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LETTER

Migratory divides coincide with reproductive barriers across replicated avian hybrid zones above the Tibetan Plateau

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Abstract

Migratory divides are proposed to be catalysts for speciation across a diversity of taxa. However, it is difficult to test the relative contributions of migratory behaviour vs. other divergent traits to reproductive isolation. Comparing hybrid zones with and without migratory divides offers a rare opportunity to directly examine the contribution of divergent migratory behaviour to reproductive barriers. We show that across replicate sampling transects of two pairs of barn swallow (*Hirundo rustica*) subspecies, strong reproductive isolation coincided with a migratory divide spanning 20 degrees of latitude. A third subspecies pair exhibited no evidence for a migratory divide and hybridised extensively. Within migratory divides, overwintering habitats were associated with assortative mating, implicating a central contribution of divergent migratory behaviour to reproductive barriers. The remarkable geographic coincidence between migratory divides and genetic breaks supports a long-standing hypothesis that the Tibetan Plateau is a substantial barrier contributing to the diversity of Siberian avifauna.

Keywords

Barn swallow, hybrid zone, migration, migratory divide, reproductive isolation, speciation.

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INTRODUCTION

Migratory divides–regions where sympatric breeding populations overwinter in different geographic locations–may facilitate completion of the speciation process by generating reproductive barriers that maintain species boundaries. Migratory divides can lead to pre-zygotic barriers via assortative mating if individuals with different wintering habitats arrive to breed at different times (Bearhop *et al*. 2005; Rolshausen *et al*. 2009; Taylor & Friesen 2017). They can also accelerate the evolution of post-mating barriers if hybrids incur survival costs associated with the use of maladaptive routes between breeding and nonbreeding locations (Helbig 1991, 1996; Berthold *et al*. 1992; Delmore & Irwin 2014; Lundberg *et al*. 2017). However, establishing a clear link between divergent migratory behaviour and reproductive isolation has been challenging. Migratory divides often occur at regions of secondary contact, where evolutionary history, divergence in traits unrelated to migratory behaviour, and ecological differences can also contribute to reproductive barriers (Ruegg 2008; Ruegg *et al*. 2012; Delmore *et al*. 2016). Isolating the effects of migratory behaviour on reproductive barriers is particularly challenging when a single region of contact is examined between taxa with broad geographic distributions, because it is not possible to assess the generality of divergent migratory behaviour in restricting gene flow across the species range. We therefore lack a comprehensive understanding of the relative importance of divergent migratory behaviour to the formation and maintenance of species boundaries (Turbek *et al*. 2018).

Here we evaluate the hypothesis that migratory divides play a central role in the maintenance of reproductive isolation in secondary contact. We specifically examine three predictions of this hypothesis. First, hybridisation should be more limited in contact zones with migratory divides compared to contact zones without migratory divides, when controlling for divergence in non-migratory traits. Second, if migratory divides *per se* limit hybridisation, migratory phenotype should explain a larger proportion of genetic variance compared to other divergent traits within migratory divides. Third, if migratory divides act as pre-mating reproductive barriers, then assortative mating by migratory phenotype should be stronger than assortative mating by other traits. Previous studies have found

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mixed evidence for assortative mating and genetic differentiation at migratory divides (Turbek *et al.* 2018), but have not assessed the relative contributions of different traits to reproductive barriers or compared reproductive isolation in hybrid zones with and without migratory divides. We evaluate these predictions in three subspecies of barn swallow (*Hirundo rustica*) that hybridise in Asia.

Barn swallows comprise six globally distributed subspecies that differ in ventral coloration and migratory behaviour (Scordato & Safran 2014). Wing length (a proxy for migratory distance) and ventral colour are associated with genome-wide differentiation among subspecies (Safran *et al.* 2016), and there is disruptive selection against intermediate migratory phenotypes within a migratory divide in the European subspecies (von Rönn *et al.,* 2016). Furthermore, previous work has revealed dramatic variation in the extent of hybridisation between subspecies, despite similarly shallow genetic differentiation. A hybrid zone in central Siberia between the *H. r. rustica* and *H. r. tytleri* subspecies is extremely narrow and coincides with differences in ventral colour and wing length (Scordato *et al.* 2017). This contrasts with extensive hybridisation and phenotypic intergradation in eastern Siberia between *tytleri* and *H. r. gutturalis* (Scordato *et al.* 2017). All three of these subspecies are long-distance migrants that diverged in allopatry but now share breeding range boundaries in secondary contact (Zink *et al.* 2006).

Here we build on previous work by evaluating whether a migratory divide explains variation in the strength of reproductive isolation among these three subspecies. The location of the narrow hybrid zone in Siberia coincides with reported migratory divides in several other pairs of avian taxa (Irwin & Irwin 2005). Convergence of migratory divides in this region may be caused by the Tibetan Plateau: small-bodied passerines tend to migrate to the west or east around this geographic barrier (Irwin & Irwin 2005). Divergent migratory behaviour has therefore been proposed to be broadly important to the evolution and maintenance of species boundaries in Siberian avifauna (Irwin & Irwin 2005). However, barn swallow subspecies differ in other traits that could also contribute to reproductive isolation (Scordato *et al.* 2017). We quantified the relative contribution of migratory behaviour to reproductive barriers via comprehensive measurement of phenotype, genomic analyses, and analysis of assortative mating. We applied these measures to replicated transects to assess the generality of our results across the species range.

MATERIALS AND METHODS

Sampling

We sampled 1288 birds from the three Eurasian barn swallow subspecies (Figs 1 and 2, Supplemental Material). We used existing range maps (Dor *et al.* 2010) to establish sampling transects across subspecies range boundaries. Because range maps were based on very few specimens and most regions had not been previously studied, we also sampled widely across Asia and allowed sampling to be guided in part by variation observed in the field. In addition to previously sampled hybrid zones between *rustica-tytleri* and *tytleri-gutturalis* in Russia (Scordato *et al.* 2017), we discovered a hybrid zone between *rustica* and *gutturalis* in western China, as well as regions of potential contact between *tytleri-gutturalis* and *rustica-tytleri* in Mongolia and China (Figs 1 and 2).

Social pair identification

Barn swallows are socially monogamous and live in groups ranging from a few nests to large colonies (Turner 2010). In smaller groups, we used targeted netting to catch one pair at a time, often at night when the male and female were roosting. For large colonies, we caught birds using passive nets. We assigned birds to a social pair if the male and female were unambiguously caught at the same nest. It was not possible to identify pairs in large colonies, and our measures of assortative mating are therefore derived from birds nesting in small groups.

Quantification of colour, identification of variants

We analysed plumage colour using a spectrophotometer. DNA was extracted, libraries were prepared for genotyping-by-sequencing, and sequencing was conducted on four replicate Illumina HiSeq lanes. Reads were aligned to a draft barn swallow reference genome (Safran *et al.* 2016) and variants called using *bcftools* and *samtools* (Li & Durbin 2009; Li *et al.* 2009). We identified 12,383 single nucleotide polymorphisms (SNPs) with a 5% minor allele frequency cutoff and median read depth of seven reads per locus. Methods for colour quantification, library preparation, sequencing, and variant calling are described elsewhere (Scordato *et al.* 2017; Liu *et al.* 2018) and in the Supplemental Material.

ANALYSIS

Evidence for a migratory divide

We assessed evidence for migratory divides by analysing stable carbon ($\delta^{13}C$) values in tail feathers (Supplemental Material). Barn swallows molt their tail feathers in winter (Turner 2010). Because feather keratin is metabolically inert after formation, feathers sampled in the summer reflect isotopic environments occupied during feather growth. Environmental $\delta^{13}C$ values vary systematically and widely with water use efficiency of plants; this differentiation is preserved through the food web, such that large differences in feather $\delta^{13}C$ between individuals suggest those individuals used resources from different environments to synthesise their feathers (Kelly 2000). We evaluated differences in the distributions of $\delta^{13}C$ values between each of the three subspecies and among hybrids. We found support for migratory divides between *rustica-tytleri* and *rustica-gutturalis* (Results, Fig. 1, Figure S1). We use $\delta^{13}C$ values (hereafter “carbon isotope values”) as proxies for an individual’s overwintering habitat in subsequent analyses.

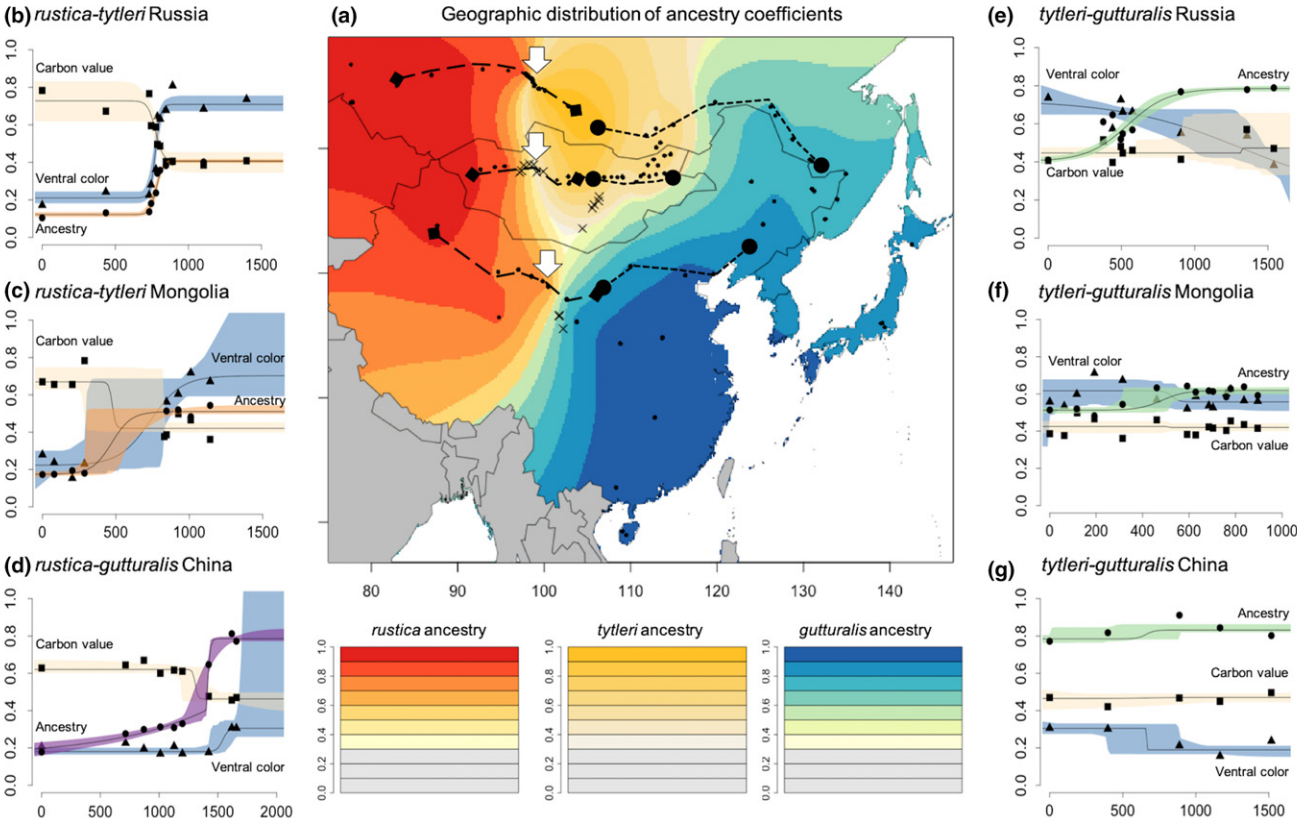

Figure 1 Geographic variation in ancestry coefficients and phenotypes across Asia. (a) ancestry coefficients from the $K = 3$ fastSTRUCTURE model. Darker colours reflect more parental ancestry (red = *rustica,* gold = *tytleri,* blue = *gutturalis;* see legend). Paler colours reflect regions with more admixture. The colour plotted at each point corresponds to the cluster with the maximum ancestry value at that point. Ancestry up to 50% is therefore plotted for each cluster; for example, the palest green colour on the map indicates 50% gutturalis ancestry and the palest orange is 50% rustica ancestry. Regions where the maximum ancestry coefficient is 40% or less for one cluster indicate three-way hybridisation or uncertainty, and have very pale-yellow colour. Small points indicate sampling locations. X's are surveyed regions with no breeding barn swallows. Dashed lines with diamond end points on western part of map show the three *rustica* transects used in geographic cline analysis. Dotted lines with circular end points on the eastern side of the map show the three *tytleri-gutturalis* transects. The geographic clines corresponding to each transect are shown in the left and right panels. Left panels: clines for genetic ancestry (circled points; orange: *rustica-tytleri*; purple: *rustica-gutturalis*), carbon isotope value (tan, square points), and ventral coloration (blue, triangle points) across the three *rustica* sampling transects (b: Russia; c: Mongolia; d: China). Clines for carbon isotope value and ancestry are steep and coincident across all three contact zones. Cline centres all occur at 98–100 degrees longitude (marked on map with white arrows), indicating migratory divides that coincide with narrow hybrid zones. Note that a mountain range separated *rustica* and *tytleri* in Mongolia; the white arrow shows the centre of the mountain range. Right panels: geographic clines for *tytleri-gutturalis* ancestry (circled points, green clines), carbon value (tan, square points), and ventral coloration (blue, triangle points) across the three eastern sampling transects (E: Russia; F: Mongolia; G: China). Ancestry clines are shallow and wide, and there is no variation in isotope values and little variation in ventral colour across the transects. All cline plots show standardised trait values (y-axis) plotted against distance from the westernmost point of the transect (x-axis).

Prediction one: extent of hybridisation across migratory divides vs. non-migratory divides

Population structure

We used three complementary methods to analyse population structure: principal components analysis (PCA); fastSTRUCTURE (Raj *et al.* 2014), which uses a variational Bayesian algorithm to assign individuals to *K* clusters; and TESS (Caye *et al.* 2016), which assigns individuals to *K* clusters but weights individual admixture proportions by geographic proximity. We ran the PCA on the genome-wide covariance matrix of 12 383 SNPs across 1288 individuals using the R function *prcomp*. We ran fastSTRUCTURE with the "simple" prior for values of K from 1 to 15 and a cross-validation of 5 repetitions per K. We ran TESS for values of K from 1 to 7, with 10 repetitions per *K*, 1000 iterations, and the regularisation parameter (alpha) = 0.001 (weak weighting by geography, Caye *et al.* 2016). Both TESS and fastSTRUCTURE showed $K = 3$ to be the best number of clusters. We assigned birds to hybrid classes (F1, later generation hybrid, or backcross) by calculating hybrid indices and average heterozygosity across subsets of differentiated loci using the R package *introgress* (Gompert & Buerkle 2009; Supplemental Material).

Geographic cline analysis

We used sigmoidal geographic clines (Szymura & Barton 1986) to determine whether geographic variation in the frequency of hybridisation coincided with differences in overwintering habitats or other divergent phenotypic traits. Geographic cline analysis requires linear transects through regions of admixture. We therefore subset our sampling to comprise six linear transects across subspecies boundaries,

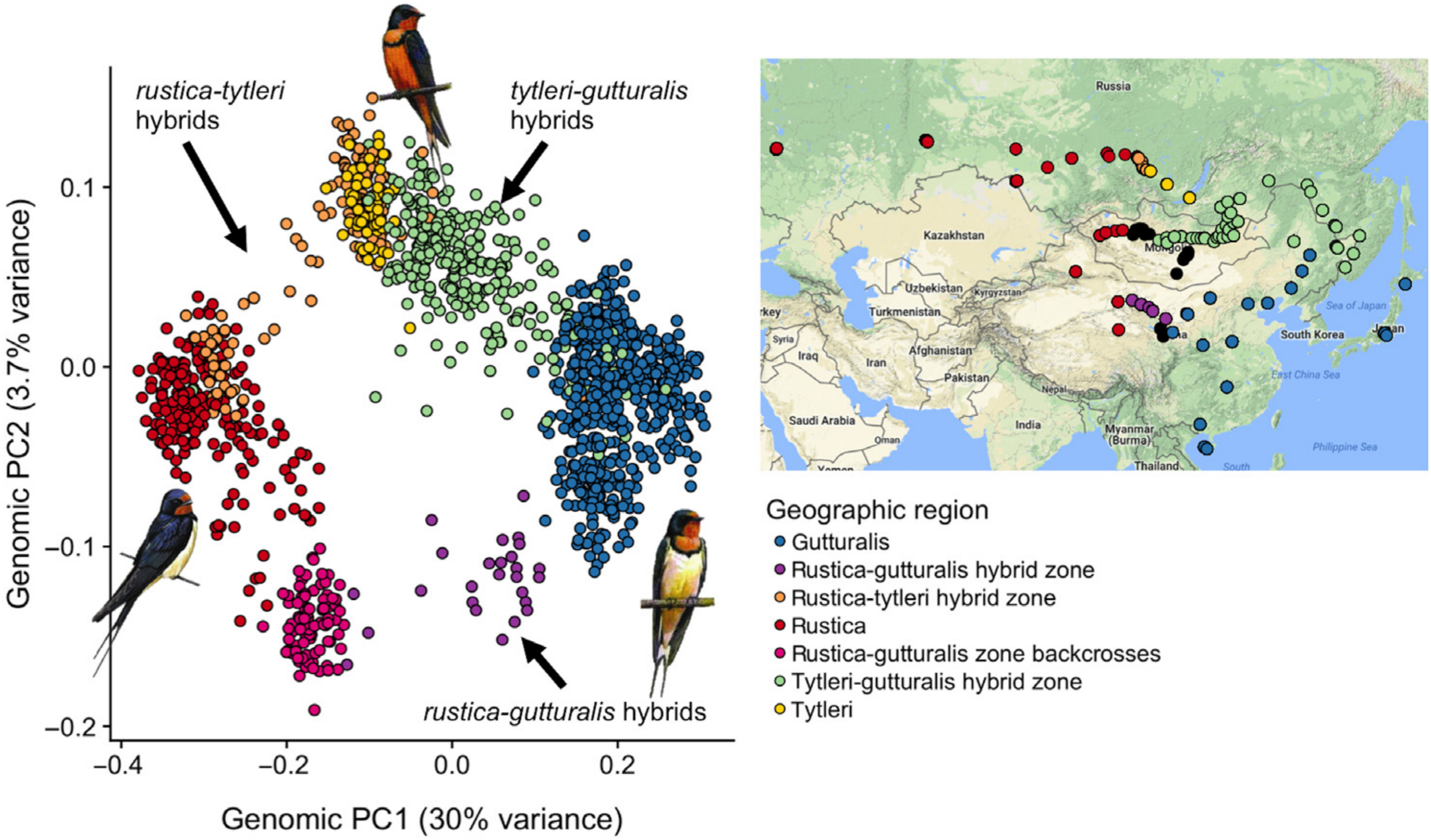

Figure 2 The first two principal components from a PCA of the genetic covariance matrix of all individual birds. Point colours correspond to geographic sampling regions, as indicated in the legend and on the inset map. The PCA generally recapitulates geography and recovers three parental clusters (*rustica* in red, *tytleri* in gold, and *gutturalis* in blue). Points connecting these clusters correspond to admixed individuals (labelled arrows). The pink cluster at PC1 = −0.2 are birds captured in the *rustica-gutturalis* hybrid zone (purple points on map) that appear to be late generation backcrosses to *rustica* and form their own discrete genetic cluster. Drawings show typical phenotype for each of the three parental subspecies. Inset: map of sampling locations with point colours corresponding to geography in the main figure legend. Black points are sampled areas where no birds were found to be breeding. Drawings courtesy of Hilary Burns.

guided by analyses of population structure (Figs 1 and 2). We fit east–west transects through contact zones between *rustica-tytleri* (two transects) and *rustica-gutturalis* (one transect, Fig. 1). Transects spanned 85–115 degrees longitude. We then identified three parallel transects through our sampling points at the same latitudes but farther-eastern longitudes (106–140 degrees). These transects ran through regions of admixture between *tytleri* and *gutturalis* (Fig. 1).

In each transect, we evaluated the extent of hybridisation by fitting clines to ancestry, measured as PC1 from the PCA (Fig. 2). PC1 explained 30% of the genetic variance and clearly separated the three subspecies as well as hybrids. To determine whether differences in overwintering habitat or phenotype coincided with variation in ancestry, we fit clines to carbon isotope values, breast chroma, throat chroma, tail streamer length, wing convexity, wing pointedness, and wing length along each of the six transects. Cline analysis was implemented in the R package HZAR (Derryberry *et al*. 2014, Supplemental Material). We applied neutral diffusion equations (Barton & Gale 1993) to determine whether cline widths were narrower than expected under a scenario of no selection or reproductive isolation. Cline widths narrower than the neutral expectation may be maintained by selection and indicate reproductive isolation (Ruegg 2008; Brelsford & Irwin 2009). Concordant clines between ancestry and phenotypic traits may indicate that those traits are associated with reproductive barriers (Gay *et al*. 2008; Gompert & Buerkle 2016).

Prediction two: variance partitioning

To test the prediction that traits associated with reproductive barriers explained comparatively large proportions of genetic variance, we used variance partitioning and redundancy analysis in the *ecodist* and *vegan* packages in R (Goslee & Urban 2007; Oksanen *et al*. 2013). This approach determines the amount of variance in a set of response variables that is due to a set of explanatory variables, while conditioning on other sets of variables. It is ideal for large data sets with intercorrelated explanatory variables (Wang 2013). We quantified the amount of variance in genomic PC1 and PC2 (Fig. 2) that could be explained by the individual and combined contributions of carbon isotope values and ventral coloration. We analysed each of the six transects separately and conditioned models on sampling location (latitude and longitude) to control for possible isolation-by-distance (Shafer & Wolf 2013; Wang 2013).

Prediction three: assortative mating

Premating reproductive isolation is maintained by assortative mating between individuals with similar genotypes. However, interpreting assortative mating is challenging when there is continuous variation in phenotypes and genotypes between interbreeding groups. We therefore measured assortative mating in two ways. First, we used phenotype networks that accommodate continuously varying genotypes and phenotypes to characterise broad-scale assortative mating across an entire sampling transect. Second, we calculated standardised indices of reproductive isolation to assess fine-scale assortative mating within individual populations.

Assortative mating: phenotype networks

We used a Partial Correlation and Information Theory (PCIT) approach (Badyaev & Young 2004; Wilkins *et al.* 2015) to identify correlations between continuously varying male and female phenotypes and genotypes. This method was developed for analysis of gene co-expression networks (Reverter & Chan 2008) but is applicable to other networks with complex correlation structures (Shizuka & Farine 2016). We began with a matrix of Spearman rank correlations between pairs of males and females. These matrices included genotype (genomic PC1), ventral colour, carbon isotope value, and sampling latitude and longitude for each member of a social pair. To remove spurious correlations, we used the *pcit* package in R (Watson-Haigh *et al.* 2009), which uses the Spearman matrix to generate a network of partial correlation coefficients. The PCIT algorithm sets a 'local threshold' for inclusion of an edge (i.e. the correlation connecting two traits) based on the average ratio of the partial to direct correlation for every trio of traits ("nodes" on the network). The algorithm begins with a network in which every pair of nodes is connected by an edge whose value is the absolute value of the correlation coefficient between the two traits. An edge between two particular nodes is discarded if the direct correlation coefficient is less than the product of the local threshold and the correlations between each node in the focal pair and the third trait in the trio.

We visualised assortative mating for each transect as a bipartite network of correlations with two categories of nodes (male and female). Each node represents a different trait, and lines (edges) connect nodes if traits are correlated within mated pairs. Analysing assortative mating along transects ensured that each network encompassed individuals with parental and admixed genotypes. Importantly, including genotype as a node allowed us to determine which aspects of phenotype might be used as proxies for genotype in the context of assortative mating. These relationships are shown as lines connecting an individual's genotype to the phenotype of its social partner. We generated networks using the R package 'qgraph' (Epskamp *et al.* 2012). To facilitate interpretation, we only show correlations between male and female pairs on the networks, but within-individual correlations were included in the PCIT analysis.

Assortative mating: strength of pre-mating isolation

To examine fine-scale assortative mating within populations, we analysed the strength of pre-mating reproductive isolation (RI) following Sobel and Chen (2014). This index requires assigning individuals to categories to determine frequencies of con- vs. heterospecific pairings. We classified each individual as a "parental" or a "hybrid" based on its genotype, its carbon isotope value, and its colour. Assignments were made using 1000 repetitions of a linear discriminant analysis (Supplemental Material). We then calculated the strength of RI based on each trait in each population across transects.

Because the proportions of parentals vs. hybrids varied between populations, we weighted observed con- and heterospecific pairings by the number of such pairings expected under random mating, given the distribution of genotypes in each population (equation 4S4 in Sobel and Chen (2014)):

$$\mathrm{RI} = 1 - \frac{2 * \left(\frac{\text{observedheterospecificpairings}}{\text{expectedheterospecificpairings}}\right)}{\left(\frac{\text{observedconspecificpairings}}{\text{expectedconspecificpairings}}\right) + \left(\frac{\text{observedheterospecificpairings}}{\text{expectedheterospecificpairings}}\right)} \tag{1}$$

To calculate expected pairings, we used the total pool of individuals in a population (not just those for which we had pairing data) to randomly generate social pairs without replacement. We counted the proportions of con- and heterospecific pairs from these random draws. We considered pairings between two hybrids to be "conspecific" and pairings between a parental and a hybrid to be "heterospecific;" this will generally underestimate the strength of reproductive isolation. The expected proportions of each pairing type under random mating were averaged over 1000 draws for each population. The observed pairings were then weighted by these expected pairings.

RESULTS

Evidence for a migratory divide

The distribution of δ^{13}C in *tytleri* feathers overlapped almost completely with *gutturalis*, whereas the distribution for *rustica* minimally overlapped the other two subspecies (Figure S1), indicating *rustica* and *tytleri*/*gutturalis* used different overwintering habitat sources for feather synthesis. Specifically, the δ^{13}C values for *rustica* reflect comparatively arid environments where food webs are based on C4 plants, whereas the values for *gutturalis* and *tytleri* are consistent with more mesic environments where food webs are based on C3 plants (Kelly 2000). Sighting records indicate that *rustica* overwinters in southern and eastern Africa and the Arabian Peninsula (Sullivan *et al.* 2009; Turner 2010). The observed δ^{13}C distributions for *rustica* are consistent with values expected for this C4-dominated region (Still *et al.* 2003). By contrast, δ^{13}C distributions and sighting records suggest *tytleri* and *gutturalis* overwinter in south and southeast Asia, a wetter region with more C3 plants (Still *et al.* 2003). Hybrid zones between *rustica* and *tytleri*/*gutturalis* exhibit intermediate means and large variances in δ^{13}C values (Figures S1, S2A), suggesting sympatry between individuals overwintering in different habitats. Distributions of stable carbon isotope values are sometimes multimodal even when individuals molt feathers in the same geographic location; this uncommon occurrence manifests when the sample comprises multiple subgroups of individuals with different migration strategies and resource use patterns (Wunder *et al.* 2012). The more parsimonious explanation for different distributions of stable carbon isotopes in feathers is that they were grown in different habitats or locations (Kelly 2000). We interpret these results as evidence for different wintering habitats, likely on different continents, and consequent migratory divides between *rustica-tytleri* and *rustica-gutturalis*.

Prediction 1: Limited hybridisation is associated with divergent migratory behaviour

We predicted that if migratory divides act as barriers to reproduction, hybridisation should be limited between subspecies pairs with migratory divides compared to pairs without migratory divides. Furthermore, clines for carbon isotope values, our proxy for different wintering habitats, should be steep and concordant with genetic ancestry clines across hybrid zones with migratory divides.

Population structure and gene flow

We identified three genetic clusters that corresponded to the three subspecies and varied dramatically in extent of admixture (Figs 1a and 2). We found limited hybridisation between *rustica-tytleri* and *rustica-gutturalis,* but extensive admixture between *tytleri* and *gutturalis* (Figs 1a and 2). There were few F1 or later generation hybrids between *rustica-tytleri* (1% F1, 13% later generation) and *rustica-gutturalis* (2% F1, 18% later generation), indicating strong isolation (Figures S1 and S3). By contrast, there were many multi-generation hybrids between *tytleri* and *gutturalis* (8% F1 and 53% later generation; Figures S1, S3). A broad region of east Asia encompassing much of the published "*gutturalis*" range (Dor *et al.* 2010) contained few parentals and was primarily comprised of admixed *tytleri-gutturalis* individuals (Fig. 1a). These analyses reveal less hybridisation overall between the subspecies pairs with migratory divides (*rustica-tytleri, rustica-gutturalis*) than the pair without a migratory divide (*tytleri-gutturalis*).

Geographic clines–rustica pairs

We next asked if narrow hybrid zones between *rustica* and *tytleri*/*gutturalis* occurred in the same geographic locations as migratory divides. Clines for ancestry (genetic PC1) were very narrow between *rustica-tytleri* in Russia and *rustica-gutturalis* in China, suggesting these hybrid zones are maintained by selection or are of unrealistically recent origin (< 1 year; Fig. 1b, d, Table 1). A mountain range separated *rustica* and *tytleri* in western Mongolia, and we found no evidence for extant interbreeding across this barrier (Fig. 1c, Table 1). Remarkably, the centres of the ancestry clines in the two *rustica* hybrid zones and the split across the mountains occurred at similar longitudes (between 98 and 101 degrees), despite spanning over 20 degrees of latitude and comprising different pairs of subspecies (Fig. 1a, white arrows). Carbon isotope clines were narrow and concordant with ancestry in all three of these transects (Fig. 1b–d, Table 1). These results indicate that narrow hybrid zones coincide with migratory divides.

Ventral coloration also varied across *rustica* transects. A narrow ventral colour cline in Russia coincided with the ancestry and carbon isotope clines (Fig. 1b, Table 1). Ventral coloration differed on either side of the mountains in Mongolia (Fig. 1c, Table 1). However, in the *rustica-gutturalis* transect in China, the colour cline was narrow and displaced to the east of the other clines (Fig. 1d, Table 1), although differences in colour were small (Figures S1 and S2).

Clines for wing pointedness were narrow and coincident with ancestry and carbon isotopes in the *rustica-tytleri* hybrid zone in Russia and across the mountains in Mongolia, but did not vary across the *rustica-gutturalis* transect in China (Table S1). Tail streamer length, throat colour, wing convexity, and wing length either did not vary clinally or exhibited very wide clines across the three *rustica* transects (Figure S2, Table S1). Thus, carbon isotope value, reflecting different wintering habitats, was the only trait consistently associated with limited hybridisation across the *rustica* transects. This result supports our prediction that narrow hybrid zones are associated with migratory divides. The convergent geographic locations of ancestry and isotope clines strongly suggest that differences in wintering habitats are influenced by the Tibetan Plateau (Fig. 1).

Geographic clines–tytleri/*gutturalis*

There was extensive admixture and no clear association between ancestry and phenotype across the three *tytleri –gutturalis* transects. Ancestry clines were wide, with only the cline in China narrower than the neutral expectation (Table 1, Fig. 1e–g). There was no variation in carbon isotope values or morphometric traits across any of the three transects (Fig. 1e–g, Table 1). The only transect with a ventral colour cline narrower than expected was in China, where the cline was concordant with ancestry (Table 1).

Prediction 2: Migratory behaviour is associated with genetic differentiation

We next assessed whether variation in carbon isotope values or ventral colour were associated with genome-wide variance. Previous studies have found wing length and shape to be associated with migratory distance (Lockwood *et al.* 1998). However, wing morphology is subject to selective pressures unrelated to migration (e.g. Desrochers 2010; Brown & Brown 2013), and no morphological trait varied in a consistent, clinal way across our migratory divides. We therefore used carbon isotope values as our sole proxy for differences in wintering habitat in subsequent analyses.

We predicted that if migratory divides are important reproductive barriers, differences in carbon isotope values would explain large proportions of among-individual genetic variance in hybrid zones with migratory divides, but not in those without migratory divides. Across the three migratory divides, carbon isotope values (alone and combined with geography) explained an average of 19.6% of the variance in PC1 and PC2 (Fig. 3a,c,e). Ventral colour, alone and with geography, explained an average of 10.6% of the variance (Fig. 3a,c,e). By contrast, in the three transects without migratory divides, carbon isotope values explained at most 2% of genetic variance (Fig. 3b,d,f).

Table 1 Best-fit geographic cline models for each trait and each transect

Transect	Ancestry Centre	Ancestry width (km)	Carbon value centre	Carbon value width (km)	Breast chroma centre	Breast chroma width (km)
RG- China	**1405.39** **(1286.03–1405.39)**	51.55* (51.55–416.46)	**1307.65** **(1199.6–1412.04)**	43.16* (0.22–496.16)	1541.49 (1454.64–1999.83)	96.97* (1.19–539.04)
RT- Mongolia	NA	NA	NA	NA	NA	NA
RT- Russia	**781.07** **(771.11–787.88)**	87.75* (72.78–114)	**775.63** **(753.85–799.19)**	102.88* (51.58–181.19)	**774.74** **(766.85–783.11)**	70.0* (47.58–102.77)
TG- China	**659.35** **(345.45–871.81)**	118.18* (4.84–256.9)	*668.16* *(364–1322.56)*	*384.63* *(32.09–2008.83)*	**662.26** **(387.93–909.19)**	2.35* (0–1167.15)
TG- Mongolia	*484.38* *(315.33–532.5)*	*201.05* *(0.45–340.66)*	*510.79* *(390.41–522.11)*	*12.61* *(0.14–172.41)*	*526.76* *(31.7–1458.11)*	*59.28* *(0–238.12)*
TG- Russia	**544.34** **(505.88–586.1)**	555.59 (450.56–693.96)	*1324.01* *(906.2–1444.22)*	*6.27* *(0–74.94)*	**1324.91** **(512.67–1997.59)**	1653.72 (258.18–2029.86)

Boldfaced clines are those that have centres coincident with the ancestry cline. Widths marked by an asterisk are narrower than expected under a neutral diffusion model assuming a dispersal distance of 42 km and a hybrid zone age older than 20 years. Italicised clines show no statistically significant variation in trait values across the transect and are consequently poorly described by cline models. Carbon clines coincide with ancestry in the *rustica* transects (top three rows) but not in the *tytleri-gutturalis* transects (bottom three rows). Cline centre units are kilometers from the westernmost transect point. Cline parameters are not given for the *rustica-tytleri* transect in Mongolia as these birds were separated by a mountain range and there was no evidence for admixture across this barrier.

The proportion of genetic variance explained by carbon isotope values, ventral colour, and geography varied among the three migratory divide transects. In the Russian *rustica-tytleri* transect, the combination of colour, carbon isotopes, and geography explained 34% of among-individual genetic variance (Fig. 3a). Geography and carbon isotope value together explained 30% of genetic variance between *rustica* and *tytleri* on either side of the mountains in Mongolia (Fig. 3c) and 23% of the genetic variance in the *rustica-gutturalis* transect in China (Fig. 3e). Carbon isotope values explained statistically significant proportions of genetic variance when controlling for the effects of colour and geography in the Russian *rustica-tytleri* and *rustica-gutturalis* transects (Table S2). Colour explained significant proportions of genetic variance in the two *rustica-tytleri* transects when controlling for geography and carbon isotope value (Table S2). Overall, these results show that overwintering habitats and, to a lesser extent, colour explain substantial genome-wide variance in migratory divides, but not in non-migratory divides.

Prediction 3: Assortative mating is based on migratory behaviour

Finally, we predicted that if migratory behaviour acts as a barrier to reproduction, we would observe assortative mating by overwintering habitats within migratory divides, but not in non-migratory divides. We assessed assortative mating using social pairing data across the *rustica-tytleri* transect in Russia, the *rustica-gutturalis* transect in China, and the *tytleri-gutturalis* transect in China (Fig. 1). Sufficient social pairing data from the hybrid zone centres (at least 10 pairs per population) were not available for the other three transects. The first two transects have migratory divides, while the third does not.

Phenotype networks

In the two transects with migratory divides, carbon isotope values were correlated within pairs (Fig. 4a and b, grey lines), indicating assortative mating by overwintering habitat. An individual's genotype also correlated with its mate's carbon isotope value (Fig. 4a and b, black lines; *rustica-gutturalis:* r_{carbon} = 0.56 and 0.36; *rustica-tytleri:* r_{carbon} = 0.56 and 0.47), indicating that overwintering habitat is an important basis for assortative mating. An individual's genotype was not correlated with it mate's carbon isotope value in the transect without a migratory divide (*tytleri-gutturalis,* Fig. 4c).

Ventral coloration was correlated within pairs and with mate's genotype in all three transects (*rustica-gutturalis*: r_{colour} = 0.35, *rustica-tytleri*: r_{colour} = 0.58, *tytleri-gutturalis*: r_{colour} = 0.38, Fig. 4). The correlations for colour were weaker than those for carbon isotopes between *rustica-gutturalis* (Fig. 4a) and similar between *rustica-tytleri* (Fig. 4b). There was assortative mating by genotype across all three transects (Fig. 4, *rustica-gutturalis* r_{genotype} = 0.82; *rustica-tytleri* r_{genotype} = 0.48; *tytleri-gutturalis* r_{genotype} = 0.50).

The phenotype networks revealed that migratory behaviour and, to a lesser extent, coloration, are broadly associated with assortative mating across migratory divides. However, genotype and phenotype were also correlated with geography in all transects (Fig. 4). The patterns of assortative mating we observe may therefore be generated in part by variation in the availability of homo- vs. heterotypic individuals as mates.

Reproductive isolation index

Applying an index of pre-mating reproductive isolation within each population allowed us to assess assortative mating at a fine scale, without potentially confounding effects of correlated geographic variables. Across the *rustica-tytleri* transect in Russia, parentals and hybrids co-occurred in several populations, but assortative mating by genotype was comparatively weak (Figure S4a). However, in all populations where both parental forms coexisted, there was evidence for assortative mating by carbon isotope value (average RI = 0.28). Isolation was strongest among *rustica* individuals (RI = 0.52); that is, individuals assigned *rustica* overwintering habitats were > 50% more likely to pair with each other than with individuals with *tytleri* overwintering habitats. Assortative mating by colour was less consistent among populations (Figure S4a).

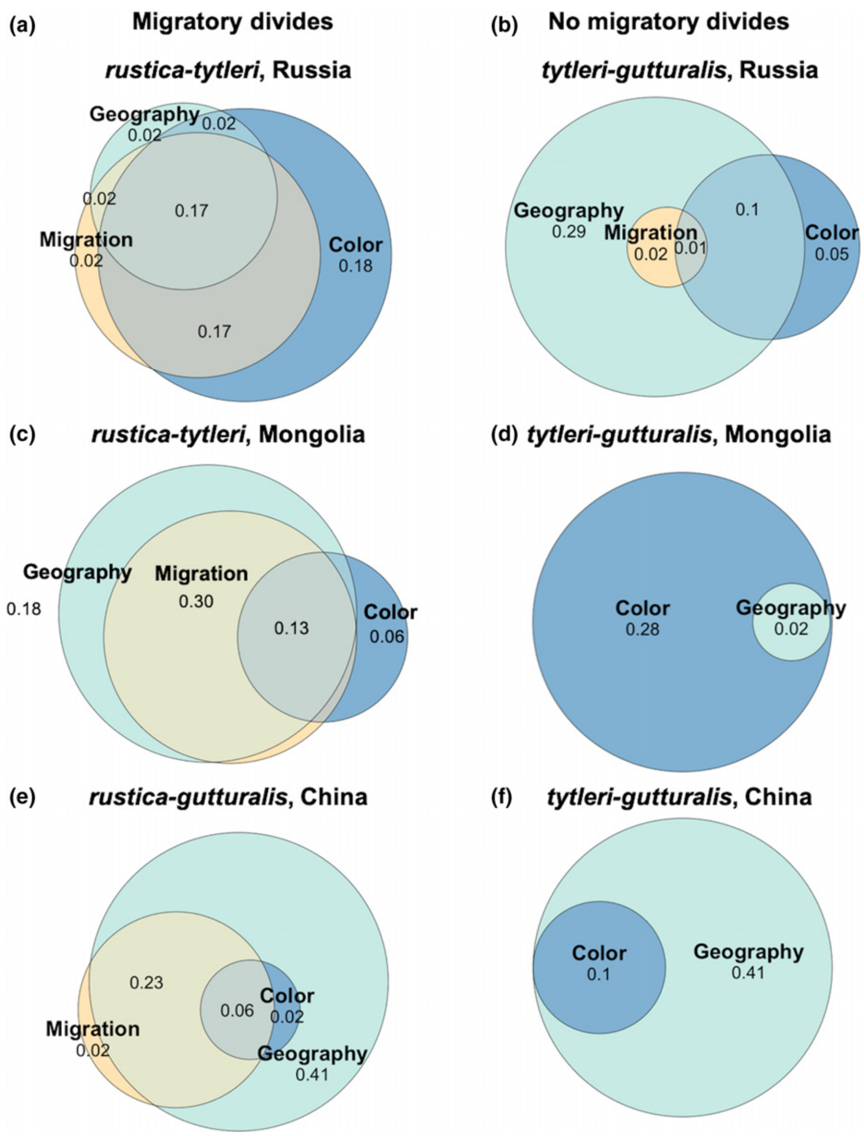

Figure 3 Genomic variance (PC1 and PC2) partitioned among traits related to migration (carbon value), sexual signalling (ventral colour), and geographic location of sampling (latitude and longitude). Variance is shown as adjusted R^2 values and is partitioned among individuals occurring along each of the six transects through regions of hybridisation (Fig. 1a). Each row shows a transect with a migratory divide on the left and the parallel transect (same latitude, different longitude) without a migratory divide on the right. Overlapping regions between circles show the amount of genetic variance explained by the combined effects of those variables; for example, the combination of carbon isotope values, colour, and geographic location explains 17% of the genetic variance in the *rustica-tytleri* transect in Russia (a) and the combination of carbon isotope values and geographic location explains 30% of the genetic variance in the *rustica-tytleri* transect in Mongolia (c). Note that migratory behaviour explains no genetic variance in the *tytleri-gutturalis* transects in Mongolia and China.

This result suggests a central role for divergent wintering habitats in mediating pre-mating reproductive isolation between *rustica* and *tytleri*.

There was some assortative mating by genotype in the *rustica-gutturalis* transect in China (average RI = 0.14, Figure S4b). However, this was due to the absence of parentals from the hybrid zone centre and consequent high pairing frequency among hybrids ("conspecific" matings); indeed, there was no population in which parental *rustica* and *gutturalis* co-occurred (Figure S4b). There was some weak assortative mating by carbon isotope value in the hybrid zone centre (Figure S4b), but mating was generally random based on phenotype.

In contrast to the two migratory divides, we did not detect assortative mating across the *tytleri-gutturalis* transect in China. Individuals within each population were homogeneously admixed, and carbon isotope values and colour varied little, making the question of pre-mating isolation less relevant (Figure S4c). Together, our measurements of pre-mating barriers suggest stronger assortative mating by carbon isotope values than colour in both migratory divides. However, distributions of parentals vs. hybrids, and hence potential mates, varied substantially. At a fine geographic scale, the mechanisms by which migratory divides contribute to reproductive barriers may differ between subspecies pairs.

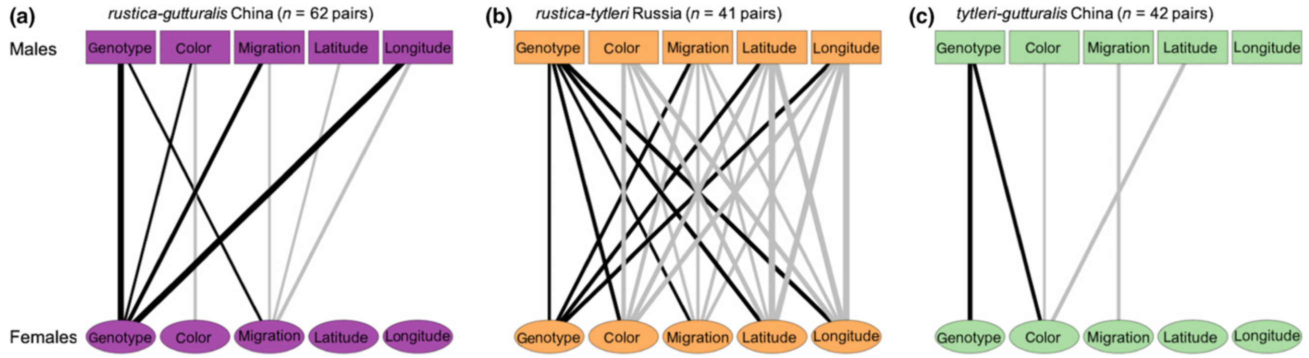

Figure 4 Bipartite phenotype networks showing traits associated with assortative mating across three transects. Black lines connecting an individual's genotype to the phenotype of its social mate indicate traits acting as proxies for assortative mating by genotype (the traits most relevant for reducing gene flow). Grey lines show traits correlated within social pairs (assortative mating, or "like mating with like"). Line width reflects the strength of the correlation. Squares (top row) are males. Circles (bottom row) are females. In the two migratory divides (a and b), an individual's genotype is correlated with migratory behaviour (carbon isotope value) and ventral colouration of its social mate (black lines between traits and genotypes). There is also strong assortative mating by genotype. In the *tytleri-gutturalis* transect in China (c), there is assortative mating by genotype, and ventral colouration is associated with social mate's genotype. This transect does not have a migratory divide, and there is no assortative mating by carbon isotope value. In the migratory divides (a and b) there are also strong correlations between geographic location and genotype, indicating geographic variation in the distribution of available mates.

DISCUSSION

We tested the hypothesis that migratory divides are broadly important to the maintenance of reproductive barriers between barn swallow subspecies. Our analyses suggest that (1) there is less hybridisation in contact zones with migratory divides compared those without migratory divides; (2) divergent overwintering habitats explain large proportions of genetic variance relative to other traits within migratory divides; and (3) divergent overwintering habitats contribute to pre-mating reproductive barriers. Further, geographic coincidence between migratory divides and narrow hybrid zones supports a long-standing hypothesis (Irwin & Irwin 2005) that divergent migratory routes around the Tibetan Plateau maintain range boundaries in Siberian and central Asian avifauna.

Many birds that breed in Asia circumnavigate the inhospitable Tibetan Plateau en route to wintering grounds in south Asia or Africa (Irwin & Irwin 2005). We found multiple migratory divides centred at the same longitude (~100 degrees) but at different latitudes and between different pairs of barn swallow subspecies. Narrow hybrid zones occurred across regions with no obvious ecological gradients or barriers to dispersal, suggesting isolation is not due to divergent ecological selection during the breeding season. Instead, the striking coincidence in width and geographic location of the hybrid zones, and the similar proportions of backcrosses in each zone (Figure S3), suggest they have independently settled in regions where selection against hybrids is symmetrical (Price 2008) or costs of long-distance migration are minimised (Toews 2017). Such observations implicate a major barrier that drives both the location and extent of hybridisation across a broad geographic region. Limited hybridisation in these areas is the pattern we would predict if the Tibetan Plateau shapes differences in migratory behaviour and contributes to the maintenance of reproductive barriers. Future work using geolocators (e.g. Turbek *et al.* 2018) to track migratory routes could further confirm this hypothesis.

Social pairing data indicate that assortative mating by overwintering habitat may be an important pre-mating barrier between *rustica* and *tytleri*. However, although migratory behaviour coincided with a narrow ancestry cline, explained large proportions of genetic variance, and was associated with broad-scale assortative mating in phenotype networks, within-population pre-mating isolation was weak between *rustica* and *gutturalis* in China. This was due to the absence of parental individuals in the hybrid zone centre. In birds, it has been proposed that pre-mating barriers often arise early in divergence, with post-mating barriers appearing later via selection against unfit hybrids (Price 2008). Different isolating mechanisms operating within the two migratory divides may reflect different lengths of time in secondary contact, as well as contributions of variables such as competitive exclusion or unmeasured ecological factors to reproductive barriers. Intrinsic post-mating barriers are unlikely given shallow divergence (Zink *et al.* 2006; Smith *et al.* 2018) and presence of backcrosses in all hybrid zones. It remains possible that as-yet-undetected loci are associated with divergent migratory behaviours and cause intrinsic genetic incompatibilities in hybrids. However, many other migratory divides lack evidence for hybrid unfitness or genetic differentiation associated with migratory phenotypes (Davis *et al.* 2006; Liedvogel *et al.* 2014; Ramos *et al.* 2017; Toews *et al.* 2017), while others demonstrate extrinsic selection against hybrids (von Rönn *et al.* 2016). It is therefore likely that assortative mating and extrinsic selection against hybridisation maintain narrow hybrid zones at migratory divides. However, we cannot assess the relative importance of pre- vs. post-mating barriers with our current data.

Here we present evidence for a central role of divergent migratory behaviour in the maintenance of reproductive barriers across multiple hybrid zones, supporting a longstanding but rarely evaluated hypothesis that migratory behaviour can be an important engine of speciation. Future work studying hybrid fitness will further clarify the mechanisms by which reproductive barriers are maintained within migratory divides.

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AUTHOR CONTRIBUTIONS

ESCS and RJS conceived of the study. ESCS carried out the fieldwork, with assistance from GS, YL, MRW, WL, AR, GS and KK. CCR did the sequence alignments and variant calling. CS processed the stable isotope data. ESCS analysed the data with input from CCR, MRW, MBW and RJS. ESCS wrote the manuscript with input from all authors, especially ST and RJS.

DATA AVAILABILITY STATEMENT

Sequence data are available on the NCBI short-read archive associated with BioProject PRJNA323498. Phenotypes and R scripts are available at Dryad Digital Repository: https://doi.org/10.5061/dryad.pvmcvdngk. Stable isotope data are available at https://doi.org/10.5066/P9C2TH2K.

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