented (red circle) and continuous (blue circle) habitats. (b) Estimates of effective migration surface depicting posterior mean migration rates (m) among individuals across the landscape, representing low (orange shades) and high gene flow (blue shades). Boxplot showing differences in (c) genomic similarity (GS residuals; decorrelated with geographic distance) and (d) inbreeding coefficient (*F*-statistic) among individuals sampled in continuous and fragmented forest habitats.

TABLE 1 The most suitable models (ΔAIC <2, with the best-fit model in italic $p = .0062$) with the contribution values of each predictor variable calculated using maximum likelihood estimation were determined for analysing the genetic distance values in the ruffed grouse population within Pennsylvania, USA.

Models	Habitat	Geographic	Elevation	logL	AIC	AICw	Conditional R^2 (CI 95%)
*Habitat**	*0.00439*	—	—	*4705.65*	*−9403.52*	*0.469*	*.591 (0.528–0.646)*
Geographic*	—	0.00413	—	4705.48	−9402.96	0.355	.217 (0.161–285)
Elevation^{ns}	—	—	0.00377	4704.69	−9401.56	0.176	—

Note: The conditional R^2 and its confidence intervals were presented for the best-fit model. Significance of chi-square contingency table tests of each predictor in the models, with * for $p < .05$, *ns* if not significant. The italic values represent p = .0062.

being one of the most well-studied North American game birds in terms of its population dynamics, behaviour and ecology, only three previous studies have investigated its pattern of population genetic structure (Honeycutt et al., 2019; Jensen et al., 2019; Perktaş, 2021). To date, this is the first study to utilize whole genome data to investigate patterns of spatial genetic variation in this species at a realistic scale for population management. The study aimed to assess the impact of long-term forest habitat fragmentation and WNV mortality on the genetic structure and connectivity of the ruffed grouse population. The main goal of this information was to support wildlife managers in spatial conservation planning by identifying areas of reduced functional connectivity.

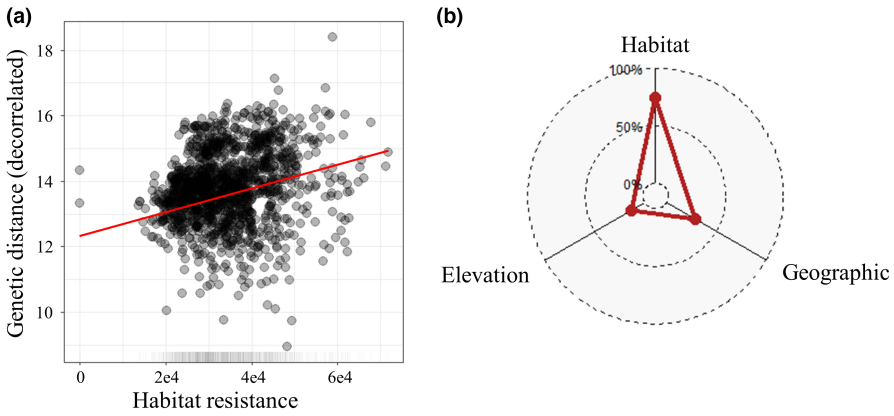

FIGURE 4 Isolation by resistance effects. (a) The relationship between habitat resistance (i.e., absence of forest cover) and genetic distance decorrelated to maximum-likelihood population effect (MLPE) correlation structure, as indicated by the best-fit model. (b) The relative importance of the three predictor variables—habitat, elevation, and geographic distance—in explaining the genetic connectivity of ruffed grouse populations in Pennsylvania, for the best-fit models (with ΔAIC <2) among the MLPE regression models.

Overall, we found ruffed grouse in Pennsylvania lacks a strong signal of genetic structure that would enable the distinction of clear subpopulation units (i.e. with independent demographic history) for conservation purposes. Instead, we found evidence that genetic differences among sampled individuals vary as a function of geographic distance, with reduced functional connectivity (i.e. migration rate) in areas with long-term habitat fragmentation and low rates of population recovery from WNV mortality (Figure 3). Additionally, we identified two large blocks (6 and 14 Mb) of high genomic differentiation located on chromosomes 4 and 20—a probable consequence of polymorphism of chromosomal inversions (Figure 2). These inversions harbour dozens of genes with contrasting patterns of nucleotide diversity and selection, in which some are potential candidate genes related to traits with important adaptive and ecological functions—offering a new avenue to use a molecular approach to track changes in individual fitness, adaptation to environmental changes, and behavioural ecology of ruffed grouse. Beyond that, we discuss what implications these findings have for ruffed grouse conservation in Pennsylvania, and across North America under the context of existing wildlife management efforts and policies.

4.1 | Genetic connectivity and associated drivers

Fragmentation of early successional forests has been suggested as a crucial factor in the reduction of ruffed grouse population connectivity in several regions of North America (Dessecker & McAuley, 2001; Sauer et al., 2014; Stauffer et al., 2018). The importance of this issue to game species population genomics cannot be overstated (Allendorf et al., 2008; Hohenlohe et al., 2021). Previous studies on ruffed grouse population genetics indicate that unsuitable habitats act as a barrier to dispersal, thereby limiting gene flow between populations on a large geographical scale within the species' distribution (Honeycutt et al., 2019; Jensen et al., 2019; Perktaş, 2021). Our genomic data also support this hypothesis, revealing that habitat fragmentation has a negative impact on the fine-scale genetic connectivity of ruffed grouse populations in Pennsylvania (Figures 3 and 4). This effect is particularly pronounced in the southern regions of the state where mixed forests are scarce, density is low and population decline rates due to WNV mortality are high (Figures 1b and S2; Nemeth et al., 2021; Stauffer et al., 2018). In contrast, areas where the persistence of mixed forests is associated with a high probability of ruffed grouse colonization and occupancy, such as on the Allegheny Plateau, exhibited the highest migration rates, demonstrating a positive correlation between functional connectivity and locality with greater continuity and habitat quality. However, nucleotide diversity analysis showed no significant differences between habitat categories when observing the average trends decrease in flush rate (Figure 1c). These results suggest that genetic diversity does not appear to be correlated with differences in WNV recovery rates in the region, indicating other contributing factors such as loss of forest habitat (Stauffer et al., 2018). These findings underscore the importance of preserving early successional forests to support population dynamics of game species such as the ruffed grouse.

In the context of the wide distribution of the ruffed grouse, mitochondrial DNA data indicates that the species forms a population subgroup in eastern North America that is genetically separate from populations in the central and western regions (Honeycutt et al., 2019). Notably, populations in Pennsylvania and Vermont play a significant role in the observed differences among populations within the eastern distribution (Honeycutt et al., 2019). Specifically considering the Pennsylvania grouse population, we find that the combination of geographic distance and habitat fragmentation appears to have a greater influence on spatial genetic variation in ruffed grouse than conspicuous barriers, such as the ridge-and-valley region (i.e. terrain elevation) within the Appalachian Mountains (Table 1; Figure 4b). This can be seen in the population genetic structure results, which indicate no genetic differentiation between opposite sides (i.e. longitudinally oriented differences) of the central Appalachian range (Figure S4). It is possible that this pattern is associated with the prevalence of mixed forest habitat at various elevations in the region, especially in northern Pennsylvania, facilitating dispersal and

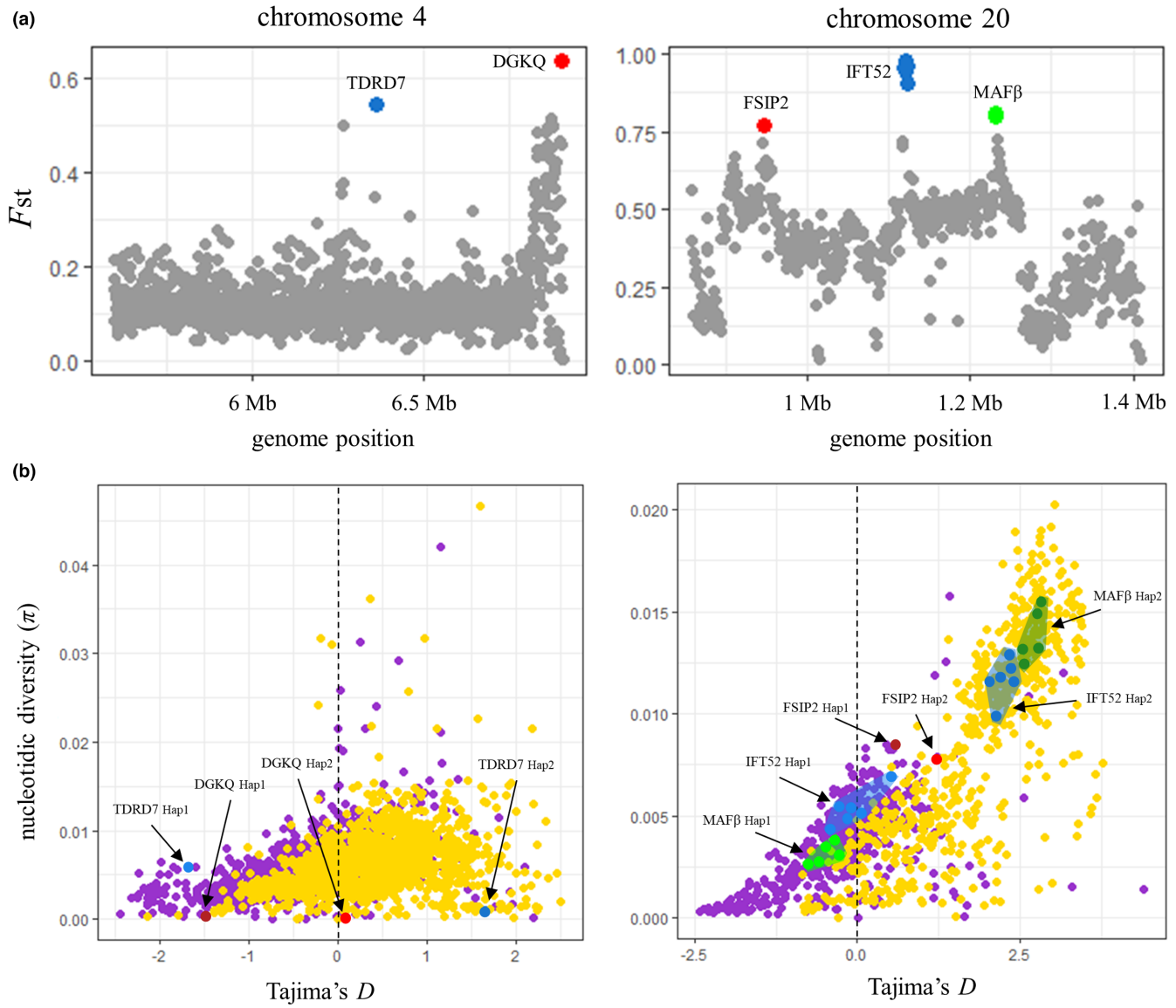

FIGURE 5 (a) Detailed visualization of regions of high differentiation comparing the two genomic clusters on chromosomes 4 and 20. (a) Grey dots depict F_{ST} estimates for 10 kb windows, and coloured dots represent annotated genes with the highest levels of differentiation within each chromosome. (b) Biplot of nucleotide diversity (π) and Tajima's D for SNPs in 10 kb windows in haplotypes 1 (purple dots) and 2 (yellow dots). Annotated candidate genes coded in red, blue, and green colours.

hence gene flow. Evidence supporting this hypothesis is that ruffed grouse population abundance tends to be higher in elevated regions in the Appalachians, where habitats tend to be more preserved and of better quality (Lewis et al., 2022). However, this relationship between terrain elevation and suitable habitat is not always linear and favourable to genetic connectivity for ruffed grouse. For instance, at broad geographic scales, the high elevation of mountain ranges is an important factor driving genetic differentiation among ruffed grouse populations (Jensen et al., 2019). From a comparative perspective, the central Appalachian region is considerably lower (~70–800 m) in contrast to the Rocky and Cascade Mountains (up to 4200 m in both cases), which represent strong barriers to gene flow between ruffed grouse populations in western North America (Jensen et al., 2019). Therefore, depending on geographic scales, the interaction between low dispersal capacity, availability of suitable habitat and landscape features can have different impacts on the spatial genetic variation of ruffed grouse populations. However, we cannot completely disregard the role of the Appalachians as a driver of genetic differentiation among populations of the ruffed grouse in the eastern USA, since we investigated populations only in the central region of the mountain range. Future studies should compare populations over broader geographic extents across eastern North America using robust landscape genetics approaches.

We also investigated whether there were differences in inbreeding levels between individuals sampled in continuous habitats compared to fragmented habitats. Our data revealed no significant differences between the inbreeding coefficients of individuals sampled in these different habitat categories (Figure 3d). This could be

the result of (1) gene flow between habitat patches possibly maintaining high levels of genetic diversity, and/or (2) high extinction rates in small, isolated populations hindering demographic stability and consequently inbreeding.

In the first case, inbreeding is prevented by occasional gene flow between habitat patches. Although possible, this hypothesis is unlikely to be plausible due to the high risks involved in the natal dispersal events of ruffed grouse. Some classic ecological studies on the movement of this species suggest limited dispersal, with average distances of approximately 4 miles (~6.4 km) (Chambers & Sharp, 1958; Small & Holzwart, 1993; Small & Rusch, 1989) and greater frequency of natal dispersal events at localities with high population density (Chambers & Sharp, 1958). Furthermore, movement through poorly suited habitats is associated with high mortality due to predation (Small & Holzwart, 1993; Yoder et al., 2004), which can reduce gene flow between forest patches. This relationship can be seen in the lower-than-average migration rates in fragmented habitats (Figure 3b), which also correspond to areas of low abundance and strong population decline (Figure 1b,c). In this sense, small habitat fragments, which support low population densities and expose individuals to high risks between movement and predation, consequently, reduce the frequency of effective dispersal (i.e., involving reproduction as a result of movement). Thereby, it is unlikely that the lack of differences in the inbreeding coefficient observed in our results between fragmented versus continuous habitats is a function of gene flow. Alternatively, small forest patches may lack the stability and environmental conditions necessary to support demographically stable populations both in time and space. This seems especially critical concerning ruffed grouse brood survival rates, which depend on forests at different stages of maturity (Dessecker & McAuley, 2001). Under these conditions, in fragmented habitats, ruffed grouse populations would be temporally ephemeral and prone to high local extinction rates, possibly preventing the formation of stable populations that would allow inbreeding.

4.2 | Genomic divergence and selection patterns

We found that the main axis of genome-wide variation in these birds localized into only two large blocks of genomic divergence, which we mapped to chromosomes 4 and 20 and encompassing more than 800 genes (Figures 2 and 5). These regions of high differentiation are often associated with a putative chromosomal inversion (Harringmeyer & Hoekstra, 2022; Küpper et al., 2016; Lundberg et al., 2017; Sanchez-Donoso et al., 2022). This is because high F_{ST} values spanning large portions of the genome can be explained by the suppression of recombination in inverted chromosomal regions, preventing homogenization by gene flow and consequently resulting in differences between haplotypes.

In the context of reduced spatial genetic connectivity and population decline due to WNV, understanding the biological consequences of chromosome inversion polymorphisms is a valuable resource for grouse management. Chromosomal inversion polymorphisms can be maintained by spatially balancing selection, clustering variants geographically due to local adaptation (Akopyan et al., 2022; Harringmeyer & Hoekstra, 2022; Wellenreuther & Bernatchez, 2018). Indeed, positive Tajima's *D* values for both chromosome inversions indicate that genetic diversity in these regions is maintained by some form of balancing selection (Figure S5). However, no obvious signs of spatial segregation of chromosomal haplotypes were found (Figure 2b), possibly due to the limited geographic scale investigated in our study. Assuming that these structural genomic variations are segregating throughout the distribution of ruffed grouse, which has a wide distribution across the USA (including Alaska) and Canada, the Pennsylvania population may be in a region where the different haplotypes are occurring in sympatry. This pattern is observed in organisms with geographically clustered chromosomal inversion polymorphisms (Harringmeyer & Hoekstra, 2022; Lundberg et al., 2017; Sanchez-Donoso et al., 2022). Testing this hypothesis will require extensive genomic sampling of extant ruffed grouse populations in North America, which can not only help to understand the spatial dynamics of this polymorphism, but its possible effects on population dynamics and susceptibility to WNV-related mortality.

The clustering patterns in PCA (Figure 2) and the high levels of nucleotide diversity (Figure S5) indicate that these putative inversions (haplotype 2) on both chromosomes 4 and 20 are only present in a heterozygous state in the ruffed grouse population analyzed here. Another notable result is the absence of individuals with alternative haplotype combinations between the chromosomes, that is birds that carry the inversion for chromosome 4 also have the inversion on chromosome 20 (Figure 2e,f; Table S2). In the case of low-frequency inversions, the homozygous state tends to be rare (Faria et al., 2019; Silva et al., 2019). However, given the relatively high frequency (~25%) of ruffed grouse specimens carrying haplotype 2 (i.e. the putative inversions), one would expect the presence of individuals homozygous for these inversions. This pattern may be due to (1) the low frequency of homozygotes for the inversion in the population, whereas they were not sampled in our study, or (2) homozygosity in these inversions may have lethal consequences.

Cases of lethality associated with homozygosity inversions have been documented in birds. For instance, in the ruff (*Philomachus pugnax*), dominant alleles that confer the satellite form of males are deleterious when in homozygosity (Küpper et al., 2016). In white-throated sparrows (*Zonotrichia albicolis*), inversions in homozygosity are deleterious, influencing aspects of disassortative mating (Tuttle et al., 2016). Therefore, managing grouse populations with potential genetic deleterious effects, especially in the context of isolation by habitat fragmentation, may reduce genetic diversity and impair adaptability, further decreasing population connectivity.

In birds, chromosomal rearrangements have been associated with meaningful biological traits, such as reduced dispersal and migration (Sanchez-Donoso et al., 2022), differences between colour morphs (Zinzow-Kramer et al., 2015), and alternative mating strategy (Küpper et al., 2016), to name a few examples. A well-known phenotypic characteristic of ruffed grouse that could be involved with chromosomal variants is the variation in plumage colour, which ranges from shades

of grey to red/brown. However, in a post hoc analysis of samples for which we have plumage information for, we found no correlation between grouse colour and inversions type. Moreover, genes commonly associated with variation in melanism expression in bird feathers, such as agouti signalling protein (*ASIP*) and melanocortin 1 receptor (*MC1R*), were not found within these highly differentiated genomic blocks, therefore excluding the possibility that inversions are responsible for differences between ruffed grouse colour morphs. Another intriguing possibility is that the haplotypes segregate with different behavioural traits. For example, ruffed grouse exhibiting hyper-territorial behaviour, also known as 'tame-grouse', have been frequently observed across Pennsylvania. This 'tame-grouse' phenomenon could suggest a potential relationship between genetic factors and behavioural traits. If the inverted genes were involved in hormone regulation, the 'tamed' behaviour observed in the grouse could be a result of the expression of these genetic differences. This implies that genetic factors can play a significant role in shaping an animal's personality, highlighting the complex interplay between genes and behaviour.

If not genes involved in the expression of outstanding phenotypic traits such as plumage polymorphism, what other genes with known biological functions are? We found that the most divergent genes within each inverted region are related to hormone regulation, cell component formation, sperm morphology and motility (Figure 5a). Furthermore, signs of the selection show that the genetic diversity in most of these genes, within each chromosomal variant, is being maintained by different evolutionary pressures (Figure 5b). Knowing aspects of variation and selection in these candidate genes may be of interest for the conservation and management of ruffed grouse concerning an individual fitness assessment, reproductive success, behaviour and responses to environmental changes and emerging disease (Fitzpatrick et al., 2005). For example, the *DGKQ* gene is responsible for adrenocortical steroidogenesis regulation (Cai et al., 2014), which plays a crucial role in behavioural responses to stressful situations such as the fight-or-flight response (Goldstein, 2010; McCarty, 2016). The *DGKQ* gene could be a potential candidate gene for investigating the relationship between the inversions and the 'tame-grouse' behaviour. The *MAFβ* gene is responsible for the formation of cellular components of blood, and mutations in this gene are related to several diseases in humans (Mahawej et al., 2013; Sato et al., 2018). Also, mutations in *FSIP2*, *TDRD7*, and *IFT52* genes can cause motility disorder and malformation in sperm, leading to infertility in males (Liu et al., 2021; Tanaka et al., 2011; Taschner & Lorentzen, 2016). This latter factor reinforces our previous hypothesis that the absence of the homozygosity state inversions may be a consequence of deleterious effects or low fertility.

4.3 | Management recommendations and prospects

Based on our genomic results, we recommend the following management measures to maintain genetic connectivity and diversity in ruffed grouse populations in Pennsylvania and across the eastern USA:

1. Specifically for the Pennsylvania population, since the availability of forested habitat was the most important driver of genetic connectivity in our results (Table 1; Figure 4b), we recommend that habitat management should occur within WMUs in southern Pennsylvania (Figure 3b) to create habitat corridors that connect forest patches to areas with higher ruffed grouse abundance. This measure can improve recruitment rates between adjacent areas, helping to maintain genetic diversity through gene flow at levels similar to those observed in areas with higher habitat connectivity, thus reducing or preventing the need for translocation of individuals (i.e. assisted gene flow). This strategy also aims to ensure natural connectivity with populations from adjacent states further south of Pennsylvania, such as Virginia and West Virginia.
2. Taking a broad perspective on ruffed grouse conservation across North America, it is crucial for wildlife managers to carefully evaluate the impact of hunting in areas with low genetic connectivity to ensure harvest is not contributing to the decline of vulnerable populations. Harvest that occurs at additive levels to other threats, such as habitat loss and WNV mortality, in regions with limited connectivity could further exacerbate the risks of disrupting natural contact and isolating small populations. Considering the Pennsylvania grouse population, our results explicitly identify the management units (Figure 3b) where reductions in harvest-related mortality would be most likely to improve local connectivity and preserve long-term genetic diversity and adaptive potential.
3. Given the threat of WNV mortality and its potential impact on genetic diversity, it is imperative that periodic genetic monitoring is implemented to track changes in population genetic variability and structure. Therefore, future studies should also aim to identify genes associated with virus susceptibility and assess their prevalence on the landscape. We also recommend that future studies aim for a broader spatial scale comparison to verify phylogeographic relationships among populations and characterize genetic diversity stock as contingency measures in case genetic rescue is needed (Whiteley et al., 2015).
4. The discovery of chromosomal inversions brings a new avenue for evolutionary, ecological, and conservation studies for ruffed grouse. The possibility that inversions in homozygosity may have lethal deleterious effects should be further investigated. To properly test this hypothesis, it will be necessary to conduct breeding experiments among birds carrying the chromosomal inversion to observe its possible deleterious effects within the brood. If this hypothesis is proven correct, this factor will have immense implications for the management of ruffed grouse, such as attempting to colonize new areas, as pairing individuals with the inversion could considerably reduce reproductive success (Küpper et al., 2016; Tuttle et al., 2016). Therefore, we recommend that any population interventions, for example, assisted gene flow, should first consider the adaptive and reproductive effects of moving birds carrying the chromosomal variants.

5 | CONCLUSION

In conclusion, the production of a very high-quality reference genome for ruffed grouse provides a valuable resource for evolution and conservation studies. Our results reveal that habitat fragmentation significantly impacts genetic connectivity of the ruffed grouse population in Pennsylvania. While the low inbreeding coefficient suggests the population is healthy, genetic monitoring should still be included as a goal in the current conservation plan. Furthermore, comparisons of genetic diversity and structure among populations across eastern North America can provide crucial insights into management strategies for ruffed grouse at a broad scale context. The identification of cryptic genetic structure due to putative chromosomal inversions is a promising finding that opens new avenues for investigating candidate genes and assessing long-term management effects on fitness and reproductive success. Taken together, our findings provide valuable information to improve current management efforts for Pennsylvania ruffed grouse populations, including genomic assessment in the context of factors such as habitat fragmentation and West Nile Virus mortality. We believe game species in general warrant the use of genomic resources and require comprehensive study in a fine-scale geographic setting to ensure appropriate harvest and management. Importantly, high-resolution datasets like this one generated for ruffed grouse will help resource managers ensure that hunted populations have the adaptive potential needed to cope with future uncertainty. Helping managers prioritize populations with high genetic diversity and strong signatures of local adaptation can help ensure population viability in the face of global change.

AUTHOR CONTRIBUTIONS

Leilton W. Luna, David P. L. Toews, and Julian D. Avery conceptualized the study with input from Lisa M. Williams, Kenneth Duren, and Reina Tyl. David P. L. Toews, Julian D. Avery, Lisa M. Williams, and Kenneth Duren gathered financial resources for project development and execution. Leilton W. Luna performed the data analysis with input and support from David P. L. Toews and Julian D. Avery. Lisa M. Williams collected and selected all the samples used in the study. Leilton W. Luna, Julian D. Avery, Lisa M. Williams, Kenneth Duren, and Reina Tyl interpreted the implications of the results for ruffed grouse conservation and management. David P. L. Toews and Julian D. Avery supervised the project development stages. Leilton W. Luna wrote the manuscript with extensive reviews and contributions from all authors.

ACKNOWLEDGEMENTS

We thank the Pennsylvania Game Commission for funding this project on grouse conservation. We also thank the Huck Institute of Life Sciences, Pennsylvania State University's Eberly College of Science, and the College of Agricultural Sciences for the availability of facilities and equipment, and administrative support. The bioinformatics analyses of this research were performed on Pennsylvania State University's Institute of Computational Data Sciences' Roar high-performance computer.

CONFLICT OF INTEREST STATEMENT

The authors declare they have no competing interests.

DATA AVAILABILITY STATEMENT

The reference genome of *Bonasa umbellus* has been deposited in GenBank (BioProject ID: PRJNA1008140). The raw sequences of the 54 specimens resequenced here have also been deposited in GenBank (BioProject ID: PRJNA1008718). Accession nos. for each sample are shown in Table S1. The inputs of the genotypes likelihood and genotypes call used in the downstream analyses are available in the Dryad digital repository (Luna et al., 2023, https://doi.org/10.5061/dryad.vx0k6djz2).

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SUPPORTING INFORMATION

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How to cite this article: Luna, L. W., Williams, L. M., Duren, K., Tyl, R., Toews, D. P. L., & Avery, J. D. (2023). Whole genome assessment of a declining game bird reveals cryptic genetic structure and insights for population management. *Molecular Ecology*, *32*, 5498–5513. https://doi.org/10.1111/mec.17129