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The Great Rift Valley is a more pronounced biogeographic barrier than the Blue Nile Valley for six Ethiopian Highland passerines in the eastern Afromontane biodiversity hotspot

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ABSTRACT

The Ethiopian Highlands are divided by lowland biogeographic barriers, including the Blue Nile Valley (BNV) and Great Rift Valley (GRV). We show that the GRV is a more pronounced phylogeographic break than the BNV for 6 focal passerines. Previous research suggests that the BNV greatly shaped phylogeographic patterns in relatively sedentary montane taxa such as frogs and rodents, whereas the GRV shaped phylogeographic patterns in volant taxa such as birds. However, no previous research simultaneously compares the impact of each valley on phylogeographic patterns in birds, and as these barriers vary in geographic extent and topography, the relative extent of their effects on gene flow is unclear. Using whole-genome resequencing, we quantified genetic variation in 6 montane forest passerines in the Ethiopian Highlands and found that their phylogeographic patterns varied, with general trends distinct from those of taxa that were previously studied across the same barriers. Genetic variation was assessed by estimating genome-wide genetic diversity (H_O), demographic history, phylogeographic structure, and phylogeographic concordance among taxa. Population pairs flanking the GRV showed higher F_{ST} and more distinct population clusters in principal component analysis than those separated by the BNV. H_O was broadly consistent across populations, excluding noticeable reductions in 2 populations (1 population each in 2 separate species). The overall phylogenetic signature and concordance across study taxa supported populations separated by the BNV as sister and populations southeast of the GRV as most distinct.

Keywords: biodiversity hotspots, Ethiopia, genetic diversity, genomics, passeriformes, phylogeography

How to Cite

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LAY SUMMARY

- The Ethiopian Highlands are exceptionally biodiverse in species and community diversity.
- Highland landscape features can isolate species' populations, causing distinct populations or new species to form over time, impacting biodiversity patterns.
- Different species may be affected by landscape features to varying degrees based on their attributes. Studies characterizing evolutionary responses to landscape features in Ethiopia are mostly limited to sedentary organisms like frogs and rodents.
- We characterized genetic variation—a component of biodiversity—in 6 Ethiopian Highland songbirds that are thought to be highly mobile.
- In these songbirds, we found genetic structure between populations separated by the Great Rift Valley and the Blue Nile Valley, showing that these valleys dissecting the Ethiopian Highlands isolate some songbird populations.
- Evolutionary relationships between populations of our focal species and between populations of all highland species studied to date differed in how genetically distinct they were in relation to each dividing barrier.

El Valle del Gran Rift es una barrera biogeográfica más pronunciada que el Valle del Nilo Azul para seis paseriformes de las Tierras Altas de Etiopía en el punto caliente de biodiversidad del este Afromontano

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RESUMEN

Las Tierras Altas de Etiopía están divididas por barreras biogeográficas de tierras bajas, incluyendo el Valle del Nilo Azul (VNA) y el Valle del Gran Rift (VGR). Mostramos que el VGR es una ruptura filogeográfica más pronunciada que el VNA para 6 paseriformes focales. Investigaciones previas sugieren que el VNA moldeó en gran medida los patrones filogeográficos en taxones montanos relativamente sedentarios como ranas y roedores, mientras que el VGR moldeó los patrones filogeográficos en taxones voladores como las aves. Sin embargo, ninguna investigación previa ha comparado simultáneamente el impacto de cada valle en los patrones filogeográficos de las aves, y dado que estas barreras varían en extensión geográfica y topografía, la magnitud relativa de sus efectos en el flujo génico no está clara. Usando la re-secuenciación del genoma completo, cuantificamos la variación genética en 6 paseriformes de bosques montanos en las Tierras Altas de Etiopía y encontramos que sus patrones filogeográficos variaban, con tendencias generales distintas de las de los taxones que se habían estudiado previamente a través de las mismas barreras. Se evaluó la variación genética estimando la diversidad genética a lo largo del genoma (H_O), la historia demográfica, la estructura filogeográfica y la concordancia filogeográfica entre los taxones. Las parejas de poblaciones a ambos lados del VGR mostraron un F_{ST} más alto y agrupamientos poblacionales más distintos en el análisis de componentes principales que aquellos separados por el VNA. El HO fue ampliamente consistente entre las poblaciones, excluyendo reducciones notables en 2 poblaciones (1 población en cada una de las 2 especies separadas). La firma filogenética general y la concordancia entre los taxones estudiados respaldaron a las poblaciones separadas por el VNA como hermanas y a las poblaciones al sureste del VGR como las más distintas.

Palabras clave: diversidad genética, Etiopía, filogeografía, genómica, paseriformes, puntos calientes de biodiversidad

INTRODUCTION

Climate stability and topography in tropical mountains contribute to high species turnover along elevation gradients (Janzen 1967, Ghalambor 2006, McCain 2009) and at global scales, diversification rate is positively associated with elevation (Quintero and Jetz 2018). As such, tropical mountains are generally characterized by high biodiversity. When there is topographic complexity, biogeographic barriers may additionally shape montane biodiversity as they restrict gene flow between isolated populations, leading to population differentiation or even speciation along phylogeographic breaks. Mountain ranges with biodiversity impacted by lowland barriers are well-characterized globally, including the southern Mexican mountain ranges separated by the lowland Isthmus of Tehuantepec in Oaxaca (Barber and Klicka 2010), the Western Ghats of India (Robin et al. 2015), the Ethiopian Highlands (Habel et al. 2015, Dessalegn et al. 2021, Manthey et al. 2022), the Black Mountain Corridor in northeastern Australia (Bryant and Krosch 2016), and portions of the Andes separated by barriers such as the Marañon Valley in Peru (Winger and Bates 2015) or the Magdalena River Valley in Colombia (Gutiérrez-Pinto 2012). However, montane communities may respond to lowland barriers differently, either through community-wide vicariance or species-specific patterns of population differentiation (Baum and Smith 2012, Satler and Carstens 2016). Therefore, it is important to characterize the effects of biogeographic barriers across a breadth of codistributed taxa to fully understand the mechanisms shaping regional biodiversity.

The tendency of tropical mountain taxa to diversify has made their habitats “cradles of biodiversity” (Kier et al. 2009). Indeed, many of the 36 globally recognized biodiversity hotspots are tropical montane regions (Mittermeier et al. 2004, 2011). These hotspots are often described at the species or community level, leaving genetic diversity uncharacterized. This presents a problem as genetic diversity is the raw material by which organisms adapt to changing environments and diseases (Frankham et al. 2002). Genetic diversity and divergence estimates are also needed to distinguish fine-scale conservation targets given limited financial resources (Moritz 1994, Fraser and Bernatchez 2001, Palsboll et al. 2007). As such, genetic diversity estimates, and how those estimates vary across a biodiversity hotspot, are fundamental to conservation.

The Ethiopian Highlands lie within the Eastern Afromontane Biodiversity Hotspot (EABH) split by lowlands that lie within the Horn of Africa Biodiversity Hotspot (Mittermeier et al. 2011). Also called the “Roof of Africa,” these highlands are the largest continuous highland system on the continent, with topographic variability ranging from ~800 to 4,533 m in elevation (Asefa et al. 2020). The positive relationship between increased rainfall and elevation in the Ethiopian Highlands results in a diversity of plant communities along its elevational gradients (Umer et al. 2004, Friis et al. 2010, Asefa et al. 2020). At the species level, Ethiopia’s highland communities host an estimated 5,200 vascular plant species (Williams et al. 2004), 40% amphibian endemicity (Mengistu 2012), 17.7% mammal endemicity (Lavrenchenko and Bekele 2017), and a higher concentration of non-passerine endemics relative to the surrounding Afrotropics (Klerk et al. 2002). However, this region’s taxa are disproportionately lacking published genetic information at the species level (Šmíd 2022), and there are few taxa with intraspecific genomic diversity sampled across multiple regions of the Ethiopian Highlands (Reyes-Velasco et al. 2018a, 2018b, Komarova et al. 2021, Manthey et al. 2017, 2022).

The Ethiopian Highlands harbor biogeographic barriers to montane taxa such as the Great Rift Valley (GRV) and Blue Nile Valley (BNV). The GRV is a wide lowland region (50–60 km in width) (Asefa et al. 2020) and its impacts as a biogeographic barrier for montane taxa are better documented than those of the BNV. The GRV acts as a barrier to gene flow in bats (Razgour et al. 2021), canids (Gottelli et al. 2004), primates (Belay and Mori 2006), birds (Manthey et al. 2022), frogs (Evans et al. 2011, Freilich et al. 2016, Manthey et al. 2017, Reyes-Velasco et al. 2018a, 2018b), and rodents (Bryja et al. 2018, Komarova et al. 2021), owing to both its physical width and its environmental conditions that contrast with the high-elevation habitats at its flanks. The BNV is geographically narrower (~40 km) and although its impacts on montane taxa are less known, its steeper escarpments limit dispersal in frogs (Evans et al. 2011, Freilich et al. 2014, Manthey et al. 2017, Reyes-Velasco et al. 2018a, 2018b) and rodents (Bryja et al. 2018, Kostin et al. 2020, Mizerovská et al. 2020, Komarova et al. 2021). These previous studies have demonstrated more consistent population structure, distinct species, and differential genetic diversity in frogs and rodents isolated by the BNV than those isolated by the GRV, likely due to the former barrier’s more extreme topography rather than its width. However, it is not clear how this pattern compares to taxa with greater dispersal potential such as birds, as only those bird populations surrounding the GRV have been studied to date (Manthey et al. 2022).

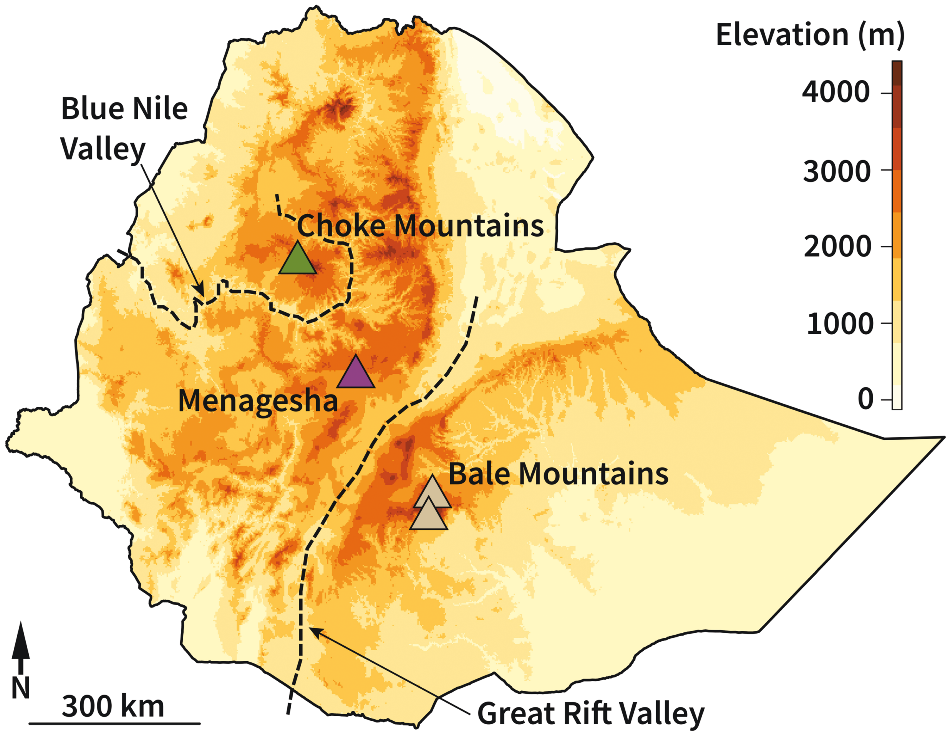

FIGURE 1. Sampling map of Ethiopia. Triangles represent sampling sites that are colored by locality (Bale Mountains National Park, Menagesha, and the Choke Mountains). Coordinates for sampling sites can be found in Supplementary Material Table 1. Dashed lines approximate lowland biogeographic barrier locations.

We used whole genome resequencing data to quantify comparative phylogeographic patterns of 6 codistributed passerines in the Ethiopian Highlands. Previous work has shown that the GRV has reduced gene flow among populations of these 6 species (Manthey et al. 2022), but the extent to which the BNV acts as a biogeographic barrier to montane birds remains unstudied. Discordance in the phylogeographic patterns between the 6 species may result from interactions between barrier attributes such as topography or geographic width, and species traits such as elevational occurrence or dispersal ability. Conversely, concordance in phylogeographic signal across the 6 study species would imply that the 2 barriers effectively isolate the species' populations in the same way, regardless of species differences in dispersal ability or elevational occurrence. Thus, we aimed to address the following questions: (1) Do each of the focal species have consistent signatures of genetic structure and regional genetic diversity across both barriers and if not, what traits might explain the discordance? (2) How do the comparative patterns of phylogeographic structure in these focal avian taxa compare to the phylogeographic patterns of other highland taxonomic groups previously revealed in similar studies that included the GRV and BNV? Most taxa from previous studies are nonvolant, potentially providing a mechanism promoting discordant phylogeographic patterns relative to our study taxa.

MATERIALS AND METHODS

Field Work and Sampling

In addition to previously published genetic samples from the Bale Mountains and Menagesha (Manthey et al. 2022), we collected samples in the Choke Mountains of the Ethiopian Highlands (Figure 1); we sampled blood from 6 species for downstream molecular analyses: *Cossypha semirufa*, *Crithagra tristriata*, *Melaenornis chocolatinus*, *Sylvia galinieri*, *Turdus abyssinicus*, and *Zosterops poliogastrus*. Of these, *S. galinieri*, *C. tristriata*, *M. chocolatinus*, and *Z. poliogastrus* are endemic to high elevations in the Horn of Africa. *C. semirufa* and *T. abyssinicus* are found throughout much of the EABH. Permit limitations led to $n \leq 3$ individuals captured per species per region. All sampling took place with permissions from the Ethiopian Wildlife Conservation Authority and local landowners.

Sequencing

We used whole-genome sequencing data from a previous study (Manthey et al. 2022) for 2 of the populations (Supplementary Material Table 1). We complemented this data with new whole-genome sequencing data from a total of 16 individuals from the Choke Mountains belonging to the same 6 study species (Supplementary Material Table 1). We extracted DNA from blood samples using the QIAGEN (Hilden, Germany) DNeasy Blood and Tissue Kit following manufacturer protocols. We sent DNA samples to the Oklahoma Medical Research Foundation Clinical Genomics Center for Illumina shotgun sequencing on either the HiSeq3500 or NovaSeq6000. We multiplexed samples on flow cells with libraries from other projects and sequenced with a target of ~10–20× genomic coverage.

Genotyping and Filtering

We used the bbduk.sh script of the *bbmap* package (Bushnell 2014) for quality filtering and adapter trimming of raw sequencing reads. Filtered reads were mapped to the Zebra Finch (*Taeniopygia guttata*) bTaeGut1.4.pri version 4 reference assembly (Warren et al. 2010, Rhie et al. 2021) using the BWA-MEM command in BWA (Li and Durbin 2009). The *T. guttata* reference assembly was optimal for mapping because it is highly contiguous, and *T. guttata* belongs to *Passeriformes*—the same order as our focal species. Analyses based on haplotype structure should not be strongly biased due to the high degree of synteny between not only members of Passeriformes but also Aves as a whole (Derjusheva et al. 2004). We used *samtools* v1.9 (Li et al. 2009) to convert SAM files to BAM format. BAM files had duplicate reads removed, read groups added, and were cleaned and sorted using the Genome Analysis Toolkit (GATK) v4.2.3.0 (McKenna et al. 2010). We measured sequencing coverage using the *samtools* depth command (Li et al. 2009) (Supplementary Material Figure 1).

We genotyped each species separately using the *HaplotypeCaller* and *GenotypeGVCFs* functions in GATK v4.2.3.0. We limited genotyping to scaffolds at least 1 mega base pairs (Mbp) in size based on limitations for some downstream analyses and consistency across datasets. We filtered genotype output using VCFtools v0.1.16 (Danecek et al. 2011) to remove indels and to only include sites with a minimum depth of coverage >6, sites with a maximum mean depth of coverage <50, and biallelic sites. Additionally, we used a maximum minor allele frequency of 0.49. For some downstream analyses, we used additional filters that are mentioned in the appropriate methods sections.

Summary Statistics

We calculated genome-wide observed heterozygosity (H_O) to quantify how genetic diversity varies among the sampled populations. H_O was calculated as the ratio of heterozygous genotypes to the total number of sites genotyped per individual. H_O does not suffer bias from the effects of uneven sampling like estimators such as nucleotide diversity (π) as it is calculated on a per individual basis.

To quantify genetic differentiation across both biogeographic barriers, we measured genomic fixation index (F_{ST}) between population pairs separated by either the GRV or BNV, using Reich and colleagues' (2009) F_{ST}. Reich's F_{ST} is unbiased at low sample sizes when using large numbers of markers (Willing et al. 2012). Because sex chromosomes may show elevated differentiation relative to the rest of the genome, we excluded the Z and 4A chromosomes. Here, we removed chromosome 4A as it is likely a neo-sex chromosome in the *Sylvioidea* superfamily, which includes both *Z. poliogastrus* and *S. galinieri* (Pala et al. 2012, Sigeman et al. 2020). We quantified associations between F_{ST} values and species traits including: mean hand-wing index (HWI; a proxy for dispersal ability) (Sheard et al. 2020), elevational occurrence (Redman et al. 2016), mean H_O, and harmonic mean effective population size (N_E) across both biogeographic barriers to test for correlations explaining differentiation disparities between species. To account for evolutionary history in differentiation-trait associations, we also quantified these associations using phylogenetically independent contrasts (Felsenstein 1985) in the R package *ape* (Paradis and Schliep 2019).

Genetic Structure

To identify population structure across the GRV and BNV, we used STRUCTURE v.2.3.4 (Pritchard et al. 2000, Falush et al. 2003). We only included sites with a minor allele count ≥2, and thinned single nucleotide polymorphisms (SNPs) to limit linkage disequilibrium (2 datasets thinned for 10 kilobase pair [kbp] or 20 kbp minimum separation between SNPs). We ran STRUCTURE with correlated allele frequencies, which assumes some level of nonindependence between populations—a likely scenario given the proximity of sampling sites. For each species and differently thinned dataset, we ran STRUCTURE for 100,000 burn-in iterations followed by an additional 500,000 recorded MCMC iterations. We ran STRUCTURE with an assumed $K = 2$ and $K = 3$ because these values are the most biologically relevant to determine the BNV and GRV contributions to population structure. We ran STRUCTURE 20 times per K value per dataset to avoid using results from runs that settled on local MCMC optima. We visually examined trace plots to ensure model convergence across replicates for each STRUCTURE analysis. For final presentation, we used the replicate with the highest likelihood to represent the ancestry assignment probabilities for each dataset. As a further nonparametric test to identify population structure with fewer assumptions, we ran a principal component analysis (PCA) with PLINK v1.90b6.21 (Chang et al. 2015). We ran PCA with the same 10 and 20 kbp thinned datasets to look for consistency between the results of STRUCTURE and PCA analyses.

Phylogenomics

To characterize evolutionary relationships among populations and individuals, we estimated phylogenies for each species. We group genotyped all individuals from all species to produce one multispecies VCF. The multispecies VCF was split into 6 single-species VCFs, each containing one outgroup individual belonging to another species from the original multispecies VCF. For analysis, we retained invariant and biallelic variant sites with a maximum of 20% missing individuals in the alignment (Supplementary Material Table 2). We used RAxML v8.2.12 (Stamatakis 2014) to estimate gene trees for nonoverlapping 50 kbp windows for each species. Only alignments with >10% of sites included after filtering (i.e., a minimum of 5,000 sites) were used to build the trees. We used gene trees as input to create species trees in ASTRAL-III v.5.7.8 (Zhang et al. 2018) with node support measured by quartet frequencies (Sayyari and Mirarab 2016). We visualized species trees using *Figtree* v1.4.4 (Rambaut 2018). A maximum-clade credibility tree was also generated using the *sumtrees.py* script in DendroPy (Sukumaran and Holder 2010) using RAxML gene trees estimated in 50 kbp windows including all individuals from all species. This maximum clade credibility tree was used in calculating phylogenetic independent contrasts as described in the summary statistics methods section. We used the maximum clade credibility tree because the terminal branch lengths are informative in contrast to the ASTRAL tree's terminal tips.

Phylogeographic Concordance

To investigate if the focal taxa exhibited a consistent evolutionary response to the biogeographic barriers, we evaluated phylogeographic concordance across our focal taxa. The concept is based on Satler and Carstens' phylogeographic concordance factors (PCFs) (2016) but uses gene trees estimated in sliding windows across the genome rather than Bayesian species tree distributions derived from single genes. Our PCF method quantifies genome-wide gene tree support for a scenario where all focal species (i.e., the "community") form clades corresponding to each sampling location due to divergence on either side of the GRV and BNV. Our PCF method also determines the best-supported relationship between the 3 sampling locations' communities, which helps uncover the biogeographic barrier that more consistently isolates the focal taxa. First, we created a file including all gene trees from all species and a mapping file whereby all individuals (gene tree tips) were assigned to their respective sampling locations. We used ASTRAL-III with these files to generate a "species" tree, forcing individuals from the same sampling location to be monophyletic in the species tree. This final species tree is hence referred to as the phylogeographic concordance tree. The phylogeographic concordance tree provided (1) branch lengths proportional to the amount of gene tree concordance for each location's community and (2) the posterior probability of the best-supported phylogeographic concordance tree topology (Sayyari and Mirarab 2016). Like Satler and Carstens, we repeated the method for each species individually to show how each species gene trees' concordance or discordance contribute to the final phylogeographic concordance tree.

The phylogeographic concordance tree presents the best-supported relationships among sampling locations considering all gene trees across species. To assess the frequency of the 2 alternative phylogeographic concordance tree topologies, we used custom R and shell scripts. Briefly, all individuals from all species were grouped according to their sampling location. For each gene tree and species, one individual (gene tree tip) was sampled at random for each sampling location, the outgroup was retained, and the remaining tips were dropped. Each sampled tip was then named according to its location. This provided thousands of sample topologies of quartet phylogenies including one outgroup and one tip per locality. Three target topologies representing the 3 possible phylogeographic concordance tree topologies were used with the *sumtrees.py* script in DendroPy to produce summary trees describing each target topology's frequency in the samples. The process was repeated 100 times and summarized again for each target topology with *sumtrees.py* to provide 95% confidence intervals.

Demography

We used MSMC2 (Multiple Sequentially Markovian Coalescent 2) (Schiffels and Wang 2020) to estimate demographic histories. MSMC2 uses mutation patterns along chromosome pairs to infer recombination events and then models coalescence rates between pairs of the non-recombining chromosomal blocks. These coalescence rates are then converted into constant N_E estimates in distinct time segments that collectively span the range of inferred coalescence times estimated from all blocks. We chose 23 distinct time segments to limit model overfitting based on previous work with some of these samples (Manthey et al. 2022). MSMC2 was run on a per individual basis because (1) masking the genome for low coverage sites across multiple individuals would vastly reduce the number of genotyped sites left for analyses in species with low coverage such as *S. galinieri*, (2) uneven sample sizes across populations could bias demographic inference when samples are pooled for one estimate, and (3) consistency across individual demographic histories within populations provides a rough measure of confidence in model outcomes. N_E estimates and time in years from MSMC2 are calculated using species-specific mutation rates and generation times. For generation times, we used double the age of sexual maturity in closely related species (2 years) (Nadachowska-Brzyska et al. 2015) with the ages of sexual maturity taken from the Animal Aging and Longevity Database (Tacutu et al. 2018). We used species-specific mutation rates calculated from a prior study (Manthey et al. 2022). We calculated harmonic mean N_E per individual to summarize N_E estimates over all time blocks as the statistic is unbiased by extreme N_E outliers.

RESULTS

Data Set Summary

Total reads ranged from 108 to 409 million yielding a total number of bases sequenced between 16.3 and 61.8 gigabase pairs (Gbp) per individual (Supplementary Material Table 1). 83%–90% of reads mapped to the reference genome across samples (mean = 88.45%; SD = 1.73%). The unmapped reads are likely attributed to each species' evolutionary divergence from the *T. guttata* reference genome used, due to poor quality reads, or ambiguous mapping to repetitive regions. Per individual mean depth of coverage was between 7.6 and 28.7 reads mapped per site. After initial filtering we kept 700 to 916 Mbp (mean = 875 Mbp, SD = 51 Mbp) for H_O, with numbers decreasing in other analyses as other filters were applied (Supplementary Material Table 2).

Population Structure and Differentiation

In PCA analyses, PC1 explained ~14%–45% of the variation in the SNP data across species and generally separated individuals on either side of the GRV (Figure 2D and H). PC2 explained ~13%–20% of the variation in the SNP data and generally distinguished Choke and Menagesha populations in most species. The 2 differently thinned datasets displayed nearly identical PCA clustering patterns and percentage of variation explained on PCs 1 and 2, suggesting that varying the thinning strategy had a negligible impact on results. In *C. semirufa*, one individual sampled from the Bale Mountains (EB024) was midway between the Bale Mountains cluster and the other population clusters separated by the BNV.

For each species, STRUCTURE at $K = 2$ generally showed a trend of high assignment probabilities of individuals to clusters separated by the GRV in all species except for *Z. poliogastrus* and *C. semirufa* (Figure 2B and F). Similarly, at $K = 3$ high assignment probabilities of individuals to a Bale Mountains cluster remained, with varying degrees of admixture in individuals from locations separated by the BNV, depending on the species (Figure 2C and G).

Interestingly, *Z. poliogastrus* showed an admixed individual (EB065) with ancestry from the Bale and Choke clusters. Running STRUCTURE with $K = 4$ for *C. semirufa* produced a fourth genetic cluster including only EB024 with an 88.3% assignment probability. STRUCTURE results were nearly identical between 10 and 20 kbp thinned datasets (not shown), suggesting consistent population structure results regardless of spacing between SNPs.

In all study species, genome-wide F_{ST} estimates were higher between the Bale Mountains and Menagesha populations separated by the GRV (F_{ST} range: 0.015–0.318) than between the Menagesha and Choke Mountains populations separated by the BNV (F_{ST} range: 0.005–0.084) (Figure 3). Disparities in F_{ST}

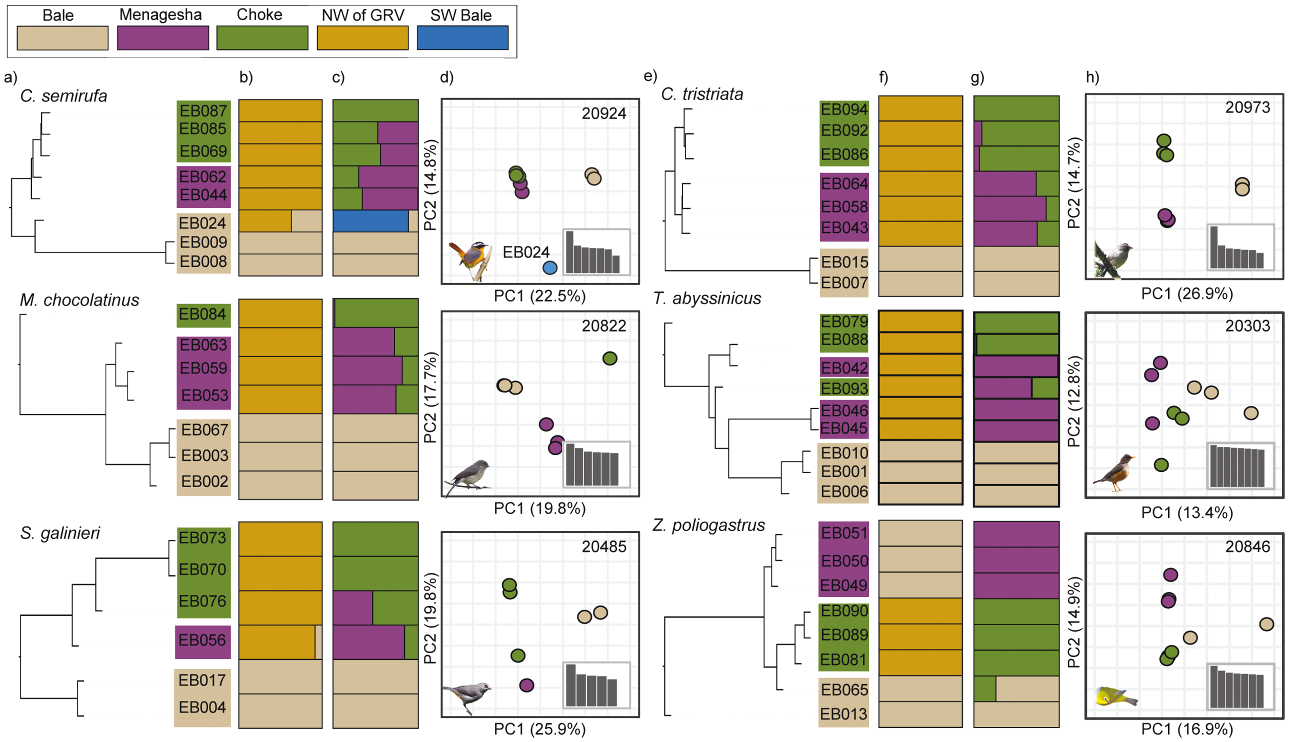

FIGURE 2. Population genetic structure. (**A, E**) ASTRAL-III species trees with tips labeled by sample ID. (**B, F**) STRUCTURE plots ($K = 2$) with bars displaying ancestry assignment probabilities of each sample corresponding to the sample ID directly adjacent to each bar. (**C, G**) STRUCTURE ($K = 3$ or 4). (**D, H**) Principal components analysis where each point represents an individual sample. Top right corners: number of SNP markers used in both PCA and STRUCTURE. Lower right corners: Scree plot denoting amount of variation explained by all principal components. Axis labels have percentage of variation explained by plotted principal components. *Cossypha semirufa* sample EB024 is labeled as it is an outlier to the other Bale samples.

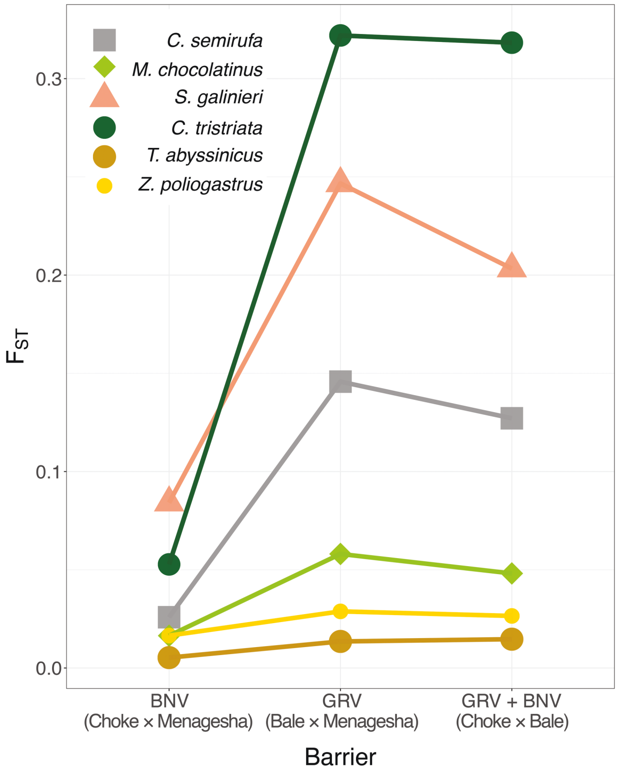

FIGURE 3. Genetic differentiation estimates (F_{ST}) for population pairs separated by the Great Rift Valley (GRV), Blue Nile Valley (BNV), or both.

between populations separated by the BNV vs. the GRV varied widely between species, with the largest disparities seen in *C. tristriata* (GRV = 0.318, BNV = 0.053). Populations separated by the GRV had F_{ST} values similar in magnitude to population pairs separated by both the BNV and GRV (Figure 3). Using PICs, species' mean HWI showed a negative association with pairwise genetic differentiation (F_{ST}) for both biogeographic barriers (GRV: $R^2 = 0.81$, $P = 0.04$; BNV: $R^2 = 0.61$, $P = 0.12$) (Supplementary Material Figure 2). Other predictors did not show strong/significant correlations with F_{ST} across either barrier. (Supplementary Material Figures 3 and 4)

Phylogenomics

ASTRAL-III species trees generated using 50 kbp windows showed a Bale clade in all species, except for *Z. poliogastrus*, which had a Bale individual nested within its Choke clade (Figure 2A and E). Sister relationships between populations varied with some species showing a sister relationship between Bale and Menagesha clades, whereas others showed Menagesha and Choke as sister. *Cossypha semirufa* had its Choke clade nested within its Menagesha clade. Three species—*C. tristriata*, *M. chocolatinus*, and *S. galinieri*—had reciprocally monophyletic clades for all localities. *Turdus abyssinicus* lacked phylogenetic structure between its Choke and Menagesha samples.

Phylogeographic Concordance

The ASTRAL-III phylogeographic concordance tree most strongly supported the Menagesha and Choke communities

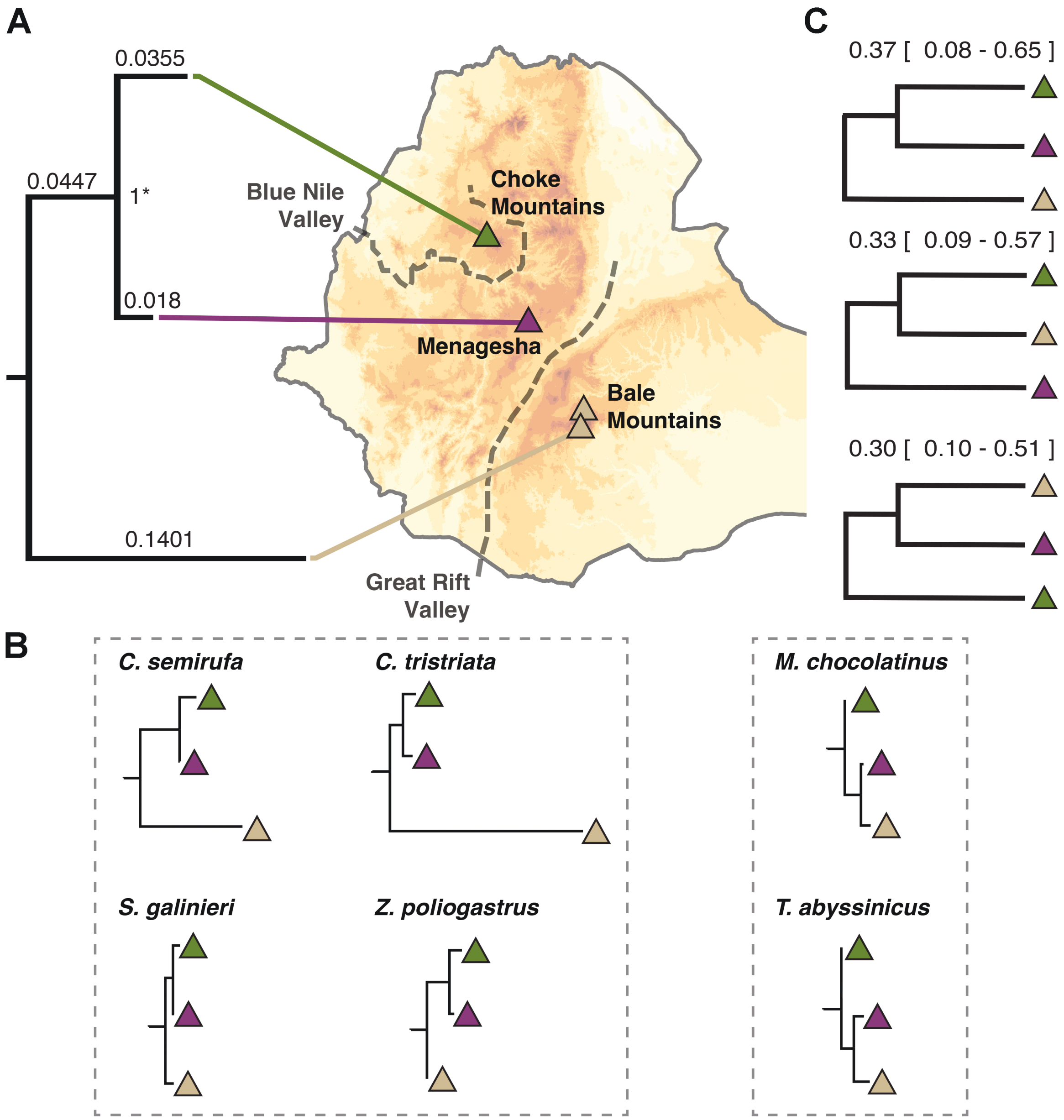

FIGURE 4. Phylogeographic concordance. (**A**) ASTRAL-III phylogeographic concordance tree. Asterisk (*) indicates the posterior probability of the topology. Branch lengths are in coalescent units and are proportional to gene tree concordance for population-scale relationships across all 6 study species. (**B**) ASTRAL-III concordance trees for each study species. Dashed boxes separate species-concordance trees by shared topology. (**C**) Three possible concordance tree topologies when all study species are monophyletic by location. Branch label indicates the confidence interval and proportion of subsampled gene trees supporting the given topology (outgroup was pruned for figure).

as sister, with the Bale community as sister to both, as indicated by the posterior probability (Figure 4A). The Bale locality had the longest branch length, indicating the most gene tree concordance for a Bale clade throughout the pooled gene trees across the genomes of all 6 species. Topologies of the concordance trees for 4 species (Figure 4B) matched the community concordance tree (Figure 4A), whereas concordance trees from *M. chocolatinus* and *T. abyssinicus* showed a divergent trend. Although concordance trees generated by gene tree sampling showed that the topology given by ASTRAL-III was most prevalent (Figure 4C), all possible topologies had comparable frequencies and overlapping confidence intervals as would be expected with recent differentiation among taxa.

Genetic Diversity and Demography

Individual genetic diversity as estimated by genome-wide H_O showed variation between species, ranging from 0.0008 in the Choke Mountains population of *S. galinieri* to 0.0054 in the Menagesha population of *C. semirufa*. (Figure 5). Mean H_O estimates remained roughly equal across populations in 4 out of the 6 species (Table 1). Exceptions to the uniform trend included *C. tristriata*, which had a lower mean H_O in its Bale Mountains population, and *M. chocolatinus* with a lower mean H_O in its Choke Mountains population.

The 4 species with consistent genetic diversity estimates across populations—*C. semirufa*, *T. abyssinicus*, *Z. poliogastrus*, and *S. galinieri*—had consistent N_e trajectories across populations (Supplementary Material Figure

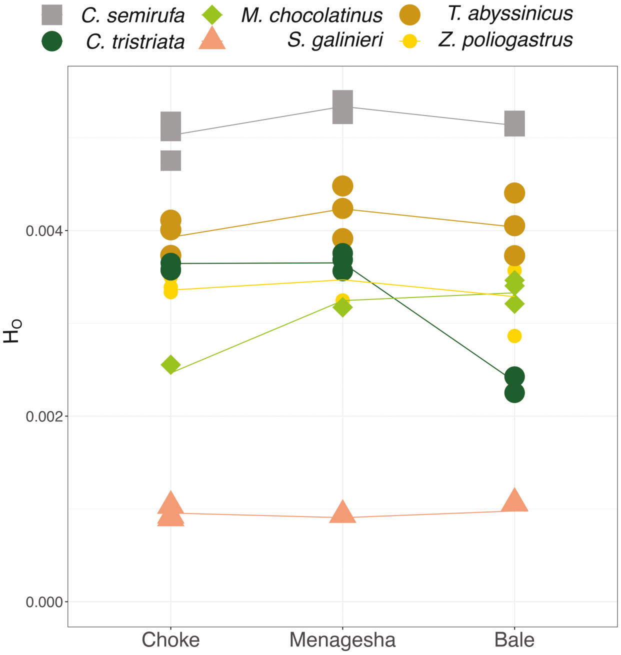

FIGURE 5. Genome-wide observed heterozygosity (H_O) for each locality per species. Points represent individual samples with lines connecting the mean heterozygosity for each locality.

8). *Crithagra tristriata* and *M. chocolatinus* showed distinct demographic histories between their populations separated by the GRV. Five species showed recent N_e declines, with 4 of these 5 species showing declines in the 2 most recent time segments. *Cossypha semirufa* reached the highest peak N_e but had the most extreme N_e fluctuations through time. Population harmonic mean N_e varied widely among species (Table 1). *T. abyssinicus* had the highest population harmonic mean N_e at 382,000—an order of magnitude higher than that of *S. galinieri*, which had the lowest population harmonic mean N_e at only 36,000.

Because gene flow may maintain genetic uniformity across populations by offsetting population-specific loss of alleles through genetic drift and local selection (Slatkin 1985), we wanted to determine if dispersal ability was correlated with genetic diversity variation across species. We found no significant relationship between dispersal ability (using HWI measurements) and genetic diversity (H_O; Supplementary Material Figure 9).

DISCUSSION

Phylogeography of the Six Focal Species

We found that the GRV has played a greater role in building phylogeographic structure relative to the BNV in the montane birds focal to this study (Figure 2). At the comparative phylogeographic scale, the phylogeographic concordance tree displayed higher gene tree concordance for a Bale clade among taxa and showed the Menagesha and Choke locations have a more recent shared history (Figure 4A). Thus, the Bale community is more distinct and likely more isolated from other sampling location's communities. F_{ST}, STRUCTURE, phylogenomic, and PCA results per species corroborated the concordance tree, as

TABLE 1. Population sampling, diversity, and demography per species. N = sample size; H_O = mean observed heterozygosity; HM N_e = harmonic mean effective population size * 1,000.

	Choke Mountains			Menagesha			Bale Mountains		
Species	N	H_O	HM N_e	N	H_O	HM N_e	N	H_O	HM N_e
Cossypha semirufa	3	0.00502	217	2	0.00534	265	3	0.00513	232
Crithagra tristriata	3	0.00364	64	3	0.00365	217	2	0.00237	210
Melaenornis chocolatinus	1	0.00247	119	3	0.00324	130	3	0.00333	137
Sylvia galinieri	3	0.00095	36	1	0.00091	39	2	0.00098	38
Turdus abyssinicus	3	0.00392	382	3	0.00423	373	3	0.00402	351
Zosterops poliogastrus	3	0.00336	165	3	0.00357	167	2	0.00313	166

there was a distinct Bale genetic cluster and elevated F_{ST} between GRV-separated populations in all species, apart from *Z. poliogastrus*. In contrast, only *M. chocolatinus* and *C. tristriata* had distinct population genetic clusters flanking the BNV in all phylogeographic analyses. The remaining species showed ambiguous support for phylogeographic structure across the BNV. Less pronounced BNV-associated phylogeographic structure corresponded to the shallow branch lengths in the Choke and Menagesha clades and the 2 locations' sister relationship on the concordance tree. The shallow branch lengths for all locations in the concordance tree and the varied clade relationships in the ASTRAL-III species trees indicate low support for a broadly shared evolutionary response to either barrier.

Genetic structure across species was partially explained by differences in dispersal ability between species, as F_{ST} was negatively correlated with HWI across the GRV and weakly across the BNV, although the small number of species sampled and limited number of locations sampled on either side of the barriers limit our ability to conclusively state that dispersal ability impacts differentiation. Additionally, populations separated by the GRV generally showed a similar magnitude of F_{ST} relative to population pairs separated by both barriers (a much greater distance), suggesting that a simple model of isolation by distance does not explain the differentiation observed across the biogeographic barriers. Differences in species ecology or diet could also have influenced long-term dispersal across either barrier and consequently genetic differentiation, as the 2 species with the greatest genetic structure around the BNV, *M. chocolatinus* and *C. tristriata*, differ in these factors relative to other species in this study. *Melaenornis chocolatinus* is the only riparian specialist, and *C. tristriata* is the only species that primarily eats seeds and is also commonly found in agricultural fields. However, using these categorical variables in models would require more extensive sampling. The highest genetic differentiation among the focal taxa in *C. tristriata* populations separated by the GRV is also likely in part due to the reduced genetic diversity in its Bale population.

Despite the idiosyncratic genetic structure across all species, the signature of genetic diversity was broadly consistent across most populations of all 6 focal species. The only inconsistencies were in the *C. tristriata* Bale and *M. chocolatinus* Choke populations with relatively lower H_O. The drivers of consistent genetic diversity within most species are likely variable. For example, *T. abyssinicus* shows weak population structure and low F_{ST} across the BNV, so the consistent H_O across that species' Choke and Menagesha populations is best explained by ongoing population connectivity. On the other hand, *S. galinieri* shows very strong phylogeographic structure and elevated F_{ST} between populations flanking the GRV, but consistent H_O between those same populations. This suggests that *S. galinieri*'s Bale population is recently diverged with a genetic diversity representing that of the ancestral population.

A positive relationship between genetic diversity and N_e is a fundamental expectation in population genetics (Hahn 2018). Unsurprisingly, we uncovered this relationship between H_O and harmonic mean N_e when accounting for phylogeny. Comparable between-population genetic diversity in two-thirds of our species coincided with their shared between-population N_e trajectories. *Crithagra tristriata* and *M. chocolatinus* were the exceptions, whose differences in population genetic diversity were directly explained by disparate population size trajectories. *Sylvia galinieri* had exceptionally low genetic diversity corresponding to its exceptionally low harmonic mean N_e.

Cossypha semirufa, *M. chocolatinus*, and *C. tristriata* showed a rise to peak N_e within the last glacial period (LGP) (115–11.5 thousand years ago (kya)) and declines within the last 50 thousand years; *T. abyssinicus* and *Z. poliogastrus* showed gradual N_e declines over the entire LGP. Regardless, all 5 of these species had N_e declines in the 2 most recent fused time segments that included the last glacial maximum (LGM) (20 kya). The LGM hailed the end of the LGP cooling trend that has been associated with marked demographic effects in high-elevation birds elsewhere in the world (Wang et al. 2013, Cheng et al. 2021) and in a wide range of avian species globally (Nadachowska-Brzyska et al. 2015). Furthermore, lacustrine sediments from Lake Tana north of the Choke Mountains, Lake Dendi to the southwest of Menagesha, and Gerba Guracha in the Bale Mountains, showed substantial climate oscillations following the LGM with subsequent effects on highland vegetation compositions and extents (Osmaston et al. 2005, Umer et al. 2007, Marshall et al. 2011, Jaeschke et al. 2020). Thus, habitat changes potentially contributed to N_e declines.

It was not possible to investigate anthropogenic effects on the N_e declines in the 2 most recent segments in 4 of our study species as these segments are fused to correct for model overfitting as suggested by the MSMC2 authors. For example, agriculture in Ethiopia did not begin until ~7 kya (Ehret 1979), and our most recent MSMC N_e estimates do not have much resolution over this time period. Interestingly, though, *S. galinieri* did show a slight increase in N_e during the narrow window encompassing the onset of agriculture. However, this result should be taken lightly as MSMC2 has limited power to accurately estimate N_e in very recent times, especially when calculating N_e trajectories on a per individual basis (Li and Durbin 2011, Schiffels and Wang 2020). Comparisons between contemporary and museum specimen genetic diversity or N_e trends could also provide resolution into recent anthropogenic impacts.

Comparative Phylogeographic Patterns

The GRV and BNV were shown to influence the phylogeography of previously studied Ethiopian species of largely limited dispersal ability relative to birds. We aimed to ask how phylogeography of volant taxa such as birds is impacted relative to that of the previously studied taxa. Like in *C. tristriata* and *M. chocolatinus*, genetic diversity differs by location in other less mobile highland taxa. For example, the proportion of private alleles was elevated for the frog *Ptychadena neumanni 5* (now *P. amharensis*) northwest of the BNV when compared to other *Ptychadena* clades (Reyes-Velasco et al. 2018a) and nucleotide diversity (π) in the Afroalpine-restricted *Canis simensis* (Ethiopian Wolf) differed amongst extant and recently extinct populations across the GRV and BNV (Gottelli et al. 2004). Even taxa with mobility rivaling that of birds can be characterized by uneven population genetic diversity in this geographic system despite their potential for connectivity. In *Plecotus balensis* (Bale long-eared bat), heightened nucleotide diversity was found in a population encompassing the Sanetti Plateau and Afroalpine moorlands of the Bale Mountains (Razgour et al. 2021).

This pattern contrasts to the general consistency in genetic diversity seen across populations in our study species.

Colonization of new areas following downward shifts in habitat during glacial maxima has been suggested to explain even nucleotide diversity in the absence of gene flow between isolated populations in *Amietia nutti*—an endemic and codistributed frog studied across the same barriers as in our study (Manthey et al. 2017). It is possible that these same historic periods of increased habitat connectivity—and potentially increased population connectivity—helped shape the consistent signatures of demography and genetic diversity in some of our focal species. Also, in contrast to *A. nutti*, the consistent genetic diversity shown in 5 of our focal species across the GRV may represent the genetic diversity of the ancestral population where there has not been sufficient time for genetic diversity shifts due to drift or selection. This is likely given that those focal populations diverged recently across the GRV (within the past ~300 ky; Manthey et al. 2022) but still show strong evidence for the GRV as a phylogeographic break in most genetic structure analyses.

Like the distribution of genetic diversity, there was no consistently shared pattern of system-wide genetic structure across our focal species. In contrast to these results, similar genomic studies involving frogs in the genera *Leptopelis* (Reyes-Velasco et al. 2018b), *Ptychadena* (Reyes-Velasco et al. 2018a), and *Amietia* (Manthey et al. 2017) showed strong phylogeographic structure across both biogeographic barriers, with the BNV arguably being a greater and shared biogeographic barrier for less mobile highland species. For example, *Leptopelis yaldenii* was only found to the northwest of the BNV, suggesting that the barrier thwarted its traversal to regions to the southeast. Similarly, *Ptychadena amharensis* was also restricted to a locality northwest of the BNV and had no clear signs of introgression with its sister species, *P. cooperi* from the other side of the BNV (Reyes-Velasco et al. 2018a, Lyra et al. 2023). Further, although *P. amharensis* was estimated to have colonized areas north of the BNV from the south 6–10 million years ago (mya), the highlands were lower during that time (~1,000 m lower at 11.7 mya), facilitating dispersal when compared to more recent conditions (Yemane et al. 1985, Adamson and Williams 1987, Lyra et al. 2023). The phylogeographic literature on Ethiopian highland frogs using genomic datasets has not shown evidence of substantial introgression between species or populations split by the BNV. *Ptychadena* species separated by the GRV, however, have shown introgression signatures such as that within *P. bekah* and *P. neumanni*—likely due to barrier traversal via changes in habitat extent during glacial maxima (Freilich et al. 2016, Reyes-Velasco et al. 2018a, Lyra et al. 2023).

Similarly, studies involving highland rodents have also suggested that the BNV is a substantial barrier to gene flow; a ddRADseq SNP phylogeny showed a clear split between 2 *Lophuromys simensis* lineages separated by the BNV (Komarova et al. 2021). The same study displayed evidence for interspecific introgression via mitonuclear discordance across the GRV between three species of *Lophuromys* (Komarova et al. 2021). In a study of 3 highland rodent species, Kostin et al. (2020) too suggested that the BNV is a relatively more substantial barrier than the GRV, based on mitochondrial phylogenies that showed strong BNV-associated phylogeographic breaks. However, some rodent species deviate from this trend such as congeners from *Stenocephalemys* that have shown introgression across both the GRV and BNV based on mitonuclear discordance (Bryja et al. 2018, Mizerovská et al. 2020). Thus, like with birds, there may be a species-specific response to these barriers among rodents, but these examples may warrant further investigation with genome-wide data.

The BNV impact on frog and rodent diversification is not surprising as the southern end of the BNV forms a steep gorge that likely presents a formidable barrier to these tropical vertebrates of relatively low mobility. Tropical species also tend to adapt to narrow environmental bands along mountains. Indeed, Bryja et al. (2018) found evidence of parapatric speciation along elevation gradients in *Stenocephalemys* rodents in the Ethiopian Highlands. In contrast, our study species' elevational occurrence range did not show a significant association with F_{ST} across either valley. So, unlike the other ground-dwelling or sometimes arboreal taxa, narrow environmental tolerances to specific elevational bands are not suggested as contributors to the genome-wide differentiation patterns of our study species. This pattern has been shared by other tropical montane birds. For example, various species of *Scytalopus* at narrow elevational bands in the Andes likely speciated allopatrically with subsequent secondary contact—rather than parapatrically through narrow environmental tolerance (Cadena et al. 2021). Similarly, a study of bird species inhabiting elevation gradients on the Islands of Buru and Seram found nonsignificant F_{ST} across elevations, yet significant F_{ST} values between islands (Pujolar et al. 2022). Our pattern does not, however, completely preclude divergence along elevational clines for our study species. Strong signals of divergent selection at adaptive loci have been found along elevational clines in an Andean hummingbird with no genetic structure (Lim et al. 2021).

Cossypha semirufa: A Fourth Population and the Sanetti Plateau Barrier

A fourth distinct genetic grouping for *C. semirufa* represented by individual EB024 came from a site to the southwest of other Bale sample collections. This individual is distinguished in PCA and STRUCTURE and peak N_e ~300,000 greater than other Bale samples. This individual's H_O was not distinctly different from other individuals in the region, lending credibility to the N_e differences inferred. These lines of evidence hint at a fourth distinct population that may be explained by the ~45 km of rugged terrain that separates the Bale sites. Much of this terrain is part of the Sanetti Plateau and ~1,000 m above the recorded elevational occurrence for *C. semirufa* (~3,200 m) (Redman et al. 2016); there are also scant eBird reports of the species strictly on the plateau (Sullivan et al. 2009). For much of the Holocene (~7,000 years), an ericaceous vegetation belt dominated the landscape of the Sanetti Plateau (Umer et al. 2007), which was likely incompatible with the species' ecological requirements. Presently, the plateau is also not predominately Afromontane forest, but Afroalpine dwarf scrub. The incompatibility was likely more pronounced during the LGM (20 kya) when the glaciated Wasama and Danka River Valleys stood between the Bale sites (Osmaston et al. 2005). The Sanetti Plateau habitat contrasts to the Afromontane Forest, where *C. semirufa* is found and likely posed a formidable barrier to migrants between the Bale sampling sites. Further testing of this hypothesis would involve sampling more *C. semirufa* individuals on either side of the Sanetti Plateau and comparing their genetic structure.

Zosterops poliogastrus: Ghost Population or Sampling Bias?

Zosterops poliogastrus had apparent gene flow across the entire highland region. Individual EB065 showed Choke and Bale ancestry and assignment to the Choke clade, although it was sampled from Bale (Figure 2G and H). This scenario would require dispersal across both the GRV and BNV, which is possible as *Z. poliogastrus* has the highest inferred dispersal ability (HWI) of the study group. Recent southward migrants from Choke could miss the small 24 km^2 of natural forest in Menagesha altogether when compared to *3,500–7,000* km^2 of native forest in the Bale Mountains National Park's south escarpment alone, which could explain why we saw no admixture in Menagesha. In more ancient times, N_e trajectories are similar across populations suggesting, in essence, a single interconnected population. This explanation is unlikely, however, as we would expect to see some evidence of admixture in another population at this genomic resolution. Another more likely explanation is an unsampled or "ghost" population (Lawson et al. 2018). Under this scenario, the individual may have migrated, or have had ancestry from, a fourth unsampled population. Further research on this topic could involve sampling *Z. poliogastrus* individuals from high-elevation locations near the Bale Mountains, such as in the Ahmar Mountains, and comparing genetic structure patterns between individuals from all sampling locations once again to determine if there is perhaps an unsampled population nearby that more strongly clusters with individual EB065.

Conclusion

We showed that topography of the Ethiopian Highlands has impacted the diversification of Ethiopian taxa with relatively high dispersal abilities and with discordant phylogeographic trends relative to nonvolant taxa of previous studies in the region. Wide lowland barriers like the GRV and BNV, as well as high elevation barriers like the Sanetti Plateau, have impacted 6 montane birds' demography, phylogeographic structure, and differentiation—albeit in an idiosyncratic fashion. The GRV is a more consistent and stronger barrier to gene flow in our study species relative to the BNV, which contrasts to the pattern seen in frogs and rodents. Although dispersal ability partially explained the differences in differentiation between populations in these study species, other species' traits not included in this study may have also contributed. Our genome-wide diversity estimates spanning the region also characterize the genetic constituent of biodiversity of these montane birds in this biodiversity hotspot and show that two species have populations of noticeably reduced genetic diversity. Through similar assessments of more species in understudied hotspots, we will better quantify genetic diversity and the drivers shaping genetic diversity through time and across the landscape.

Supplementary material

Supplementary material is available at *Ornithology* online.

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Ethics statement

All fieldwork was done in accordance with NYU IACUC accepted procedures (IACUC Protocol Number 15-00002A1) and permissions from the Ethiopian Wildlife Conservation Authority, the Oromia Forest and Wildlife Enterprise, and local landowners.

Conflict of interest statement

Authors declare no conflicts of interest.

Author contributions

G.J.B. led the writing of the manuscript; all authors contributed to writing and editing the manuscript; J.D.M. and G.J.B. concieved project ideas; J.D.M. conducted fieldwork; and G.J.B. analyzed the data.

Data Availability

Fastq reads for the Choke Mountains samples are accessioned at NCBI under Bioproject PRJNA948542. Fastq reads for the Bale Mountains and Menagesha samples are accessioned at NCBI under Bioproject PRJNA605410. Scripts used for analyses are available at Behrends et al. (2024).

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