

doi:10.1111/evo.14448



A migratory divide spanning two continents is associated with genomic and ecological divergence

Sheela P. Turbek,^{1,2} Drew R. Schield,¹ Elizabeth S. C. Scordato,^{1,3} Andrea Contina,⁴ Xin-Wei Da,⁵ Yang Liu,⁶ Yu Liu,⁷ Emilio Pagani-Núñez,⁸ Qing-Miao Ren,⁹ Chris C. R. Smith,¹ Craig A. Stricker,¹⁰ Michael Wunder,⁴ David M. Zonana,^{1,11} and Rebecca J. Safran¹

Received February 4, 2021 Accepted December 29, 2021

Migratory divides are contact zones between breeding populations with divergent migratory strategies during the nonbreeding season. These locations provide an opportunity to evaluate the role of seasonal migration in the maintenance of reproductive isolation, particularly the relationship between population structure and features associated with distinct migratory strategies. We combine light-level geolocators, genomic sequencing, and stable isotopes to investigate the timing of migration and migratory routes of individuals breeding on either side of a migratory divide coinciding with genomic differentiation across a hybrid zone between barn swallow (*Hirundo rustica*) subspecies in China. Individuals west of the hybrid zone, with *H. r. rustica* ancestry, had comparatively enriched stable-carbon and hydrogen isotope values and overwintered in eastern Africa, whereas birds east of the hybrid zone, with *H. r. gutturalis* ancestry, had depleted isotope values and migrated to southern India. The two subspecies took divergent migratory routes around the high-altitude Karakoram Range and arrived on the breeding grounds over 3 weeks apart. These results indicate that assortative mating by timing of arrival and/or selection against hybrids with intermediate migratory traits may maintain reproductive isolation between the subspecies, and that inhospitable geographic features may have contributed to the diversification of Asian avifauna by influencing migratory patterns.

KEY WORDS: Barn swallow, geolocator, hybrid zone, seasonal migration.

¹Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, Colorado 80309

²E-mail: sheela.turbek@colorado.edu

³Department of Biological Sciences, Cal Poly Pomona, Pomona, California 91768

⁴Department of Integrative Biology, University of Colorado, Denver, Colorado 80217

⁵College of Life Science, Wuhan University, Wuhan 430072, China

⁶School of Ecology, Sun Yat-sen University, Guangzhou 510275, China

⁷Key Laboratory for Biodiversity Sciences and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing 100875, China

⁸Department of Health and Environmental Sciences, Xi'an Jiaotong-Liverpool University, Suzhou 215123, China

⁹School of Life Sciences, Lanzhou University, Lanzhou 730000, China

¹⁰U.S. Geological Survey, Fort Collins Science Center, Fort Collins, Colorado 80526

¹¹Department of Biological Sciences, University of Denver, Denver, Colorado 80210

Every year, billions of fish, mammals, insects, and birds undergo spectacular journeys in pursuit of seasonal changes in resource abundance that carry them vast distances from their breeding grounds (Dingle 2014). These annual migrations between breeding and nonbreeding habitats require an integrated suite of morphological, physiological, sensory, and behavioral adaptations necessary for long-distance movement, as well as survival and reproduction in disparate environments (Milner-Gulland et al. 2011; Dingle 2014). Given the complex nature of seasonal migration, divergence in migratory strategies between closely related taxa has been proposed to promote and maintain genetic divergence and population differentiation (Irwin and Irwin 2005; Winker 2010; Turbek et al. 2018). However, the difficulty of tracking seasonal movements across space and time has historically limited our understanding of the ways in which seasonal migration can contribute to patterns of population divergence (Kays et al. 2015).

Migratory divides are contact zones between breeding populations with divergent migratory strategies during the nonbreeding season (O'Corry-Crowe et al. 1997; Bensch et al. 1999; McDevitt et al. 2009; Delmore et al. 2012; Dodson et al. 2013; Alvarado et al. 2014). These regions offer a unique opportunity to study the role of migratory behavior in reproductive isolation and speciation (Turbek et al. 2018). Although migratory divides in some taxa, particularly fish, have likely arisen through primary divergence (Wood et al. 2008; Dodson et al. 2013), they often form in birds through secondary contact between populations that were isolated in distinct glacial refugia during the Pleistocene. Following the last glacial maximum, these populations expanded along divergent pathways and have retained their ancestral migration routes (Irwin and Irwin 2005; Ruegg 2008; Bensch et al. 2009). Regardless of their biogeographic history, migratory divides are associated with divergent migratory behavior that is thought to restrict genetic exchange and/or maintain reproductive isolation upon secondary contact through assortative mating by timing of reproduction and/or selection against hybrids that inherit intermediate migratory traits (Bearhop et al. 2005; Irwin and Irwin 2005; Delmore and Irwin 2014; Pujolar et al. 2014), leading to an accumulation of genomic divergence over time (Rolshausen et al. 2009; Dodson et al. 2013; Jacobsen et al. 2014). Although most studies have used indirect tracking methods, such as mark recapture or stable isotopes, to infer the contribution of migratory behavior to the maintenance of population differentiation and reproductive isolation at migratory divides, animal-borne tracking methods offer information on the timing of migration and migratory routes of individuals, and are therefore needed to investigate the nature of isolating barriers that may restrict interbreeding between organisms with distinct migratory strategies.

In Asia, the high-elevation Qinghai-Tibetan Plateau has been proposed as a hostile geographic barrier to animal migration (Delany et al. 2017; Liu et al. 2018a) that has potentially contributed to the divergence of Asian avifauna (Irwin and Irwin 2005). According to compiled data from banding records and species range distributions, a disproportionate number of passerine species that breed in Siberia migrate either to the east or west of the Qinghai-Tibetan Plateau en route to their wintering grounds in southern Asia (Irwin and Irwin 2005). In addition, many species that circumvent the barrier in either direction exhibit migratory divides between eastern and western forms potentially associated with reproductive isolation on the breeding grounds (Irwin and Irwin 2005). By influencing the migratory routes of passerine species, major geographic features, such as the Qinghai-Tibetan Plateau and the nearby low-altitude Gobi and Taklamakan deserts, may have shaped the biogeographic patterns of songbird diversification in northern Asia. However, direct tracking methods are necessary to fully understand the nonbreeding locations, timing of migration, and migratory routes of passerines as they navigate the Qinghai-Tibetan Plateau and similarly inhospitable features in central Asia (Delany et al. 2017). Given that animal-borne tracking devices, such as geolocators, must be recovered following deployment, thereby limiting the number of individuals that can be tracked across the full annual cycle, using geolocators to ground truth stable isotope data can provide this critical information while improving inferences of nonbreeding locations made from isotope data alone.

Barn swallows (Hirundo rustica) are widely distributed throughout the Palearctic and comprise six subspecies that exhibit substantial variation in migratory behavior (Turner 2010; Scordato and Safran 2014). Two subspecies, H. r. rustica, which breeds throughout northern Africa, Europe, and western Asia, and H. r. gutturalis, which breeds in southern and eastern Asia, share a hybrid zone in northwestern China that was likely formed through secondary contact (Fig. 1) (Scordato et al. 2020). The two subspecies differ in body size, as well as ventral coloration and tail streamer length, known sexually selected traits, and exhibit a narrow cline for genomic ancestry, suggesting that the hybrid zone between H. r. rustica and H. r. gutturalis is maintained by selection (Scordato et al. 2020). Previous work, which used genotyping-by-sequencing (GBS) to examine patterns of differentiation in barn swallows across Asia, documented low genomic differentiation (mean genome-wide $F_{ST} = 0.028$) between the subspecies and detected backcrossed individuals but few F1 hybrids in the hybrid zone in Gansu Province, China, providing evidence of limited but ongoing gene flow between H. r. rustica and H. r. gutturalis (Scordato et al. 2020). The study also found limited support for within-population premating isolation between the subspecies when analyzing patterns of social pairing and indicated that parental individuals of H. r. rustica and H. r. gutturalis are largely confined to either end of the hybrid zone and do not overlap in the zone's center, which is composed of

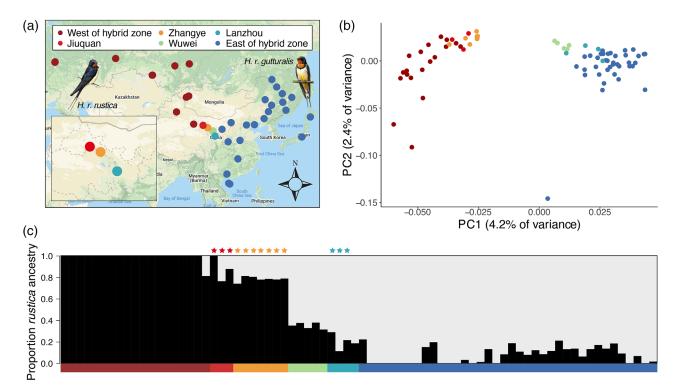


Figure 1. (A) Sampling locations, (B) principal component analysis (PCA) of the genome-wide covariance matrix, and (C) admixture proportions generated using whole-genome data for two subspecies of barn swallow (*Hirundo rustica rustica* and *H. r. gutturalis*) breeding across Asia (n = 78). Points are colored by sampling location. The inset in panel A shows the three cities, Jiuquan, Zhangye, and Lanzhou, where geolocators were deployed. Individuals west of the hybrid zone in China between *H. r. rustica* and *H. r. gutturalis* were sampled from allopatric populations of *H. r. rustica* (dark red in all panels), whereas individuals east of the hybrid zone were sampled from allopatric populations of *H. r. gutturalis* (dark blue in all panels). The admixture proportions of the 13 individuals with geolocator tracks are indicated in panel C with stars.

admixed individuals (Scordato et al. 2020). Thus, extrinsic selection against hybrids may contribute to the maintenance of subspecies boundaries between H. r. rustica and H. r. gutturalis. Finally, the subspecies exhibit substantial differentiation in the stable-carbon isotope values of their tail feathers, which are molted on the wintering grounds (Turner 2010; Scordato et al. 2020). Stable isotopes values (e.g., carbon $[\delta^{13}C]$) are incorporated into metabolically inert tissues, such as feathers, through diet and remain constant across the annual cycle, reflecting the isotopic composition of the local environment in which the feathers were grown (Hobson 1999; Rubenstein and Hobson 2004). Carbon isotopes, in particular, are influenced by the relative abundance of C3 and C4 plants in the area of feather growth (Hobson 1999; Rubenstein and Hobson 2004). Thus, strong differentiation in δ^{13} C values between the subspecies has been used to infer the presence of a migratory divide (Scordato et al. 2020). However, variation in carbon isotope values can arise due to a variety of factors, including divergence in dietary preferences or habitat selection over small spatial scales (Bearhop et al. 2003). In addition, stable isotopes do not provide information on divergence in the timing of migration or migratory routes of individuals, which is

critical to understand the isolating mechanisms that restrict interbreeding between barn swallow subspecies. Direct tracking methods are therefore necessary to verify the presence of a migratory divide between *H. r. rustica* and *H. r. gutturalis* and assess the role that divergent migratory strategies play in maintaining reproductive isolation upon secondary contact.

To investigate the possibility that a migratory divide between H. r. rustica and H. r. gutturalis is involved in the maintenance of this subspecies boundary, we followed the workflow detailed in Turbek et al. (2018) aimed at testing whether divergent migratory phenotypes contribute to reproductive isolation at migratory divides. First, we documented differences in migratory phenotype by attaching light-level geolocators to barn swallows breeding on either end of the hybrid zone. These animal-borne tracking devices recorded light intensity levels at specific intervals throughout the annual cycle. In addition, we used stable-carbon (δ^{13} C) and hydrogen (δ^{2} H) isotopes to examine a larger sample of individuals concentrated in the area of putative hybridization and verify patterns observed in the geolocator data. To assess genomic differentiation between barn swallows breeding on either side of the migratory divide, we analyzed published high-resolution

whole-genome sequencing (WGS) data from the individuals that returned with geolocators and generated lower resolution double digest restriction site-associated DNA (ddRAD) sequencing data from the larger sample of individuals. Finally, we assessed correlations between timing of migration, migratory route, phenotypic traits, and genomic ancestry to identify traits associated with patterns of genomic differentiation across the migratory divide.

If migratory behavior contributes to the maintenance of population divergence and reproductive isolation between barn swallow subspecies, we predicted that individuals on either side of the hybrid zone would overwinter in geographically distinct areas. In addition, we expected to detect (1) strong concordance between geolocator tracks/isotope values and ancestry estimates, (2) an association between migratory route and ancestry-related traits (e.g., ventral coloration and tail streamer length), and (3) an association between migratory route and timing of arrival on the breeding grounds.

Materials and Methods

FIELD SAMPLING

We sampled 130 individuals of H. r. rustica and H. r. gutturalis in three cities on either side of the hybrid zone in Gansu Province, China (Lanzhou: n = 58, Zhangye: n = 58, Jiuquan: n = 14; Table S1) across two sampling trips that took place during the barn swallow breeding season (July 2016 and May 2017). In each region, we captured barn swallows with mist nets, fit each bird with an individually numbered aluminum band, collected the two inner-most tail rectrices, and took a blood sample from the brachial vein prior to release. Blood samples were stored in lysis buffer and DNA was extracted with the DNeasy blood and tissue kit (Qiagen, CA, USA). In addition, we took three measurements of the left and right tail streamer (to the nearest mm) per individual and averaged across both. Finally, we collected 10-15 feathers from four plumage patches (throat, breast, belly, and vent) to quantify coloration in each ventral region. We used a spectrometer (USB 4000, Ocean Optics, FL) with a pulsed xenon light source (PX-2, Ocean Optics) and Spectrasuite software to measure the hue, chroma, and brightness of each plumage patch as described in Safran et al. (2010). These 130 individuals were included in the double digest restriction site-associated DNA sequencing (ddRAD-seq) dataset focused on the hybrid zone (see below).

GENOME-WIDE ANCESTRY: WHOLE-GENOME SEQUENCING

To assess patterns of genomic differentiation across the hybrid zone, we analyzed published whole-genome sequence data from Smith et al. (2018) (NCBI BioProject PRJNA323498). Although the published dataset included 168 individuals from six barn swallow subspecies, which were retained during variant calling to improve genotyping accuracy, we limited the final dataset to the 80 birds that were relevant to the hybrid zone in China (individuals of H. r. rustica and H. r. gutturalis that were sampled in Asia; i.e., Russia, China, Mongolia, and Japan; Table S2). Fourteen of these individuals were sampled during the July 2016 trip described above and included in the ddRAD-seq dataset (described below). We used Trimmomatic (version 0.36) to remove bases at the ends of each read with quality scores below 30 and trim reads with an average quality score less than 30 or length less than 50 bp (Bolger et al. 2014). We then aligned the trimmed reads to a barn swallow reference genome (Formenti et al. 2018) with BWA-MEM (version 0.7.12) and called variants with SAMtools (version 1.5) and BCFtools (version 1.5) (Li et al. 2009; Li 2013). To avoid sex-linked loci, we filtered out sex chromosomes by aligning the barn swallow scaffolds to collared flycatcher (Ficedula albicollis) autosomes (Ellegren et al. 2012) and the chicken W chromosome (International Chicken Genome Sequencing Consortium 2004) using the program MashMap (Jain et al. 2017, Jain et al. 2018). We set the mapping segment length parameter to 10,000 for scaffolds shorter than 1 Mb and 50,000 for scaffolds longer than 1 Mb. Like other alignment programs, MashMap outputs many short alignment segments. We assigned individual scaffolds to a chromosome if (1) more than half of the alignment segments landed on the same chromosome, and (2) more than half of the total scaffold length aligned to the same chromosome, or else excluded the scaffold from further analysis. A total of 173 scaffolds were assigned to flycatcher autosomes, totaling \sim 1.01 Gbp, or 91% of the barn swallow genome. For the retained barn swallow scaffolds, we approximated the base pair position by subtracting half of the scaffold length from the median alignment position among the short alignments mapping to the assigned chromosome.

We removed variants that had a read depth below five reads per locus, a minor allele frequency less than 0.05, or were present in fewer than 80% of individuals with VCFtools (version 0.1.17) (Danecek et al. 2011). In addition, we removed two individuals with more than 50% missing data and only included one SNP per 10 kb to remove loci that were tightly linked. This pipeline produced 32,029 SNPs derived from 78 individuals across the breeding range of H. r. rustica and H. r. gutturalis in Asia (Fig. 1A).

GENOME-WIDE ANCESTRY: DOUBLE DIGEST RESTRICTION SITE-ASSOCIATED DNA SEQUENCING

We used ddRAD-seq (Peterson et al. 2012), following the protocol in Safran et al. (2016) and Scordato et al. (2017), to generate a reduced representation genomic dataset focused on the hybrid zone in Gansu Province, China for the 130 individuals

sampled in July 2016 and May 2017 (Fig. 1A; Table S1). Briefly, we used restriction enzymes Msel and EcoRI to fragment loci and ligated sample-specific 8-10 bp barcode sequences for library preparation. Libraries were generated at the University of Colorado Boulder BioFrontiers Institute, size-selected for a 350- to 400-bp region, and sequenced on two replicate Illumina HiSeq 2500 lanes using 100-bp single-end sequencing at the University of Texas Austin Genomic Sequencing and Analysis Facility.

Following sequencing, we detected a lane effect issue stemming from an unintentional difference in fragment size selection from previous libraries. As a result, too few loci were represented by both the current and previous GBS datasets during initial variant calling steps. We were therefore unable to combine data from the newly generated libraries with previously generated GBS data for H. rustica from Scordato et al. (2020), which included allopatric individuals of H. r. rustica and H. r. gutturalis needed to infer ancestry within the hybrid zone. To leverage data from individuals sampled outside of the putative hybrid zone for ancestry inference, we selected eight individuals that had nearly 100% assignment to H. r. rustica or H. r. gutturalis from the broader WGS dataset and included these individuals when calling variants for the newly generated ddRAD data.

We used the Stacks (version 2.5) process radtags module (Catchen et al. 2013; Rochette et al. 2019) to demultiplex raw sequencing files and trimmed reads for quality using Trimmomatic (version 0.39) (Bolger et al. 2014), removing bases from read ends with a quality score <20 and filtering any reads with an average quality score <30 or final length <32 bp. We mapped the resulting ddRAD data, along with WGS data from the eight individuals with high H. r. rustica or H. r. gutturalis ancestry, to the *H. rustica* reference genome using BWA (version 0.7.17) with program defaults (Li and Durbin 2009). We combined information from all mapping files and called SNPs using BCFtools (version 1.10.2). Raw SNP calls were filtered with BCFtools to remove any sites with a median depth among samples <7. We also used BCFtools to recode any sites with individual depth <5 or individual genotype quality <30 as missing data. We used VCFtools (Danecek et al. 2011) to only retain biallelic SNPs that were called in at least 80% of individuals and had a minor allele frequency >0.05, as well as remove any SNP calls that overlapped with the H. rustica genome repeat annotation or were located on the Z chromosome. In addition, we only retained a single SNP per RAD locus to reduce the effects of linkage. Three samples (two from Zhangye and one from Lanzhou) had >80% missing data after filtering steps and were therefore removed from analysis. The final filtered VCF table, which included 3974 SNPs from 127 individuals, was converted to readable input for ancestry inference using Plink (version 1.9) (Purcell et al. 2007).

POPULATION GENETIC ANALYSES

We used ADMIXTURE (version 1.3) to infer the number of genetic clusters and ancestry proportions of individuals breeding on either side of the hybrid zone in Gansu Province, China relative to allopatric populations of H. r. rustica and H. r. gutturalis (Alexander et al. 2009). We ran ADMIXTURE analyses on both the WGS and ddRAD datasets for a series of K values 1-5, using the default cross-validation approach to compare models of K genetic clusters. In both cases, the cross-validation procedure indicated similar support for the K = 1 and K = 2 models, as these models had a lower cross-validation error than the next bestperforming model (K = 3). Because this analysis was designed in part to examine the relative proportion of H. r. rustica versus H. r. gutturalis ancestry in birds breeding across this region, we interpret ancestry proportions under the K=2 model. A principal component analysis (PCA) of the genome-wide covariance matrix derived from the WGS data using the R function prcomp supported the inference of ancestry from two genetic clusters.

To confirm that the ddRAD- and WGS-based inferences of ancestry were congruent, we performed a post hoc comparison of ancestry proportions for the 14 individuals that returned with geolocators, which were sequenced using both WGS and ddRAD approaches. We found a significant positive correlation between the proportion of H. r. rustica ancestry inferred using the two datasets (Pearson's r = 0.718, P = 0.004, n = 14; Fig. S1), indicating that the two methods provide largely consistent estimates of ancestry.

MIGRATORY STRATEGY: GEOLOCATOR TRACKS

During the July 2016 sampling trip, we captured breeding adults in three cities on either side of the hybrid zone between H. r. rustica and H. r. gutturalis in Gansu Province, China (Lanzhou: n = 33, Zhangye: n = 33, and Jiuquan: n = 10) and attached Intigeo P55B1-7 geolocators (0.6 g; Migrate Technology, Cambridge, UK) to all captured birds using a Rappole-Tipton leg-loop backpack harness (Rappole and Tipton 1991) made of 0.7-mm elastic cord. The total weight of the tag and harness was less than 5% of average body mass (15.26 \pm 1.22 g; mean \pm SD) at the time of attachment. In May 2017, we re-sighted 16 of the 76 tagged individuals (21%). However, we only obtained geolocator data from 13 birds (7 males and 6 females), as the battery of one device from Lanzhou stopped working prior to migration and we were unable to capture two individuals. One of the 13 recovered geolocators containing migratory data stopped recording in March 2017 but was included in subsequent analyses because it collected data throughout the wintering period. Information on the return rates of banded individuals that were not fit with geolocators is unavailable, as we deployed geolocators on all captured birds during the 2016 sampling trip.

We downloaded light intensity data with IntigeoIF (Migrate Technology) and preprocessed the light readings with the R package TwGeos (Wotherspoon et al. 2016), setting a light threshold of 1.5 to automatically determine times of sunrise and sunset. We then visually inspected each twilight period to exclude twilights that exhibited obvious effects of shading. We manually removed twilights that showed atypical light patterns to maintain day-to-day consistency in the shape of the light curve near sunrise and sunset during the calibration period. We analyzed the light intensity data with the R package FLightR following the workflow detailed in the supporting information of Rakhimberdiev et al. (2017). Briefly, we derived calibration periods from the data using the plot_slopes_by_location function in FLightR and optimized the model with 1 million particles. The FLightR model included a spatial mask that prohibited residency over water, allowed individuals to fly a maximum distance of 1500 km between twilights (as in Rakhimberdiev et al. 2016), and automatically excluded outliers when generating daily estimates of latitude and longitude. We detected stationary periods by running the stationary.migration.summary function in FLightR with 0.25 as the minimum probability of movement and 3 days as the minimum duration of the stationary period.

To assess the accuracy of the geolocator estimates, we calculated the distance between the true deployment location and the location estimated by the geolocators during the calibration period. In addition, we estimated migratory distance during fall and spring migration by importing the geolocator tracks into QGIS (version 2.18.20) and calculating the length of each track using the length measuring tool (QGIS Development Team 2019). To estimate mean arrival and departure dates from the breeding grounds, we calculated the date by which half of the particles in the FLightR model crossed a spatial boundary of one degree longitude west of the breeding site, as in Rakhimberdiev et al. (2016).

MIGRATORY STRATEGY: STABLE ISOTOPES

We analyzed the stable-carbon (δ^{13} C) and hydrogen isotope $(\delta^2 H)$ values of the inner-most tail rectrices from the 130 individuals included in the ddRAD dataset to estimate wintering locations for individuals breeding in the three cities on either side of the hybrid zone (i.e., Jiuquan, Zhangye, and Lanzhou). Following the protocol in Scordato et al. (2020), we cleaned tail feathers with a 2:1 mixture of chloroform and methanol to remove oils and surface contaminants, cut 0.5-1 mg from the center of the feather vane, avoiding the tip and base, and rolled the feather into 4×6 mm capsules (tin for carbon and silver for hydrogen isotope analysis; Costech Analytical Technologies, Valencia, CA). For carbon isotope measurements, samples were combusted in an elemental analyzer (Carlo Erba NC2500, Milan, Italy) interfaced to an Optima mass spectrometer (VG Micromass, Manchester, UK) (Fry et al. 1992). Nonexchangeable hydrogen isotope measurements were measured on a thermal conversion elemental analyzer (Thermo Scientific, Bremen, Germany) interfaced to a Delta V mass spectrometer (Thermo Scientific, Bremen, Germany), following conventional methods for carbon reduction and comparative equilibration (Wassenaar and Hobson 2003). All measurements were taken at the U.S. Geological Survey Stable Isotope Laboratory (Denver, CO). We report data in standard delta notation with respect to internationally accepted scales (V-PDB and V-SMOW). Carbon isotope data were normalized to USGS 40 (δ^{13} C = -26.24 %) and 41 $(\delta^{13}C = 37.76 \%)$, and hydrogen isotope data were normalized to caribou hoof ($\delta^2 H = -157\%$) and kudu horn ($\delta^2 H = -35.3\%$). Analytical precision of replicate standards, including two additional secondary standards, was better than $\pm 0.2\%$ for δ^{13} C and \pm 4% for δ^2 H. Two individuals, both sampled in Zhangye, had biologically unrealistic δ^{13} C and δ^{2} H values, respectively, and were therefore excluded from subsequent analyses.

We assigned geographic locations of origin for individuals breeding in Jiuquan, Zhangye, and Lanzhou from feather δ²H values and a geographic model for δ^2 H in precipitation (Bowen and Revenaugh 2003) using methods based on Wunder (2010), as the δ^2 H isoscape derived from precipitation was more informative in eastern Africa and southern Asia than the δ¹³C isoscape for geographic assignments. Feathers collected from birds that returned with geolocators were assumed to be of known origin because tail feathers are grown during the winter and the geolocators provided estimates of a single stationary wintering location per individual. We used these known-origin feathers (n = 13) to calibrate the precipitation model for barn swallow feathers with the function calRaster in the R package assignR (Ma et al. 2020), and subsequently used the function pdRaster to generate a posterior probability of origin raster (i.e., assignment raster) for each of the 116 feathers from birds of unknown wintering location. We limited the spatial extent of the assignment rasters to the extent of the wintering range. We further subdivided the assignment rasters based on recognized Asian flyways for either an African or Asian wintering region (Fig. S2). Because this subdivision resulted in different numbers of raster cells for each subregion (Africa: n = 20,210 cells; Asia: n = 13,882 cells), we computed the average of the cell values in the assignment raster for each region. If the average for one subregion was more than twice that for the other region, the individual was assigned to the region with the higher average. We assigned individuals as "undetermined" (12/116 individuals; 10%) if there was less than a twofold difference between the averages.

STATISTICAL ANALYSIS

We ran unpaired t-tests to compare migratory distance, timing of migration, WGS-based ancestry estimates, and δ²H values for

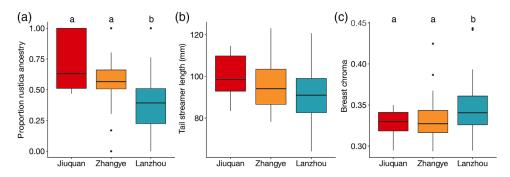


Figure 2. (A) Proportion of *Hirundo rustica rustica* ancestry derived from double digest restriction site-associated DNA (ddRAD) data, (B) mean tail streamer length, and (C) breast chroma of barn swallows (*H. r. rustica* and *H. r. gutturalis*) breeding across a hybrid zone in China (n = 127). Different lowercase letters indicate significant differences between locations (Dunn test with Holm's correction for ancestry and one-way ANOVA with Tukey's HSD for tail length and ventral coloration, adjusted P < 0.05) and colors correspond to cities on the map in Figure 1. Box plots depict the minimum, first quartile, median, third quartile, and maximum, with outliers shown as single points.

individuals with geolocators traveling from separate continents. In addition, we ran a Kruskal-Wallis test with Dunn's post hoc test to compare the ddRAD-based ancestry estimates of individuals breeding in the three cities on either side of the hybrid zone. We calculated Pearson's correlation coefficient to determine whether ancestry values inferred from ddRAD data were associated with the stable-carbon and stable-hydrogen isotope values of tail feathers. Finally, we ran a Fisher's exact test to examine whether wintering continent (Africa vs. Asia) differed by breeding location (eastern vs. western side of the hybrid zone) and a Kruskal-Wallis test with Dunn's post hoc test to compare distributions of ancestry estimates for individuals that were assigned to Africa, Asia, or unassigned based off of their $\delta^2 H$ values. All statistical analyses were performed in R version 3.6.2 (R Core Team 2018).

Results

GENOME-WIDE ANCESTRY DIFFERS ACROSS THE HYBRID ZONE

The PCA of the genome-wide covariance matrix derived from WGS data, which included individuals from the hybrid zone as well as allopatric populations of both subspecies, grouped individuals from Jiuquan and Zhangye with allopatric populations of H. r. rustica and individuals from Wuwei and Lanzhou with allopatric populations of H. r. gutturalis (Fig. 1B). These results were further confirmed by the ancestry proportions estimated using ADMIXTURE on the WGS data, which indicated that birds sampled in Jiuquan and Zhangye had a large proportion of H. r. rustica ancestry (0.81 \pm 0.08; mean \pm SD), whereas those sampled in Lanzhou had predominantly H. r. gutturalis ancestry (0.20 \pm 0.07; mean \pm SD; Fig. 1C). In contrast to the PCA, ADMIXTURE detected additional introgression in Wuwei, indicating that individuals breeding near the center of the hybrid zone

possess a combination of *H. r. rustica* and *H. r. gutturalis* ancestry (Fig. 1C).

Ancestry estimates generated using ADMIXTURE on SNPs from the ddRAD dataset, which included individuals sampled in Jiuquan, Zhangye, and Lanzhou, broadly corresponded to the WGS-based estimates (Fig. S1). The proportion of H. r. rustica ancestry significantly differed between individuals breeding on either side of the migratory divide (Kruskal-Wallis: $\chi^2 = 31.55$, df = 2, P < 0.0001, n = 127; Fig. 2A). Birds in Jiuquan and Zhangye had a significantly higher proportion of H. r. rustica ancestry than individuals breeding in Lanzhou (Dunn's post hoc test: Jiuquan vs. Lanzhou: P < 0.0001; Zhangye vs. Lanzhou: P < 0.0001; Jiuquan vs. Zhangye: P = 0.28). Relative to the WGS data (Fig. 1C), the ddRAD data revealed more intermediate individuals and greater variance in ancestry estimates in the area of putative hybridization (Figs. 2A and S3); this variation could be due to differences in the hybrid classes of sampled individuals in each dataset, as well as the smaller number of SNPs and lower resolution of the ddRAD data (ddRAD: 3974 SNPs vs. WGS: 32,029 SNPs). Ancestry-related traits that are known to differ between the subspecies (Scordato and Safran 2014) also varied across the migratory divide (Fig. 2B, C). Individuals that spent the breeding season in Jiuquan and Zhangye had longer tail streamers on average and less saturated ventral coloration than individuals that spent the breeding season in Lanzhou (tail streamer length: ANOVA with Tukey's HSD: P = 0.05, n = 116 [Fig. 2B]; ventral coloration: ANOVA with Tukey's HSD, P = 0.008, n = 128 [Fig. 2C]).

GEOLOCATORS DOCUMENT A MIGRATORY DIVIDE IN THE HYBRID ZONE

The location estimates derived from the geolocators during the calibration period differed from the true deployment locations in Jiuquan, Zhangye, and Lanzhou by 109 ± 96 km, which is

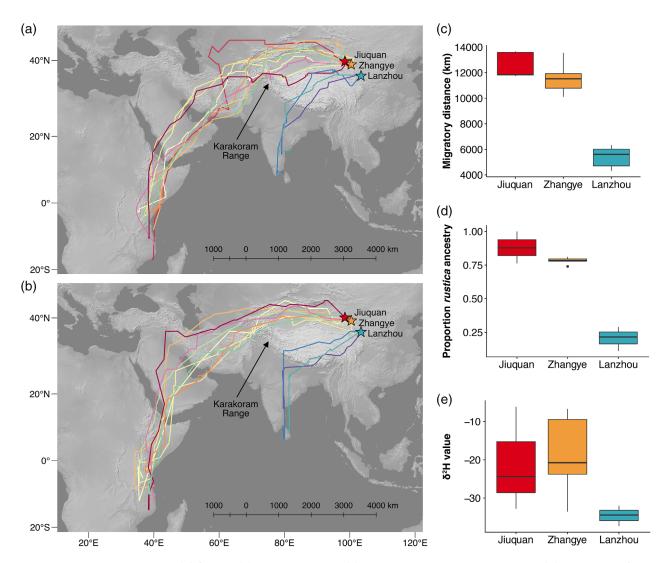


Figure 3. Geolocator tracks during (A) fall and (B) spring migration, (C) distance traveled during migration, (D) proportion of *Hirundo rustica rustica* ancestry generated using whole-genome data, and (E) stable-hydrogen (δ^2 H) isotope value from tail feathers of barn swallows (*H. r. rustica* and *H. r. gutturalis*) breeding across a hybrid zone in China (n = 13). The tracks were constructed from median geographic position estimates generated twice per day. Individuals that traveled to Africa are shown in warm colors, birds that migrated to India are shown in cool colors, and cities where geolocators were deployed are depicted as stars. Colors correspond to cities on the map in Figure 1.

comparable to estimates of similar geolocator studies (Delmore et al. 2012) and smaller than the width of the hybrid zone (620 km from Jiuquan to Lanzhou). Although tagged individuals that returned to Jiuquan and Zhangye were slightly heavier at the time of tagging than those that did not return (returned: 17.01 ± 0.81 g vs. no return: 16.05 ± 1.21 g [mean \pm SD]; unpaired t-test: P = 0.01, n = 43), we did not detect a significant difference in mass between tagged birds that returned to Lanzhou and those that did not (returned: 16.10 ± 1.11 g vs. no return: 15.21 ± 1.01 g (mean \pm SD); unpaired t-test: P = 0.21, n = 33). Nonetheless, biased sampling due to higher return rates in heavier individuals could have influenced the findings of this study (e.g., if heavier birds follow different migratory routes than lighter

birds). Additional research will be needed to evaluate whether mass influences migratory behavior in barn swallows.

The two subspecies exhibited a striking migratory divide, with individuals on either side of the hybrid zone overwintering over 5000 km from one another. Individuals breeding on the western side of the hybrid zone (Jiuquan: n=3 and Zhangye: n=7) flew north of the Taklamakan Desert and crossed the Arabian Peninsula to overwinter in eastern Africa (Fig. 3A, B). In contrast, birds breeding on the eastern side (Lanzhou: n=3) traveled southward across the Qinghai-Tibetan Plateau and overwintered in southern India (Fig. 3A, B). The geolocator tracks indicated that the two subspecies took divergent migratory routes around the Karakoram Range, a high-altitude mountain range that

extends along the borders of India, Pakistan, and China (Fig. 3A, B). On average, individuals migrating to Africa traveled over twice as far as swallows traveling to India (India: $5407 \pm 834 \text{ km}$ vs. Africa: 11,829 \pm 1073 km [mean \pm SD]; unpaired *t*-test: P < 0.001, n = 13, Fig. 3C). Migratory distance did not differ between fall and spring migration for individuals that overwintered in India (Fall: 5369 \pm 1012 km vs. Spring: 5446 \pm 866 km [mean \pm SD]; paired *t*-test: P = 0.69, n = 13) or Africa (Fall: $11,370 \pm 506$ km vs. Spring: $12,085 \pm 1265$ km [mean \pm SD]; paired t-test: P = 0.18, n = 12). Birds traveling to and from Africa tended to stop over more frequently and for a longer period of time than those migrating to and from India during both the fall (Africa: 6 stopovers, 16 days vs. India: 3 stopovers, 7 days) and spring (Africa: 3 stopovers, 10 days vs. India: 2 stopovers, 8 days).

The WGS ADMIXTURE results revealed that individuals with geolocators that are present during the breeding season in Jiuquan and Zhangye and migrated to Africa had predominantly H. r. rustica ancestry, whereas birds that spent the breeding season in Lanzhou and migrated to India had a much lower proportion of H. r. rustica ancestry (Africa: 0.81 ± 0.08 vs. India: 0.21 ± 0.09 [mean \pm SD]; unpaired *t*-test: P = 0.002, n = 13, Fig. 3D). In addition, individuals with geolocators that overwintered in Africa had higher δ^2 H values relative to birds that overwintered in India (Africa: $-19.07 \pm 10.44\%$ vs. India: $-34.59 \pm 2.69\%$ [mean \pm SD]; unpaired *t*-test: P = 0.002, n = 13, Fig. 3E). Although uncertainty in location estimates increased around the two equinoxes, and latitudinal estimates were less accurate than longitudinal estimates, the two subspecies showed clear differences in migratory behavior throughout the annual cycle (Fig. S4).

STABLE ISOTOPE DATA FROM A LARGER SAMPLE OF INDIVIDUALS PROVIDE ADDITIONAL EVIDENCE FOR A MIGRATORY DIVIDE

The proportion of H. r. rustica ancestry for individuals breeding on either side of the hybrid zone (i.e., Jiuquan, Zhangye, and Lanzhou) was positively correlated with the δ^{13} C (r = 0.152, P = 0.089, n = 126) and $\delta^2 H$ (r = 0.290, P = 0.001, n = 126) values of their tail feathers, indicating that individuals with predominantly H. r. rustica ancestry molt their feathers in areas characterized by higher δ^{13} C and δ^{2} H values, although the relationship for carbon was only marginally significant (Fig. S5). Isotope measurements obtained from individuals that were captured in multiple years (n = 14) were highly repeatable, with a mean absolute difference between years of 1.06 \pm 1.01% SD (range = 0.1-3.3%) for δ^{13} C and $7.80 \pm 5.68\%$ SD (range = 1-17‰) for δ^2 H.

Of the 13 geolocator birds used to calibrate the geographic assignment model, eight of the 10 birds that overwintered in Africa were assigned to Africa and the remaining five individuals, including the three birds that overwintered in India, could not be assigned to either location with a greater than twofold difference between the average assignment probabilities for the two regions. Of the 116 birds without geolocators breeding in Jiuquan, Zhangye, and Lanzhou, 70 were assigned to Africa, 34 to Asia, and 12 could not be assigned to either location. Wintering location differed by breeding location (i.e., eastern vs. western side of the hybrid zone) for assigned individuals (P < 0.0001, n = 104, Fig. 4A). In particular, the odds of an individual breeding on the western side of the hybrid zone (Jiuquan and Zhangye) being assigned to Africa was 148 times greater than the odds of a western-breeding bird being assigned to an Asian wintering locale.

GENOME-WIDE ANCESTRY IS ASSOCIATED WITH MIGRATORY BEHAVIOR

Individuals assigned to different wintering continents on the basis of δ^2 H values differed in their ancestry distributions (Kruskal-Wallis: $\chi^2 = 27.32$, df = 2, P < 0.0001, n = 116; Fig. 4B). Pairwise comparisons (Dunn test with Holm's correction) revealed differences between the ancestry estimates of birds assigned to Africa versus Asia (P < 0.0001), and individuals assigned to Africa versus unassigned (P = 0.002), but not between individuals assigned to Asia versus unassigned (P = 0.85; Fig. 4B). Swallows assigned to Africa had a median ancestry estimate (i.e., proportion H. r. rustica ancestry) of 0.57, whereas those assigned to Asia had a median value of 0.39, which is consistent with the geolocator results that demonstrate that individuals traveling to eastern Africa had predominantly H. r. rustica ancestry.

TIMING OF ARRIVAL ON THE BREEDING GROUNDS DIFFERS ACROSS THE MIGRATORY DIVIDE

One mechanism by which distinct migratory strategies may contribute to reproductive isolation at migratory divides is divergence in migratory timing and subsequent assortative mating by timing of arrival on the breeding grounds. Barn swallows on either side of the migratory divide departed from the breeding grounds at similar times (India: September 5 ± 10 days vs. Africa: September 6 ± 6 days [mean \pm SD]; unpaired *t*-test: P = 0.83, n = 13). However, swallows that overwintered in India arrived on the breeding grounds almost a month earlier on average than individuals traveling from Africa (India: April 9 \pm 6 days vs. Africa: May 8 ± 6 days [mean \pm SD]; unpaired t-test: P = 0.004, n = 12, Fig. 5).

Discussion

Divergence in migratory strategy is thought to promote and maintain population differentiation and reproductive isolation by exposing organisms to different environments and restricting

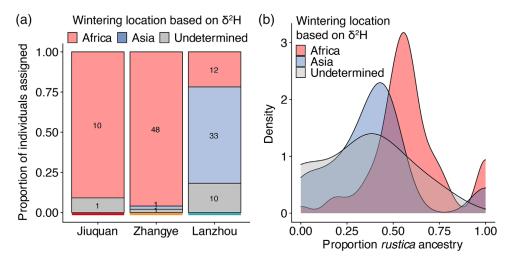


Figure 4. (A) Proportion of individuals and (B) double digest restriction site-associated DNA (ddRAD)-derived ancestry distributions of barn swallows (*Hirundo rustica rustica* and *H. r. gutturalis*) assigned to Africa (n = 70), Asia (n = 34), or unassigned (n = 12) on the basis of the stable-hydrogen (8^2 H) isotope values of their tail feathers. Colors at the base of the bars in panel A correspond to cities on the map in Figure 1. The number of individuals assigned to each wintering continent are shown inside the bars in panel A.

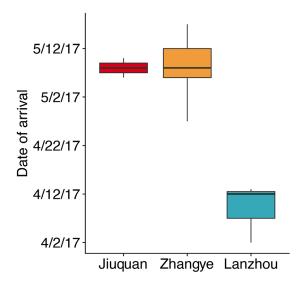


Figure 5. Timing of arrival on the breeding grounds derived from geolocators for barn swallows (*Hirundo rustica rustica* and *H. r. gutturalis*) breeding across a hybrid zone in China (n = 13). Colors correspond to cities on the map in Figure 1.

gene flow between divergent groups. However, direct tracking data, which provide information on the timing of migration and migratory routes of individuals, are necessary to investigate the role of seasonal migration in the origin and maintenance of species boundaries. Combining data from light-level geolocators, genomic sequencing, and stable isotopes, we documented a geographic migratory divide that is associated with genomic differentiation across a hybrid zone between barn swallow subspecies. Previous studies have proposed that divergence in migratory strategy may help maintain genetic differentiation in barn

swallows (Safran et al. 2016; Scordato et al. 2017, 2020) and tracked migratory routes within *H. r. rustica* using ringing data (Ambrosini et al. 2009), stable isotopes (von Rönn et al. 2016, 2020), and geolocators (Liechti et al. 2015). However, this is the first study to reveal when barn swallows migrate and how they navigate inhospitable barriers in central Asia in a hybrid zone context by directly tracking individuals on either side of a migratory divide.

Although ongoing gene flow has been reported between H. r. rustica and H. r. gutturalis (Scordato et al. 2020), and we detected elevated admixture in Wuwei, located near the center of the hybrid zone, all individuals that returned with geolocators possessed ancestry primarily from one parental subspecies or the other. In addition, the PCA results from the WGS data indicated a lack of hybridization between the subspecies, which suggests fairly strong reproductive isolation and is consistent with the absence of F1 hybrids and steep cline for genomic ancestry previously documented in the hybrid zone (Scordato et al. 2020). Although the lower resolution ddRAD data revealed greater variance in ancestry estimates in the area of putative hybridization, the ddRAD-based admixture proportions broadly corresponded to the WGS-based estimates, further supporting genomic differentiation across the migratory divide. We note, however, that the exact admixture proportions estimated from the ddRAD data should be interpreted with caution, as they have relatively low

In general, barn swallows show remarkably little ecological or genomic differentiation across large geographic regions (Scordato et al. 2017). Nonetheless, we found that *H. r. rustica* migrated longer distances from the breeding grounds to overwinter in eastern Africa, whereas *H. r. gutturalis* traveled

shorter distances to southern India. These results were further supported by the stable isotope signatures of a larger number of individuals sampled on either end of the hybrid zone. Barn swallows breeding on the western side, with predominantly H. r. rustica ancestry, had higher stable-carbon and hydrogen isotope values and were assigned to Africa more frequently than those breeding on the eastern side, with H. r. gutturalis ancestry. In terrestrial systems, δ^{13} C values vary systematically with mechanisms of water use efficiency in C3 plants and the proportion of C3 and C4 plants in the environment (Hobbie and Werner 2004). In particular, higher δ^{13} C values are associated with arid environments dominated by C4 plants, whereas lower δ¹³C values are found in mesic environments with a higher proportion of C3 plants (Dawson et al. 2002; Still et al. 2003). Environmental δ^2 H values, in contrast, vary spatially in accordance with deuterium patterns in local precipitation (Bowen and Revenaugh 2003), which can be exploited for large-scale geographic assignments of migratory species (Hobson 1999; Rubenstein and Hobson 2004). Overall, the observed δ^{13} C and δ^{2} H distributions on either end of the hybrid zone, assignment results, and sighting records of the two subspecies (Turner 2010; Sullivan et al. 2014) are consistent with the geolocator data, which indicate that H. r. rustica overwinters in eastern Africa, a drier region dominated by more C4 plants, whereas H. r. gutturalis migrates to southern Asia (Still et al. 2003; Scordato et al. 2020).

Differences in migratory distance appeared to affect timing of arrival on the breeding grounds. In particular, individuals on the eastern side of the hybrid zone returned to the breeding grounds over 3 weeks earlier than individuals on the western side, likely due to their shorter migratory route. Divergence in timing of arrival is a widespread pattern at migratory divides, where populations on either side often migrate different distances to their nonbreeding grounds (Bensch et al. 1999; Ruegg et al. 2012; Pujolar et al. 2014). For example, since the evolution of a novel migratory route in Eurasian blackcaps (Sylvia atricapilla), northwest migrants that overwinter in the United Kingdom arrive on sympatric breeding grounds in Germany significantly earlier on average than blackcaps that migrate along their historical route to the Mediterranean (Bearhop et al. 2005; Rolshausen et al. 2010). In addition, in a well-studied hybrid zone between subspecies of Swainson's thrush (Catharus ustulatus) in British Columbia, the short-distance coastal migrant (C. u. ustulatus) arrives earlier on the breeding grounds than the longer distance inland migrant (C. u. swainsoni) (Ruegg et al. 2012).

Differential timing of migration as a result of divergent migratory strategies can promote reproductive isolation if individuals mate assortatively by timing of arrival on the breeding grounds (Turbek et al. 2018). Eurasian blackcaps that follow the novel northwestern migratory route and arrive earlier on the breeding grounds are more likely to pair with other northwestern migrants than southwestern migrants that overwinter in the Mediterranean (Bearhop et al. 2005). Since the 1960s, when the new migratory route in blackcaps was first documented, genetic and phenotypic differences have accumulated between the two migratory forms (Rolshausen et al. 2009), supporting the hypothesis that premating isolation due to divergence in arrival times can contribute to population differentiation.

Although we found that divergent migratory behavior corresponds to differences in genome-wide ancestry in barn swallows, we were unable to analyze whether assortative mating by migratory timing limits gene flow between the subspecies, as geolocators were only deployed on either side of the migratory divide. Barn swallows immediately establish territories upon arrival in China and raise up to two broods in a single breeding season (Liu et al. 2018b). Thus, a 3-week delay in arrival time could limit the availability of mates, reduce access to high-quality territories, and restrict opportunities for double brooding. However, parental individuals of H. r. rustica and H. r. gutturalis do not come into direct contact in the center of the hybrid zone, which consists solely of admixed individuals, and previous work within the hybrid zone found little support for assortative mating by carbon isotope value (Scordato et al. 2020). Further population-level studies from the hybrid zone center will help determine whether the lack of overlap in arrival times between individuals on either side of the migratory divide contributes to the maintenance of subspecies boundaries.

In addition to assortative mating by timing of arrival, divergent migratory behavior could also prevent gene flow through postmating isolation. For example, hybrids at migratory divides may inherit maladaptive trait combinations or intermediate migratory routes that expose them to increased mortality during migration (Rohwer and Irwin 2011; Delmore and Irwin 2014). Hybrids may also follow maladaptive stopover schedules or select stopover sites that do not provide them with sufficient resources to fuel their migratory journeys. Migratory orientation is at least partially genetically determined in songbirds (Berthold 1991; Liedvogel et al. 2011), and several studies have identified genomic regions associated with divergent migratory phenotypes between closely related populations (Delmore et al. 2016; Lundberg et al. 2017). In addition, experimental crosses in Eurasian blackcaps, as well as natural hybridization in Swainson's thrushes, have revealed that hybrid individuals can inherit intermediate migratory directions relative to parental populations (Helbig 1991; Delmore and Irwin 2014; Delmore et al. 2016).

Uncertainty in latitudinal estimates derived from the geolocators increased around the equinoxes, when birds migrate to and from the wintering grounds. However, the geolocator tracks clearly indicated that the two subspecies took divergent migratory routes around the Karakoram Range, which contains eight peaks over 7500 m and constitutes the second highest mountain range in the world. In addition, birds that migrated to Africa tended to stop over more frequently and spend a longer time refueling than individuals that migrated shorter distances to India. All of the barn swallows that returned with geolocators had a large proportion of ancestry from one parental subspecies or the other, and the migratory routes of F1 hybrids remain unknown. However, if hybrid individuals within the contact zone attempt to directly cross arid and mountainous regions that offer few opportunities for refueling, or fail to stop at appropriate stopover sites, elevated mortality during migration could lead to postmating isolation and maintain genetic differentiation between the subspecies. Nonetheless, barn swallows migrate in flocks and congregate in communal roosts during the migratory and nonbreeding periods (Rubolini et al. 2002). Social learning and cultural transmission of migratory routes could thus limit the strength of postmating isolating barriers. Further research evaluating the heritability of migratory phenotypes and the role of social learning in barn swallow migration will provide insight into the potential for postmating isolation due to divergent migratory phenotypes. In addition, direct tracking data from juveniles could shed additional light on the contribution of postmating isolation to the maintenance of subspecies boundaries, as juveniles lack any prior experience to guide them and postmating selection due to nonadaptive migratory routes may therefore be strongest at the juvenile stage (McKinnon et al. 2014).

Although several geographic features in central Asia, particularly the Qinghai-Tibetan Plateau, have been proposed as major barriers to migration, no previous study has directly tracked Asian passerines to examine how small-bodied birds navigate these inhospitable barriers. Barn swallows breeding at this migratory divide face numerous geographic features that provide limited opportunities for refueling, including the Taklamakan Desert, the high-altitude Qinghai-Tibetan Plateau, and the Himalayas, which contain over 50 mountains that exceed 7200 m in elevation. Barn swallows have been observed above 3000 m during the summer at various locations along the Qinghai-Tibetan Plateau (Farrington 2016). However, flying across the Himalayas poses a physiological challenge for migratory birds, as oxygen consumption, and therefore flight costs, increases and the partial pressure of oxygen diminishes at higher altitudes (Hawkes et al. 2013). Bar-headed geese (Anser indicus) are known for their high-altitude migrations across the southern edge of the Qinghai-Tibetan Plateau, reportedly reaching altitudes greater than 8000 m. Nonetheless, these birds have evolved several physiological adaptations that allow them to tolerate hypoxia during their high-altitude journeys, including larger lungs, increased capillary densities in their heart, and hemoglobin with a higher O₂ affinity than their low-altitude relatives (Scott et al. 2011; Natarajan et al. 2018). Further investigations into the flight altitudes and physiological limits of small-bodied passerines may help determine the implications of possible intermediate migratory routes in hybrids.

Conclusions

By combining genomic sequencing, stable isotopes, and lightlevel geolocators, we investigate the timing of migration and migratory routes of barn swallows breeding on either side of a migratory divide that is closely associated with genomic differentiation between two subspecies. This study provides insight into the ways in which migratory behavior may maintain subspecies boundaries in barn swallows, particularly through assortative mating by timing of arrival and/or selection against hybrids that inherit intermediate migratory traits. Our findings are consistent with the long-standing hypotheses that (1) divergent migratory behavior can prevent gene flow and facilitate differentiation and (2) inhospitable geographic features may have contributed to the diversification of Asian songbirds by influencing migratory patterns.

AUTHOR CONTRIBUTIONS

SPT and RJS conceived of the study. SPT carried out the field work with assistance from RJS, ESCS, XD, YL, YL, EP, QR, and DZ. DRS and CCRS analyzed the genomic data. CS processed the stable isotope data. SPT processed the geolocator data. AC and MW analyzed the stable isotope data. SPT wrote the manuscript with input from all authors.

ACKNOWLEDGMENTS

We thank Lanzhou University (particularly Dr. Bo Du), Sun Yat-sen University, the State Darwin Museum in Moscow, Hainan Normal University, National University of Mongolia, the Mongolian Ornithological Society, and the Japan Bird Research Association for sponsoring our research. We thank Z. Gompert for his assistance with the ddRAD lane effect issue. This work was supported by a National Science Foundation Graduate Research Fellowship and a University of Colorado EBIO Grant to SPT, a National Science Foundation Postdoctoral Fellowship to DRS (DBI-1906188), a National Geographic Society Committee on Research and Exploration grant to ESCS, and National Science Foundation grants to RJS (DEB-CAREER 1149942, IOS 1856266) and MW (DBI-1564947). Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS

Field work was approved by University of Colorado Institutional Animal Care and Use Committee (IACUC) protocol no. 2498 and conducted in accordance with USFWS permit MB12129A-10 and USDA permit 112702.

DATA ARCHIVING

The whole-genome sequencing data (BioProject PRJNA323498) and double digest restriction site-associated DNA sequencing data (BioProject PRJNA796590) are archived in the NCBI Sequence Read Archive. Raw light level data, filtered VCF files, and associated metadata are available on Dryad (https://doi.org/10.5061/dryad.tx95x6b0c).

LITERATURE CITED

- Alexander, D. H., J. Novembre, and K. Lange. 2009. Fast model-based estimation of ancestry in unrelated individuals. Genome Res. 19:1655-
- Alvarado, A. H., T. L. Fuller, and T. B. Smith. 2014. Integrative tracking methods elucidate the evolutionary dynamics of a migratory divide. Ecol. Evol. 4:3456-3469.
- Ambrosini, R., A. P. Møller, and N. Saino. 2009. A quantitative measure of migratory connectivity. J. Theor. Biol. 257:203-211.
- Bearhop, S., W. Fiedler, R. W. Furness, S. C. Votier, S. Waldron, J. Newton, G. J. Bowen, P. Berthold, and K. Farnsworth. 2005. Assortative mating as a mechanism for rapid evolution of a migratory divide. Science 310:502-504.
- Bearhop, S., R. W. Furness, G. M. Hilton, S. C. Votier, and S. Waldron. 2003. A forensic approach to understanding diet and habitat use from stable isotope analysis of (avian) claw material. Funct. Ecol. 17:270-275.
- Bensch, S., T. Andersson, and S. Åkesson. 1999. Morphological and molecular variation across a migratory divide in willow warblers, Phylloscopus trochilus. Evolution 53:1925-1935.
- Bensch, S., M. Grahn, N. Müller, L. Gay, and S. Åkesson. 2009. Genetic, morphological, and feather isotope variation of migratory willow warblers show gradual divergence in a ring. Mol. Ecol. 18:3087-3096.
- Berthold, P. 1991. Orientation in birds. Birkhäuser Verlag, Basel, Switzerland. Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114-2120.
- Bowen, G. J., and J. Revenaugh. 2003. Interpolating the isotopic composition of modern meteoric precipitation. Water Resour. Res. 39:1299.
- Catchen, J., P. A. Hohenlohe, S. Bassham, A. Amores, and W. A. Cresko. 2013. Stacks: an analysis tool set for population genomics. Mol. Ecol. 22:3124-3140.
- Danecek, P., A. Auton, G. Abecasis, C. A. Albers, E. Banks, M. A. DePristo, R. E. Handsaker, G. Lunter, G. T. Marth, S. T. Sherry, et al. 2011. The variant call format and VCFtools. Bioinformatics 27:2156-2158.
- Dawson, T. E., S. Mambelli, A. H. Plamboeck, P. H. Templer, and K. P. Tu. 2002. Stable isotopes in plant ecology. Annu. Rev. Ecol. Syst. 33:507-
- Delany, S., C. Williams, C. Sulston, J. Norton, and D. Garbutt. 2017. Passerine migration across the Himalayas. Pp. 58-81 in H. H. T. Prins and T. Namgail, eds. Bird migration across the Himalayas: wetland functioning amidst mountains and glaciers. Cambridge Univ. Press, Cambridge,
- Delmore, K. E., J. W. Fox, and D. E. Irwin. 2012. Dramatic intraspecific differences in migratory routes, stopover sites and wintering areas, revealed using light-level geolocators. Proc. R. Soc. B Biol. Sci. 279:4582-4589.
- Delmore, K. E., and D. E. Irwin. 2014. Hybrid songbirds employ intermediate routes in a migratory divide. Ecol. Lett. 17:1211-1218.
- Delmore, K. E., D. P. L. Toews, R. R. Germain, G. L. Owens, and D. E. Irwin. 2016. The genetics of seasonal migration and plumage color. Curr. Biol. 26:2167-2173.
- Dingle, H. 2014. Migration: the biology of life on the move. Oxford Univ. Press, New York.
- Dodson, J. J., N. Aubin-Horth, V. Thériault, and D. J. Páez. 2013. The evolutionary ecology of alternative migratory tactics in salmonid fishes. Biol. Rev. 88:602-625.
- Ellegren, H., L. Smeds, R. Burri, P. I. Olason, N. Backström, T. Kawakami, A. Künstner, H. Mäkinen, K. Nadachowska-Brzyska, A. Qvarnström,

- et al. 2012. The genomic landscape of species divergence in Ficedula flycatchers. Nature 491:756-760.
- Farrington, J. D. 2016. A survey of the autumn 2009 and spring 2010 bird migrations at Lhasa, Tibet Autonomous Region, China. Forktail 32:14-
- Formenti, G., M. Chiara, L. Poveda, K.-J. Francoijs, A. Bonisoli-Alquati, L. Canova, L. Gianfranceschi, D. S. Horner, and N. Saino. 2018. SMRT long reads and Direct Label and Stain optical maps allow the generation of a high-quality genome assembly for the European barn swallow (Hirundo rustica rustica). Gigascience 8:giy142.
- Fry, B., W. Brand, F. J. Mersch, K. Tholke, and R. Garritt. 1992. Automated analysis system for coupled .delta.13C and .delta.15N measurements. Anal. Chem. 64:288-291.
- Hawkes, L. A., S. Balachandran, N. Batbayar, P. J. Butler, B. Chua, D. C. Douglas, P. B. Frappell, Y. Hou, W. K. Milsom, S. H. Newman, et al. 2013. The paradox of extreme high-altitude migration in bar-headed geese Anser indicus. Proc. R. Soc. B Biol. Sci. 280:20122114.
- Helbig, A. J. 1991. Inheritance of migratory direction in a bird species: a cross-breeding experiment with SE-and SW-migrating blackcaps (Sylvia atricapilla). Behav. Ecol. Sociobiol. 28:9-12.
- Hobbie, E. A., and R. A. Werner. 2004. Intramolecular, compound-specific, and bulk carbon isotope patterns in C3 and C4 plants: a review and synthesis. New Phytol. 161:371-385.
- Hobson, K. A. 1999. Tracing origins and migration of wildlife using stable isotopes: a review. Oecologia 120:314-326.
- International Chicken Genome Sequencing Consortium. 2004. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 432:695-716.
- Irwin, D. E., and J. H. Irwin. 2005. Siberian migratory divides: the role of seasonal migration in speciation. Pp. 27-40 in R. Greenberg and P. Marra, eds. Birds of two worlds: the ecology and evolution of migration. Johns Hopkins Univ. Press, Baltimore, MD.
- Jacobsen, M. W., J. M. Pujolar, L. Bernatchez, K. Munch, J. Jian, Y. Niu, and M. M. Hansen. 2014. Genomic footprints of speciation in Atlantic eels (Anguilla anguilla and A. rostrata). Mol. Ecol. 23:4785-4798.
- Jain, C., A. Dilthey, S. Koren, S. Aluru, and A. M. Phillippy. 2017. A fast approximate algorithm for mapping long reads to large reference databases. J. Comput. Biol. 25:66-81.
- Jain, C., S. Koren, A. Dilthey, A. M. Phillippy, and S. Aluru. 2018. A fast adaptive algorithm for computing whole-genome homology maps. Bioinformatics 34:i748-i756.
- Kays, R., M. C. Crofoot, W. Jetz, and M. Wikelski. 2015. Terrestrial animal tracking as an eye on life and planet. Science 348:aaa2478.
- Li, H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv1303.3997.
- Li, H., and R. Durbin. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754-1760.
- Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, and 1000 Genome Project Data Processing Subgroup. 2009. The sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078-2079.
- Liechti, F., C. Scandolara, D. Rubolini, R. Ambrosini, F. Korner-Nievergelt, S. Hahn, R. Lardelli, M. Romano, M. Caprioli, A. Romano, et al. 2015. Timing of migration and residence areas during the non-breeding period of barn swallows Hirundo rustica in relation to sex and population. J. Avian Biol. 46:254-265.
- Liedvogel, M., S. Åkesson, and S. Bensch. 2011. The genetics of migration on the move. Trends Ecol. Evol. 26:561-569.
- Liu, D., G. Zhang, H. Jiang, and J. Lu. 2018a. Detours in long-distance migration across the Qinghai-Tibetan Plateau: individual consistency and habitat associations. Peer I 6:e4304

- Liu, Y., E. S. C. Scordato, R. Safran, and M. Evans. 2018b. Ventral colour, not tail streamer length, is associated with seasonal reproductive performance in a Chinese population of Barn Swallows (Hirundo rustica gutturalis). J. Ornithol. 159:675-685.
- Lundberg, M., M. Liedvogel, K. Larson, H. Sigeman, M. Grahn, A. Wright, S. Åkesson, and S. Bensch. 2017. Genetic differences between willow warbler migratory phenotypes are few and cluster in large haplotype blocks. Evol. Lett. 1:155-168.
- Ma, C., H. B. Vander Zanden, M. B. Wunder, and G. J. Bowen. 2020. assignR: an R package for isotope-based geographic assignment. Methods Ecol. Evol. 11:996-1001.
- McDevitt, A. D., S. Mariani, M. Hebblewhite, N. J. Decesare, L. Morgantini, D. Seip, B. V. Weckworth, and M. Musiani. 2009. Survival in the Rockies of an endangered hybrid swarm from diverged caribou (Rangifer tarandus) lineages. Mol. Ecol. 18:665-679.
- McKinnon, E. A., K. C. Fraser, C. Q. Stanley, and B. J. M. Stutchbury. 2014. Tracking from the tropics reveals behaviour of juvenile songbirds on their first spring migration. PLoS ONE 9:e105605.
- Milner-Gulland, E. J., J. M. Fryxell, and A. R. E. Sinclair. 2011. Animal migration: a synthesis. Oxford Univ. Press, New York.
- Natarajan, C., A. Jendroszek, A. Kumar, R. E. Weber, J. R. H. Tame, A. Fago, and J. F. Storz. 2018. Molecular basis of hemoglobin adaptation in the high-flying bar-headed goose. PLoS Genet. 14:e1007331.
- O'Corry-Crowe, G. M., R. S. Suydam, A. Rosenberg, K. J. Frost, and A. E. Dizon. 1997. Phylogeography, population structure and dispersal patterns of the beluga whale Delphinapterus leucas in the western Nearctic revealed by mitochondrial DNA. Mol. Ecol. 6:955–970.
- Peterson, B. K., J. N. Weber, E. H. Kay, H. S. Fisher, and H. E. Hoekstra. 2012. Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. PLoS ONE 7:e37135.
- Pujolar, J. M., M. W. Jacobsen, T. D. Als, J. Frydenberg, E. Magnussen, B. Jónsson, X. Jiang, L. Cheng, D. Bekkevold, G. E. Maes, et al. 2014. Assessing patterns of hybridization between North Atlantic eels using diagnostic single-nucleotide polymorphisms. Heredity 112:627-637.
- Purcell, S., B. Neale, K. Todd-Brown, L. Thomas, M. A. R. Ferreira, D. Bender, J. Maller, P. Sklar, P. I. W. De Bakker, M. J. Daly, et al. 2007. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81:559-575.
- QGIS Development Team. 2019. QGIS Geographic Information System. Open Source Geospatial Foundation Project.
- R Core Team. 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.
- Rakhimberdiev, E., A. Saveliev, T. Piersma, and J. Karagicheva. 2017. FLightR: an r package for reconstructing animal paths from solar geolocation loggers. Methods Ecol. Evol. 8:1482-1487.
- Rakhimberdiev, E., N. R. Senner, M. A. Verhoeven, D. W. Winkler, W. Bouten, and T. Piersma. 2016. Comparing inferences of solar geolocation data against high-precision GPS data: annual movements of a double-tagged black-tailed godwit. J. Avian Biol. 47:589-596.
- Rappole, J. H., and A. R. Tipton. 1991. New harness design for attachment of radio transmitters to small passerines (Nuevo Diseño de Arnés para Atar Transmisores a Passeriformes Pequeños). J. F. Ornithol. 62:335–337.
- Rochette, N. C., A. G. Rivera-Colón, and J. M. Catchen. 2019. Stacks 2: analytical methods for paired-end sequencing improve RADseq-based population genomics. Mol. Ecol. 28:4737-4754.
- Rohwer, S., and D. E. Irwin. 2011. Molt, orientation, and avian speciation. Auk Ornithol. Adv. 128:419-425.
- Rolshausen, G., K. A. Hobson, and H. M. Schaefer. 2010. Spring arrival along a migratory divide of sympatric blackcaps (Sylvia atricapilla). Oecologia 162:175-183.

- Rolshausen, G., G. Segelbacher, K. A. Hobson, and H. M. Schaefer. 2009. Contemporary evolution of reproductive isolation and phenotypic divergence in sympatry along a migratory divide. Curr. Biol. 19:2097-2101.
- Rubenstein, D. R., and K. A. Hobson. 2004. From birds to butterflies: animal movement patterns and stable isotopes. Trends Ecol. Evol. 19:256–263.
- Rubolini, D., A. Gardiazabal Pastor, A. Pilastro, and F. Spina. 2002. Ecological barriers shaping fuel stores in barn swallows Hirundo rustica following the central and western Mediterranean flyways. J. Avian Biol. 33:15-22.
- Ruegg, K. 2008. Genetic, morphological, and ecological characterization of a hybrid zone that spans a migratory divide. Evolution 62:452–466.
- Ruegg, K., E. C. Anderson, and H. Slabbekoorn. 2012. Differences in timing of migration and response to sexual signalling drive asymmetric hybridization across a migratory divide. J. Evol. Biol. 25:1741-1750.
- Safran, R. J., K. J. McGraw, M. R. Wilkins, J. K. Hubbard, and J. Marling. 2010. Positive carotenoid balance correlates with greater reproductive performance in a wild bird. PLoS ONE 5:e9420.
- Safran, R. J., E. S. C. Scordato, M. R. Wilkins, J. K. Hubbard, B. R. Jenkins, T. Albrecht, S. M. Flaxman, H. Karaardıç, Y. Vortman, A. Lotem, et al. 2016. Genome-wide differentiation in closely related populations: the roles of selection and geographic isolation. Mol. Ecol. 25:3865–3883.
- Scordato, E. S. C., and R. J. Safran. 2014. Geographic variation in sexual selection and implications for speciation in the Barn Swallow. Avian Res. 5:8.
- Scordato, E. S. C., C. C. R. Smith, G. A. Semenov, Y. Liu, M. R. Wilkins, W. Liang, A. Rubtsov, G. Sundev, K. Koyama, S. P. Turbek, et al. 2020. Migratory divides coincide with reproductive barriers across replicated avian hybrid zones above the Tibetan Plateau. Ecol. Lett. 23:231-241.
- Scordato, E. S. C., M. R. Wilkins, G. Semenov, A. S. Rubtsov, N. C. Kane, and R. J. Safran. 2017. Genomic variation across two barn swallow hybrid zones reveals traits associated with divergence in sympatry and allopatry. Mol. Ecol. 26:5676-5691.
- Scott, G. R., P. M. Schulte, S. Egginton, A. L. M. Scott, J. G. Richards, and W. K. Milsom. 2011. Molecular evolution of cytochrome c oxidase underlies high-altitude adaptation in the bar-headed goose. Mol. Biol. Evol.
- Smith, C. C. R., S. M. Flaxman, E. S. C. Scordato, N. C. Kane, A. K. Hund, B. M. Sheta, and R. J. Safran. 2018. Demographic inference in barn swallows using whole-genome data shows signal for bottleneck and subspecies differentiation during the Holocene. Mol. Ecol. 27:4200-4212.
- Still, C. J., J. A. Berry, G. J. Collatz, and R. S. DeFries. 2003. Global distribution of C3 and C4 vegetation: carbon cycle implications. Global Biogeochem. Cycles 17:6-14.
- Sullivan, B. L., J. L. Aycrigg, J. H. Barry, R. E. Bonney, N. Bruns, C. B. Cooper, T. Damoulas, A. A. Dhondt, T. Dietterich, A. Farnsworth, et al. 2014. The eBird enterprise: an integrated approach to development and application of citizen science. Biol. Conserv. 169:31-40.
- Turbek, S. P., E. S. C. Scordato, and R. J. Safran. 2018. The role of seasonal migration in population divergence and reproductive isolation. Trends Ecol. Evol. 33:164-175.
- Turner, A. 2010. The barn swallow. T & A D Poyser, Lond.
- von Rönn, J. A. C., M. U. Grüebler, T. Fransson, U. Köppen, and F. Korner-Nievergelt. 2020. Integrating stable isotopes, parasite, and ringreencounter data to quantify migratory connectivity—a case study with Barn Swallows breeding in Switzerland, Germany, Sweden, and Finland. Ecol. Evol. 10:2225-2237.
- von Rönn, J. A. C., A. B. A. Shafer, and J. B. W. Wolf. 2016. Disruptive selection without genome-wide evolution across a migratory divide. Mol. Ecol. 25:2529-2541.
- Wassenaar, L. I., and K. A. Hobson. 2003. Comparative equilibration and online technique for determination of non-exchangeable hydrogen of

- keratins for use in animal migration studies. Isotopes Environ. Health Stud. 39:211–217.
- Winker, K. 2010. On the origin of species through heteropatric differentiation: a review and a model of speciation in migratory animals. Ornithol. Monogr. 69:1–30.
- Wood, C. C., J. W. Bickham, R.hn. Nelson, C. J. Foote, and J. C. Patton. 2008. Recurrent evolution of life history ecotypes in sockeye salmon: implications for conservation and future evolution. Evol. Appl. 1: 207–221.
- Wotherspoon, S., M. Sumner, and S. Lisovski. 2016. TwGeos: basic data processing for light-level geolocation archival tags. Version 0.0-1.

Wunder, M. B. 2010. Using isoscapes to model probability surfaces for determining geographic origins. Pp. 251–270 in J. B. West, G. J. Bowen, T. E. Dawson, and K. P. Tu, eds. Isoscapes: understanding movement, pattern, and process on Earth through isotope mapping. Springer Netherlands, Dordrecht, The Netherlands.

Associate Editor: M. E. Maan Handling Editor: T. Chapman

Supporting Information

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

- **Figure S1.** Comparison of whole genome sequencing (WGS) and double digest restriction-site associated DNA (ddRAD)-based estimates of *Hirundo rustica rustica* ancestry for barn swallows tracked using geolocators in Gansu, China (one device failed to record data).
- **Figure S2.** Map showing the migratory flyways used to restrict the possible wintering locations of the barn swallow subspecies *Hirundo rustica rustica* and *H. r. gutturalis* in the assignment model.
- **Figure S3.** Admixture proportions generated using double digest restriction-site associated DNA (ddRAD) data for two subspecies of barn swallow (*Hirundo rustica rustica and H. r. gutturalis*) breeding on either end of a hybrid zone in China (n = 127).
- Figure S4. (A) Latitudinal and (B) longitudinal estimates throughout the annual cycle for 13 barn swallows tracked using geolocators.
- Figure S5. The relationship between proportion of *Hirundo rustica rustica* ancestry derived from double digest restriction-site associated DNA (ddRAD) data and the feather ($\bf A$) stable-carbon isotope (d13C) and ($\bf B$) stable-hydrogen isotope (d2H) values of barn swallows ($\bf H.~r.~rustica$ and $\bf H.~r.~gutturalis$) breeding across a hybrid zone in China ($\bf n=126$).
- **Table S1.** Sample locations for the 130 barn swallows (*Hirundo rustica*) included in the double digest restriction-site associated DNA (ddRAD) dataset, which included the 13 individuals with geolocator tracks.
- Table S2. Sample locations for the 168 barn swallows (Hirundo rustica) for which we generated whole-genome sequencing (WGS) data.