

VOLUME 25
NUMBER 16
AUGUST
2016

MOLECULAR ECOLOGY



Genome-wide differentiation in closely related populations: the roles of selection and geographic isolation

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Abstract

Population divergence in geographic isolation is due to a combination of factors. Natural and sexual selection may be important in shaping patterns of population differentiation, a pattern referred to as ‘isolation by adaptation’ (IBA). IBA can be complementary to the well-known pattern of ‘isolation by distance’ (IBD), in which the divergence of closely related populations (via any evolutionary process) is associated with geographic isolation. The barn swallow *Hirundo rustica* complex comprises six closely related subspecies, where divergent sexual selection is associated with phenotypic differentiation among allopatric populations. To investigate the relative contributions of selection and geographic distance to genome-wide differentiation, we compared genotypic and phenotypic variation from 350 barn swallows sampled across eight populations (28 pairwise comparisons) from four different subspecies. We report a draft whole-genome sequence for *H. rustica*, to which we aligned a set of 9493 single nucleotide polymorphisms (SNPs). Using statistical approaches to control for spatial autocorrelation of phenotypic variables and geographic distance, we find that divergence in traits related to migratory behaviour and sexual signalling, as well as geographic distance, together explain over 70% of genome-wide divergence among populations. Controlling for IBD, we find 42% of genome-wide divergence is attributable to IBA through pairwise differences in traits related to migratory behaviour and sexual signalling alone. By (i) combining these results with prior studies of how selection shapes morphological differentiation and (ii) accounting for spatial autocorrelation, we infer that morphological adaptation plays a large role in shaping population-level differentiation in this group of closely related populations.

Keywords: climate variability, genomic divergence, genotyping by sequencing, population genetics, reproductive isolation, speciation

Received 1 March 2016; revision received 8 June 2016; accepted 15 June 2016

Introduction

Species are thought to arise primarily as populations diverge in spatial isolation and accumulate differences

that preclude their reproduction upon secondary contact (Mayr 1963; Coyne & Orr 2004). This has been argued to be particularly true for birds (Price 2008). There is also long-standing interest in the extent to which population divergence can arise or increase in the context of incomplete spatial isolation, coupled with selection against

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immigrants or hybrids to offset homogenizing genetic exchange ('divergence with gene flow': Nosil *et al.* 2008; 2009). More generally, spatial isolation is a continuum, and divergence can arise as a consequence of spatial isolation and genetic drift (isolation by distance, IBD; Wright 1943; Slatkin 1993); spatially varying natural and sexual selection (isolation by adaptation, IBA, or a subset of IBA: isolation by environment, IBE; Rundle & Nosil 2005; Crispo *et al.* 2006; Nosil 2012, Schluter 2001, 2009; Shafer & Wolf 2013); or both (Lande 1980; Shafer & Wolf 2013; Wang & Bradburd 2014). While the contributions of spatial isolation and divergent selection to population differentiation have been research areas in evolutionary biology since the beginning of empirical population genetics (Fisher 1930), only more recently have we begun to characterize genomic divergence by simultaneous tests of IBA and IBD (e.g. Wang & Summers 2010; Bradburd *et al.* 2013; Wang *et al.* 2013; Wang & Bradburd 2014).

In comparison with accumulating evidence for the role of ecological adaptation (one form of IBA) and geographic isolation (IBD) in population divergence and speciation (Shafer & Wolf 2013; Wang & Bradburd 2014), there is little empirical data on the contribution of sexual selection to population genetic divergence (e.g. Parchman *et al.* 2013; Baldassarre *et al.* 2014; Morgans *et al.* 2014). Adaptation to the local environment may come in the form of sexual selection if competition over mates differs among closely related populations, or signal traits reflect variation in the environmental context in which these traits are developed or advertised (Ingleby *et al.* 2010; Maan & Seehausen 2011; Baldassarre *et al.* 2013; Safran *et al.* 2013; Matute 2014; Seehausen *et al.* 2014). The lack of such data represents a major gap in our understanding of speciation because divergence in sexual traits is a common form of phenotypic differentiation among populations that may strongly relate to reproductive isolation (e.g. West-Eberhard 1983; Panhuis *et al.* 2001; Irwin *et al.* 2001; Ritchie 2007; Curie Network 2012; Safran *et al.* 2012; Langerhans & Riesch 2013; Seddon *et al.* 2013). Moreover, previous work has shown that sexual signals can evolve more rapidly than traits related to ecological adaptation (e.g. Kingsolver *et al.* 2001; Svensson *et al.* 2006; Kingsolver & Pfennig 2007; Siepielski *et al.* 2011; Seddon *et al.* 2013). Collectively, these observations suggest that sexual signals could be an important feature of biological diversification, especially in the early, formative stages of speciation (Kraaijeveld *et al.* 2010). Here, a testable prediction for a role of sexual selection in divergence is that divergence in aspects of phenotype related to mate selection is positively associated with genetic divergence, either due to increased local adaptation, reduced immigration, or both. This is similar to evidence for ecological speciation, where divergence in

traits related to ecological adaptation predicts population genetic differentiation (Shafer & Wolf 2013).

In its most general form, IBA predicts associations between genomic divergence and differentiation in traits related to both ecology and mating success. Note that ecological adaptations and mating adaptations are not mutually exclusive, and indeed, may even be related, as sexual signals necessarily evolve in an ecological context (Ingleby *et al.* 2010; Maan & Seehausen 2011; Baldassarre *et al.* 2013; Safran *et al.* 2013; Seehausen *et al.* 2014). Thus, important questions remain about the relative contributions and likely interactions of sexual selection, natural selection and geographic distance to genomic differentiation among closely related populations (e.g. van Doorn *et al.* 2009; Maan & Seehausen 2011; Wagner *et al.* 2012; Safran *et al.* 2013). For example, sexual selection has been shown to interact with ecological context in the early stages of population divergence and the formation of premating barriers to reproduction (Scordato *et al.* 2014), yet we know very little about the relative importance of each selective process in the accumulation of biologically relevant genomic differences among populations.

Closely related populations with variation in geographic distance are advantageous for identifying spatial and trait-based predictors of genomic divergence. To address the extent to which sexual selection and natural selection either interact or singly influence population divergence, we analysed patterns of genomic divergence as a function of variation in aspects of phenotype known to be involved in mate selection and migration, as well as relevant measures of environmental variation (using elevation data and long-term climate databases), in a widespread, phenotypically divergent, yet young group of subspecies: barn swallows (*Hirundo rustica*). Barn swallows include six subspecies worldwide (Fig. 1), with populations varying in (i) geographic distance from one another, (ii) trait combinations with known importance in sexual signalling, (iii) breeding and nonbreeding environments and (iv) migratory behaviour (reviewed in Scordato & Safran 2014). A recent phylogenetic reconstruction for this group of closely related subspecies indicates that they are monophyletic with respect to other members of the genus *Hirundo* (Dor *et al.* 2010). Additionally, recent mtDNA, phylogeographic and microsatellite analyses suggest this group formed rapidly (between 100 000 and 27 000 years ago; Zink *et al.* 2006) and is not strongly genetically differentiated, despite marked sexual signal and behavioural differentiation among populations (Dor *et al.* 2010, 2012). Phenotypic and genomic divergence among barn swallow populations are likely due to a combination of selection and drift in the context of variable degrees of geographic isolation. For example,

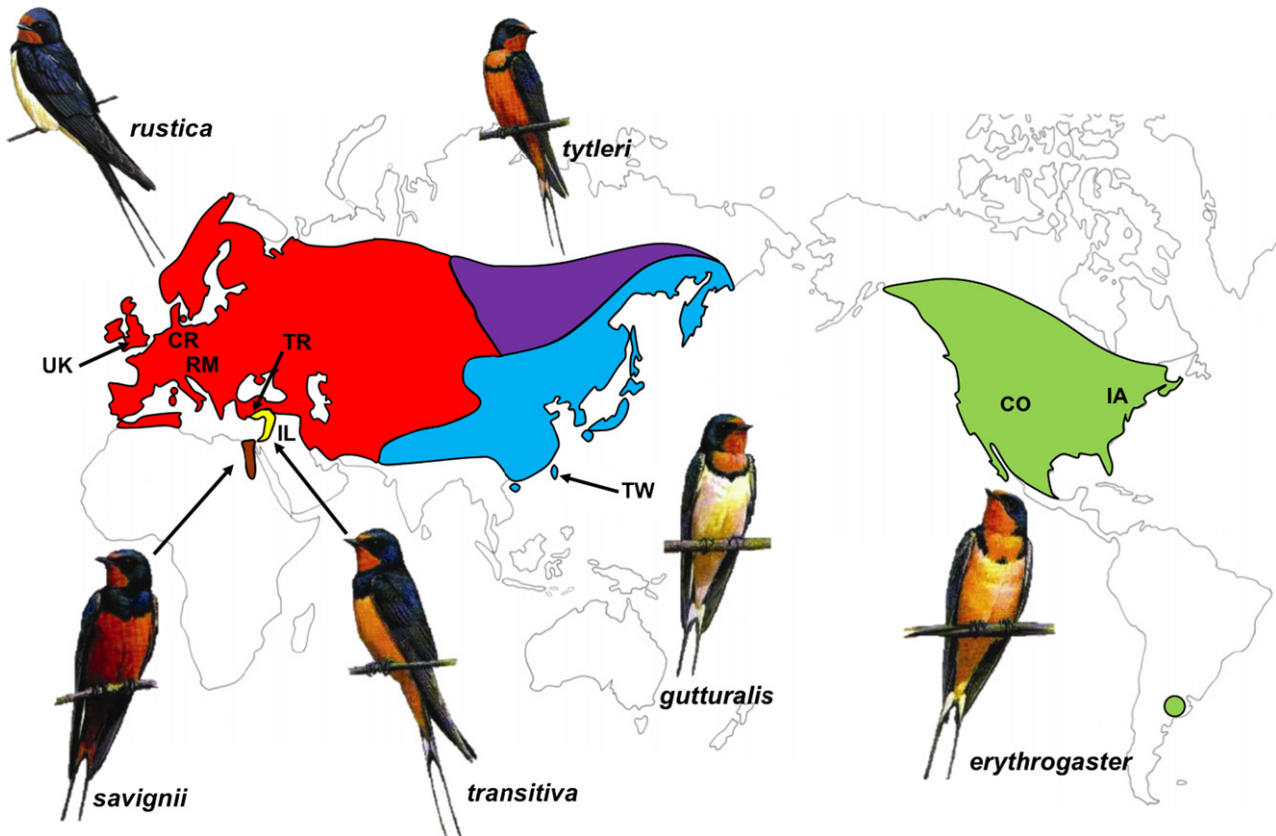


Fig. 1 *Hirundo rustica* complex breeding distribution map indicating eight sampling locations. Cartoons of male phenotypes are shown for each subspecies (with permission by artist Hilary Burn). Acronyms for sampling sites are as follows: CO, Colorado, USA; CR, Czech Republic; IA, New York, USA; IL, Israel; RM, Romania; TR, Turkey; TW, Taiwan; UK, United Kingdom.

populations in North America and Eurasia have likely diverged without gene flow, whereas evidence suggests that populations in the Middle East have experienced recent historical or ongoing gene flow with populations in Europe (Dor *et al.* 2010, 2012).

Previous research in barn swallows has demonstrated phenotypic divergence in traits important to both sexual selection (morphological and behavioural sexual signals; e.g. Safran & McGraw 2004; Safran *et al.* 2005; Vortman *et al.* 2011, 2013; Scordato & Safran 2014) and natural selection (traits related to flight, foraging and migration; reviewed in Turner 2006). One of six well-characterized subspecies within the larger *H. rustica* complex, the European barn swallow (*H.r. rustica*), has been the subject of intense research activity over the last twenty years, with an emphasis on sexual selection (Turner 2006). This work clearly demonstrates that females mate preferentially with long-tailed males, and their offspring experience advantages over those of shorter-tailed males (e.g. Møller 1994). By contrast, in two North American populations of *H.r. erythrogaster*, males with darker plumage colour have greater social and extra-pair mating success (Safran *et al.* 2005; Safran *et al.*, in

Press). In the east Mediterranean distributed *H.r. transitiva*, males with a combination of darker ventral plumage and longer streamer lengths are favoured by females through social and extra-pair mating decisions (Vortman *et al.* 2011, 2013).

Both experimental and observational data suggest that tail streamer length and ventral colour are under varying degrees of sexual selection in different populations of barn swallows, and previous work has defined characteristics of wing shape, such as wing length, as being associated with migratory behaviour (von Rönne *et al.* 2016). We therefore use these traits to examine the roles of sexual and natural selection in contributing to genome-wide divergence among populations of barn swallows. Further, previous work has shown that variation in ventral colour (Saino *et al.* 2013; Hubbard *et al.* 2015) and variation in tail streamer length have heritable components (Møller 1994), indicating that they are subject to evolutionary change and thus relevant aspects of phenotype to investigate in terms of their influence on genomewide divergence.

Here, we genotyped thousands of SNPs (using genotyping-by-sequencing, GBS) in 354 barn swallows from

eight populations, representing four of the six subspecies, distributed across the Northern Hemisphere. Additionally, we report a draft whole-genome sequence for a male *Hirundo rustica erythrogaster*, which was constructed to ensure that GBS sequences cleanly assembled to a reference and to reduce problems with duplicates/paralogs. We use these population genomic data to examine the extent to which population genomic divergence (based on average genome-wide F_{ST}) is associated with divergence in (i) sexual signals, (ii) wing length, (iii) features of climate and/or (iv) geographic distance. The first three factors all relate to IBA, while the fourth relates to IBD. A specific objective of this study is to partition variance in pairwise genomic divergence as a function of geographic distance (IBD); population differences in ecology (IBA, influenced by natural selection); wing morphology related to flight and migratory behaviour (IBA, influenced by natural selection); and differences in morphology related to sexual communication (IBA, influenced by sexual selection).

Methods

Field data collection

We sampled individuals from eight locations representing four subspecies across the barn swallow breeding range (Table 1). The following samples or measures were taken from male barn swallows known to be breeding at each site: (i) a sample of ventral feathers for objective colour quantification, (ii) length of wing and tail streamers (outer rectrices; see Table 2 for a complete description) and (iii) blood samples as a DNA source

from each bird (approx 50 μ l, stored in 2% SDS lysis buffer). See Fig. 1 and Table 1 for sampling location and final sample size information.

Genomics methods

Genome assembly. A draft genome assembled with moderate to high coverage enables the identification of single-copy portions of the genome, reduces the challenges associated with distinguishing close paralogs from alleles at the same locus, and, depending on quality and scaffold sizes, can identify the genomic location of GBS loci. The DNA for our genome assembly came from a male barn swallow with a well-known reproductive history in our Boulder, Colorado study site (ID 2540-44680). This male was first captured in 2008 and had five successive breeding seasons at the same location (from 2008 through 2012). To generate a draft reference genome, we obtained sequences from four lanes of Illumina HiSeq platform, two lanes of 101-base pair reads from two paired-end libraries and two lanes of 101-base pair reads from a mate-pair library from Macrogen (www.macrogenusa.com). One paired-end library had an insert size of 176 bp, while a second paired-end library had an insert size of 454 bp. The mate-pair average insert size was 1458 bp. We obtained a total of 27 Gbp from paired-end library one, and 17.4 Gbp from library two. From the two lanes of mate-pair sequence, we obtained 43.4 Gbp. After cleaning to remove low-quality reads (lower than a base quality of 20 on either end of the read) and common contaminants, 129.4 million pairs of reads remained in library one, 80.0 million pairs in library two and 95.8 million pairs in the mate-pair library remained, for a total of 61.7 Gb of sequence.

Table 1 Sampling locations across four subspecies of *Hirundo rustica*. Acronyms are given for each location that match with locations on Figs 1 and 2

Sampling location (abbreviation for location on map)	Subspecies	Lat/long	Final sample size	Sampling dates: year (sample size)
Boulder, Colorado, USA (CO)	<i>Hirundo rustica erythrogaster</i>	40.17, -105.10	144	2008 (50) 2009 (72) 2010 (22)
Czech Republic (CR)	<i>Hirundo rustica rustica</i>	49.06, 14.76	24	2010
Israel (IL)	<i>Hirundo rustica transitiva</i>	32.93, 35.54	45	2008 (3) 2009 (37) 2010 (5)
Ithaca, New York, USA (IA)	<i>Hirundo rustica erythrogaster</i>	42.44, -77.50	27	2002
Romania (RM)	<i>Hirundo rustica rustica</i>	46.75, 23.83	16	2010
Taiwan (TW)	<i>Hirundo rustica gutturalis</i>	25.09, 121.56	18	2010
Turkey (TR)	<i>Hirundo rustica rustica</i>	36.85, 31.16	50	2010 (50)
United Kingdom (UK)	<i>Hirundo rustica rustica</i>	50.50, -4.65	26	2009
Totals				
8 populations	4 subspecies		350	

Table 2 Morphological and environmental traits measured in 350 individuals, across 8 sampling locations

Types of Traits	Traits
Traits related to reproductive performance among subspecies of barn swallows	Tail streamer length, ventral colour (throat breast and vent, % brightness)
Trait related to flight behaviour and migratory distance	Wing length (mm)
Features of the environment that affect food availability	Mean, variability and seasonal minima in breeding temperatures; elevation

Based on an estimated genome size of 1.3 Gb (Andrews *et al.* 2009), this is an average of 47× coverage.

Reads were assembled using SOAPdenovo 2.04, using a k-mer length of 47, an edge coverage cut-off of 3, a k-mer frequency cut-off of 3 and an arcweight filter of 3. Repeats were resolved with reads. Otherwise, parameters were as defaults. After removing short scaffolds (below 1000 bp), we had a total of 100 153 scaffolds and a total assembly length of 1.1 Gb, 85% of the estimated 1.3 Gb genome size (Andrews *et al.* 2009). The average scaffold length was 11 010 bp, the longest scaffold was 732 517 bp, the N50 was 38 844 bp and N90 was 3718. Of this 1.1 Gb of assembled sequence, a total of 1.06 Gb could be conservatively mapped to the *Ficedula albicollis* genome (Ellegren *et al.* 2012), using blastn with a minimum *e*-value cut-off of 10^{-80} and a minimum sequence similarity of 80%.

The alignment of our *Hirundo rustica* genome assembly to the most closely related, well-annotated genome assembly for Collared Flycatcher *Ficedula albicollis* (Ellegren 2012) shows a high degree of sequence conservation. Over 91% of our single-copy, assembled sequence could be uniquely placed onto the genome. Thus, although we do not have a genetic or physical map placing our sequences onto chromosomes, we can provisionally place virtually all of our assembled genome using sequence similarity to existing avian genome sequences. Given the high degree of synteny found across passerines (Backström *et al.* 2008; Ellegren 2013; Kawakami *et al.* 2014), and among much more distant avian lineages (Derjusheva *et al.* 2004; Zhang *et al.* 2014), taking advantage of existing related genomes enabled us to infer which markers were likely to be linked on chromosomes. We also identified markers likely to be on autosomes and on the sex-determining Z chromosome. As the barn swallow populations we focus on in this paper exhibit only slight genome-wide differentiation (see Results), this draft genome is ideal

for use as a reference for aligning the GBS SNPs described below.

Population genomics. We generated DNA sequence data from 354 individual barn swallows sampled across eight populations (see Table 1). We constructed reduced genomic complexity libraries for each individual using a restriction fragment-based procedure (Gompert *et al.* 2012; Parchman *et al.* 2012). We first digested genomic DNA with two restriction endonucleases (EcoRI and MseI) and ligated double-stranded adaptor oligonucleotides to the digested fragments. These oligonucleotides consisted of the priming sites for Illumina sequencing, followed by eight, nine or ten base pair barcode sequences that allow for the unique identification of sequences from each individual. This method has been successfully used to generate population genetic data for a large number of projects (Gompert *et al.* 2012, 2014; Nosil *et al.* 2012; Parchman *et al.* 2012, 2013; Mandeville *et al.* 2015); a full version of the protocol is available at dryad (doi: <http://dx.doi.org/10.5061/dryad.17hr9>). We used 354 unique barcoded adaptors, which allowed us to sequence all individuals in one Illumina HiSeq sequencing lane. After the restriction and ligation reactions, we pooled all samples and used standard Illumina PCR primers to amplify the barcode-adapted fragments. We separated the amplification products on a 2% agarose gel and excised fragments between approximately 350 and 450 bp in length. We purified these fragments using Qiagen's Qiaquick Gel Extraction Kit (Qiagen Inc.). Concentration and quality of the pooled library was evaluated on an Agilent BioAnalyzer qPCR. Sequencing was performed by the National Center for Genome Research (Santa Fe, NM, USA) with 100-base pair single-end reads, yielding 98 401 301 reads following standard contaminant filtering.

We used a custom Perl script (dryad doi: <http://dx.doi.org/10.5061/dryad.17hr9>) to remove bases associated with the barcode and EcoRI cut site for all 98 401 301 sequences and to replace the sequence IDs with individual IDs for each DNA sample. This script also corrected barcodes with potential single or double base mismatches due to sequencing or oligonucleotide synthesis errors. Sequences were then split by individual for further analysis. Four of the individuals had fewer than 2000 reads and were disregarded, which left 350 individuals for further analysis. We used BWA v 0.7.12 (Burrows–Wheeler Aligner; Li & Durbin 2009) to assemble data for each individual against the barn swallow genome assembly, with an edit distance of 4 and the remaining parameters set as default. This edit distance ensures that even with 1% sequencing errors typical of Illumina sequencing, distant alleles from

divergent subspecies will map to the genome, while nevertheless preventing nonspecific alignments. Across all individuals, an average of 61.66% of reads assembled, with 97.5% of individuals having 57.9% or greater percentage of reads assembling. We used SAMTOOLS v 1.2 and BCFTOOLS v 1.2 (Li *et al.* 2009) to identify variant sites in the assembled sequences and obtain genotype likelihoods for variable sites. To identify variants, we required data for 60% or more individuals. This resulted in 67 773 single nucleotide variants. A complete list of parameters used for assembly and variant calling are available from the authors by request.

We used point estimates of allele frequencies in all 350 individuals to separate this set into 22 328 common variants (minimum minor allele frequency of 5% or greater) that were used for further analysis and 45 445 rare variants that were disregarded. We focused on comparatively common variants because they are more likely to occur in more than one location and contain information about population histories (Gompert *et al.* 2012). To avoid analysing SNPs with highly correlated allele frequencies, we randomly sampled a single SNP from each of the 100-bp regions in the assemblies (GBS loci are 100 bases in length). For the resulting final set of 9493 single nucleotide polymorphisms (SNPs), the average coverage depth per individual per site was $1.5 \times$ (0.09–3.57, 2.5% and 97.5% quantiles). This kind of low coverage genomic data is appropriate for population-level inferences when analysed with models that incorporate uncertainty arising from variability in sequencing coverage across individuals and loci (e.g. Nielsen *et al.* 2011; Gompert *et al.* 2012; Buerkle and Gompert 2013).

Population genetic analyses. We used a Bayesian model to estimate population allele frequencies for each of 9493 variable nucleotides based on the point estimates of genotypes from BCFTOOLS (described in Gompert *et al.* 2012). Genotypes and the population allele frequencies were treated as unknown model parameters, and genotype probabilities and allele frequencies were simultaneously estimated for each sampling locality (separately from estimates for other localities). Importantly, this model incorporates uncertainty from stochastic variation in sequencing coverage depth across individuals and loci into the estimation process and provides a sound approach for estimating population-level parameters for low coverage sequencing approaches. Population allele frequencies serve as prior information, and genotype probabilities are inferred for each locus in each individual. We obtained posterior probabilities for parameters using Markov chain Monte Carlo (MCMC). Each analysis consisted of a single chain iterated for 2000 steps following a 1000-step burn-in, with samples retained every other step, yielding 1000 samples from

the posterior distributions. For these data and this simple model, mixing and convergence were clearly evident in plots of MCMC histories.

We obtained the mean genotype (scale of 0–2) from the posterior distributions for each SNP in each individual. Furthermore, we calculated the mean genotype across all individuals to centre the genotypes at each locus before calculating the genetic covariance between the genotype vectors for all pairs of individuals. We summarized the genetic covariance matrix using principal component analysis (PCA). Because the PCA will reflect all genetic covariances and will be affected by uneven sampling (e.g. 144 samples from Colorado and 16 from Romania), we performed a second PCA in which we randomly down-sampled all localities to the smallest sample size from any locality ($N = 16$). We also obtained mean allele frequencies from the posterior distributions and transformed these to estimates of mean F_{ST} (Hudson *et al.* 1992) and Nei's D (Nei *et al.* 1983; Takezaki & Nei 1996) between all pairs of populations. F_{ST} and Nei's D were highly correlated ($r = 0.996$), so we only present results for F_{ST} . We performed the PCA and calculated F_{ST} and Nei's D in R (using *prcomp* and custom functions, R Core Team, 2015).

Phenotypic and environmental variables

Our goal was to compare divergence among traits known to be related to sexual signalling (plumage colour and streamer lengths) and migratory behaviour (i.e. wing length). We also compared aspects of environmental variability known to affect the aerial insect populations that barn swallows prey upon. These variables include elevation and several measures of temperature variation (e.g. Thomsen *et al.* 2016).

Quantifying feather colour. We collected 5–10 feathers from the throat and breast (upper ventral region) and vent (lower ventral region, below the attachment of tail streamers) and stored them in small envelopes in a dark, dry environment prior to measurement (following Safran & McGraw 2004). We assessed the colour of these samples by measuring plumage brightness using an Ocean Optics USB4000 spectrometer (Dunedin, FL). Reflectance data were generated relative to a white standard (Ocean Optics WS-1) and a dark standard (all light excluded), and spectra were recorded with the SPECTRASUITE software package (version 2.0.125, Ocean Optics Inc.). We find no evidence of UV reflectance in the ventral plumage colour of barn swallows (Safran & McGraw 2004) and thus used three traditional axes of colour for objective measurement of colour variation. We used average brightness, which was calculated from three separate measurements of the collected throat and

breast feathers, as a representative metric of overall ventral plumage colour. Average brightness is a good colour metric, as all three traditional axes of colour (hue, chroma and brightness) were previously found to be highly correlated across the ventral region of individual barn swallows (McGraw *et al.* 2005, Safran & McGraw 2004, J.K. Hubbard, unpublished data), and brightness is the most variable dimension of colour in this region (J.K. Hubbard, unpublished data). Lower brightness scores (% reflectance) indicate plumage colour that appears darker, redder and more saturated when compared to feathers with higher brightness scores.

Estimates of phenotypic divergence. To determine pairwise distance in phenotypes among environmental and morphological traits, we used an unbiased effect size statistic (ΔP ; Safran *et al.* 2012) to calculate trait distance for each trait in pairwise comparisons among the 8 sampled populations. ΔP is calculated based upon a joint cumulative distribution function (CDF) from all populations in the data set. Distances were calculated for each pairwise comparison using the population median percentile in the overall CDF. ΔP was developed specifically to analyse phenotypic distance among closely related populations, as it easily accommodates simultaneous comparisons of any number of traits across any number of populations, and is relatively insensitive to unequal variances and sample sizes among populations (Safran *et al.* 2012). For all analyses, we use the absolute value of pairwise distances.

Climate and elevation data. Temperature and elevation data were obtained using the CRUTEM database maintained by the Climatic Research Unit and available at <http://www.cru.uea.ac.uk/cru/data/crutem/ge/>. Using this database, we downloaded the last 50 years of temperature data from the three weather stations closest to each of our sampling sites (see Supporting information). From these data, we derived the mean, minimum and coefficient of variation in temperature during the breeding season. Breeding seasons vary among our populations, and, accordingly, we used the following months for climate data collection for each site: Colorado, USA: April–September; New York, USA: May–August; UK: April–August; Israel, January–April; and Czech Republic, Romania, Turkey and Taiwan, April–July. These measurements have been routinely employed in studies of other avian taxa with wide-spread geographic ranges (Rubenstein & Lovette 2007, 2007; Botero *et al.* 2009, 2014).

Estimates of geographic distance. Geographic distances between study sites were calculated in the R package 'geosphere' using the Haversine great circle distance

between points. This is the shortest 'as the crow flies' distance, assuming a spherical earth.

Associations of geographic distance, environmental context and phenotype with population genomic divergence

To assess the degree to which genome-wide divergence is associated with geographic and phenotypic distances, we analysed correlations between pairwise trait distance and both pairwise geographic and genetic distances (F_{ST}), and assessed their significance (999 permutations) using Mantel tests using the R package 'VEGAN'. Next, to quantify the relative strength of association among geographic, phenotypic and genomic variables, accounting for correlations between phenotypic and geographic distances, we used two complementary statistical approaches. First, we applied multiple matrix regression (Wang 2013). This performs multiple regression on distance matrices and uses permutation tests ($n = 10\,000$) to obtain P and R^2 values using the MRM function in the R package 'ecodist' (Wang 2013). In the second approach, we employed constrained redundancy analysis and variance partitioning to analyse the relative contributions of traits related to natural and sexual selection and environmental context in explaining pairwise genetic divergence (Legendre and Fortin 2010) using the R package 'VEGAN' (Oksanen *et al.* 2015 v. 2.2). Redundancy analysis is a type of constrained ordination that quantifies how much variation in a set of variables is explained by a second set of variables, with the option of conditioning on a third set. This analysis is ideal for correlated matrices, as is often the case with the matrices related to phenotype, genotype, environmental variables and geographic distance among closely related populations (Shafer & Wolf 2013; Wang & Bradburd 2014). Using these methods, variance can then be partitioned between the constrained, conditioned and joint variable sets. Our data were structured in two different formats, depending on the analysis. For Mantel tests, we employed matrices of pairwise differences for each population. For MRM and the variance partitioning analyses, these matrices were transformed into vectors of pairwise differences for each variable and each pair of populations. When the response variable is a single vector (as here, using pairwise differences in mean F_{ST} between populations), variance partitioning is done by partial regression.

Results

Population genomics

Principal component analysis of genetic covariances between an even sample of individuals from each

population ($n = 16$) revealed clear genomic differences between some localities, but greater genomic similarity among the nearby sampling locations in Czech Republic, Romania, Turkey and Israel (Fig. 2). The first two principal components explained 52% of the genomic variation among individuals in each locality (Fig. 2). The separation of populations on PC1 is consistent with the phylogenetic hypothesis (Dor *et al.* 2010) that separates eastern and western barn swallows, with samples from Israel and Europe recoverable as a distinct lineage from samples in North America (Colorado and New York) and southern Asia (Taiwan). Interestingly, while Israel is considered a distinct subspecies based on phenotype, it cannot be differentiated from European populations based on genomic covariance. PC2 explains a relatively small amount (10.3%) of genetic variation compared to PC1 (41.9%), and further separates subspecies within the eastern and western clades. The PCA based on all 350 individuals gave considerable weight in the first axis to distinction between the large sample from North America ($N = 144$ from Colorado and 27 from New York) and samples from elsewhere, with PC1 accounting for 67.9% of the genetic variation. Pairwise F_{ST} ranged from 0.024 to 0.073 (Table 3).

Phenotypic and environmental variables

Phenotypic and environmental divergence. Populations differed in the extent of both phenotypic variation and environmental context (Tables 4 and 5). Mean percentiles within a cumulative frequency distribution varied considerably among populations; the distribution of variables measured from each population differed in terms of its placement on the overall cumulative frequency distribution (Table 4). For example, populations in the UK have the longest wing length, whereas populations in Taiwan have the shortest. Populations in North America (Colorado and Ithaca) have the lightest throat colour yet the darkest breast colour; thus, there is variation in the extent and direction in which colour patches differ among subspecies. The spread of percentile values is indicative of the degree of pairwise divergence for each trait or climate variable. For example, of all phenotypic traits, populations exhibited the most extreme differences in wing length, with a range of percentiles from 9.02 to 91.16. Note that the percentile values for the phenotypic measures are based on multiple individuals within populations (Table 1), whereas the percentile values for the environmental measures are based on one measure within each

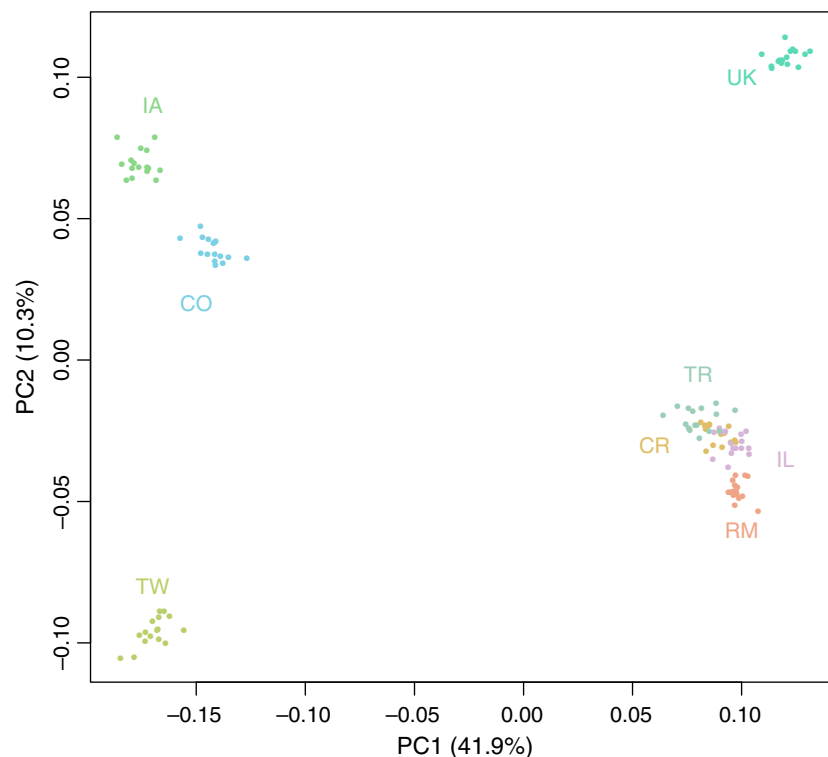


Fig. 2 Statistical summary of population genetic structure based on principal component axes one (PC1), and two (PC2) derived from genotype probabilities. Acronyms for sampling sites are as follows: CO, Colorado, USA; CR, Czech Republic; IA, New York, USA; IL, Israel; RM, Romania; TR, Turkey; TW, Taiwan; UK, United Kingdom.

Table 3 Average pairwise F_{ST} (upper diagonal in italicized text) and geographic distance (km; lower diagonal)

Sampling location	Colorado	Czech Republic	New York	Israel	Romania	Turkey	Taiwan	UK
Colorado	NA	<i>0.049</i>	<i>0.024</i>	<i>0.054</i>	<i>0.053</i>	<i>0.046</i>	<i>0.046</i>	<i>0.059</i>
Czech Republic	853.948	NA	<i>0.059</i>	<i>0.038</i>	<i>0.041</i>	<i>0.030</i>	<i>0.062</i>	<i>0.048</i>
New York	2397.257	6726.714	NA	<i>0.062</i>	<i>0.063</i>	<i>0.055</i>	<i>0.055</i>	<i>0.067</i>
Israel	10959.901	2454.926	9169.197	NA	<i>0.044</i>	<i>0.031</i>	<i>0.065</i>	<i>0.051</i>
Romania	9131.466	700.843	7399.893	1828.448	NA	<i>0.037</i>	<i>0.067</i>	<i>0.053</i>
Turkey	10381.422	1864.741	8578.322	591.36	1255.353	NA	<i>0.059</i>	<i>0.045</i>
Taiwan	11321.578	9083.006	12305.315	8199.932	8582.409	8425.255	NA	<i>0.073</i>
UK	7337.099	1443.954	5361.685	3818.056	2144.723	3230.591	10145.824	NA

Table 4 Percentiles for male morphological traits across all populations used for calculating ΔP differences. Lower wing and tail streamer percentiles indicate shorter wings and streamers, respectively. Higher colour percentiles indicate *lighter* colour. Greater elevation percentiles are indicative of higher elevations at breeding locations. Greater percentiles for variation, minima and average temperatures are indicative of more variable, hotter climates measured over the period of data collection (1965–2015)

Trait	Population sampled								Range in percentile
	Colorado	Czech Republic	Israel	New York	Romania	Taiwan	Turkey	UK	
Wing length	24.08	70.73	61.00	30.22	61.87	9.02	61.00	91.16	9.02–91.16
Streamer length	21.82	72.23	60.22	15.05	79.79	28.66	67.16	60.22	15.05–79.79
Throat colour	77.74	59.42	39.96	80.77	18.35	28.26	47.12	44.62	18.35–80.77
Breast colour	21.03	58.63	27.61	21.99	58.72	80.77	55.41	70.39	21.03–80.77
Vent colour	18.10	27.01	16.02	20.32	21.45	32.99	25.32	30.14	16.02–30.14
Elevation	100	62.5	75	37.5	50	12.5	87.5	25	12.5–100
Var temp	37.5	75	87.5	62.5	50	25	100	12.5	12.5–100
Min temp	62.5	25	12.5	87.5	50	100	87.5	37.5	12.5–100
Mean temp	62.5	25	12.5	75	50	100	87.5	37.5	12.5–100

population. Analyses of variance reveal that all of the phenotypic traits we measured in this study showed statistically significant differences among populations (adjusted R -squared values range from 0.25 to 0.52; Table 5).

Associations of geographic distance, environmental context and phenotype with population genomic divergence

Geographic distance. Geographic distance between pairs of populations predicted genome-wide divergence, consistent with a model of isolation by distance ($r = 0.628$, Mantel P value < 0.008 , Fig. 3, Table 3).

Phenotypic divergence and environmental context. Distance matrices (based on ΔP) for various features of phenotype, including measures of ventral colour, tail and wing length, were positively correlated with genome-wide divergence among pairs of eight geographically isolated populations (Mantel tests; Fig. 4 and Table 6). None of the pairwise differences in environmental variables (Mantel tests; elevation and various measures of breeding season temperature) were significantly

associated with genome-wide divergence among our study populations (Fig. 5 and Table 6).

Geographic distance, environment, phenotype. Several features of phenotypic divergence also covaried with geographic distance (Mantel tests; Fig. 6 and Table 6), whereas environmental and climate features did not (Mantel tests; Fig. 7 and Table 6). Thus, genome-wide divergence among closely related populations was associated with both geographic and phenotypic trait distances, and the two are sometimes correlated with each other. It is therefore necessary to adequately control for correlations between phenotypic and geographic distances in order to infer the relative significance of IBA and IBD. We did this using two complementary approaches.

First, to investigate the associations of specific traits with pairwise, genome-wide F_{ST} while accounting for correlations among variables in our model (e.g. phenotypes correlated with one another and with geographic distance), we used multiple matrix regression (Wang 2013). We started with a maximal model that included the pairwise distance matrix of F_{ST} values as the response variable and distance matrices (based on ΔP

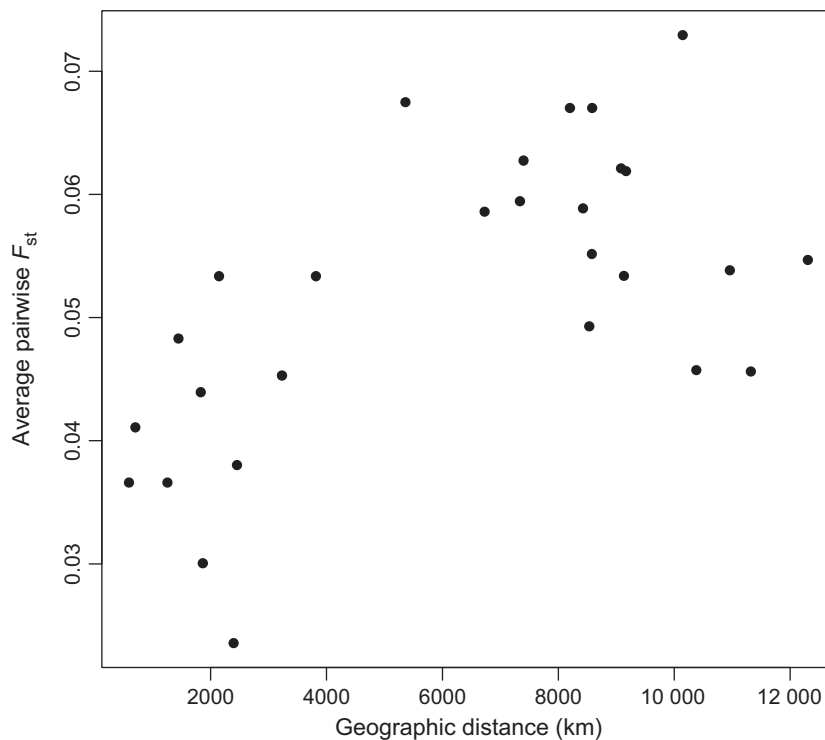
Table 5 Results of ANOVAS to show geographic variation in morphological traits among eight closely related populations of barn swallows

Trait	F	P	Adj R-squared
Wing length	54.11 _{7,353}	<0.001	0.52
Streamer length	54.27 _{7,352}	<0.001	0.52
Throat colour	18.84 _{7,346}	<0.001	0.25
Breast colour	28.16 _{7,345}	<0.001	0.37
Vent colour	24.97 _{7,345}	<0.001	0.34

values) for all nine phenotypic and ecological variables as predictors. We then used backwards stepwise model selection, sequentially deleting the least significant term and rerunning the model, until coefficients for all remaining predictor variables were significantly different from zero. The final multiple matrix regression model included two aspects of phenotype that explained significant variation in pairwise F_{ST} while controlling for spatial autocorrelation: wing length and throat colour (full model $F_{2,17} = 24.95$, $P = 0.002$, R -squared = 0.757; parameter estimate and t -test: wing length coefficient = 0.82, $T = 6.12$, $P = 0.002$, throat colour coefficient = 0.22, $T = 1.79$, $P = 0.03$, geographic distance coefficient = 0.01, $T = 0.09$, $P = 0.94$).

We used the significant predictor variables from the multiple matrix regression in variance partitioning and redundancy analyses to further examine the relative contributions of wing length, throat colour and geographic distance in explaining variation in genome-wide divergence among the populations in our data set. The variance partitioning approach enabled us to test hypotheses about the relative significance of IBA and IBD, taking into account correlations among phenotypic, environmental and geographic distance variables.

The best model fit from the variance partitioning analyses (Table 7) included the effects of all three matrices (geographic distance, throat colour distance and wing length distance). These models, in which various combinations of matrices are conditioned upon one another, demonstrated that collectively 73% of genome-wide divergence is attributable to three variables: wing length, throat coloration and geographic distance between populations (Fig. 8). Further analyses enabled us to analyse the association between each matrix (geographic distance, throat colour, wing length) separately by conditioning each variable on the others in all possible combinations (Table 7). For example, when the matrix containing pairwise distance in wing length is conditioned on the matrices containing pairwise differences in geographic distance and throat colour, the

**Fig. 3** Average pairwise genetic distance (F_{ST}) as a function of geographic distance (km) among pairwise comparisons of eight barn swallow populations, consistent with a pattern of isolation by distance.

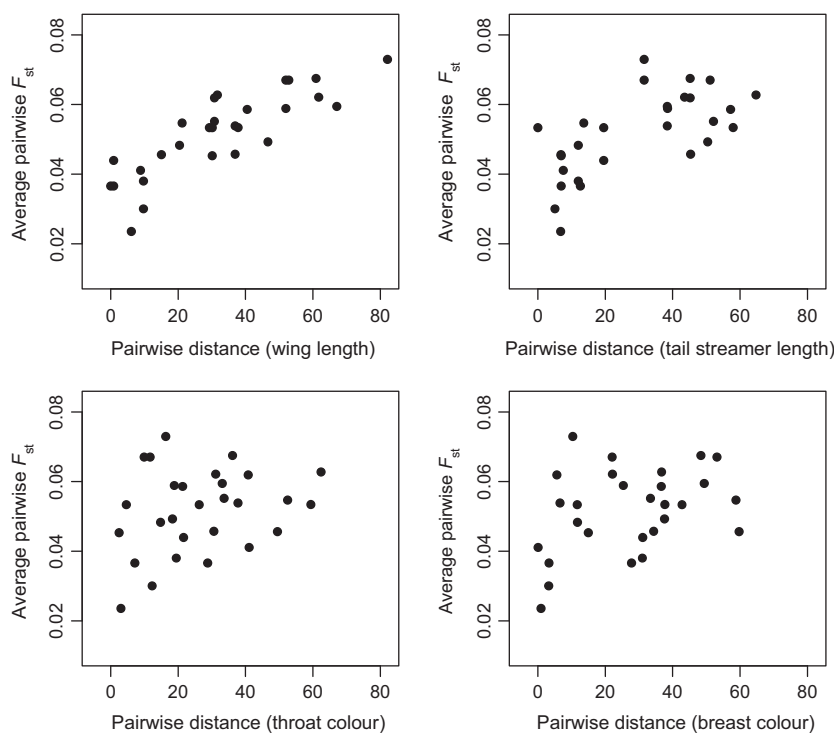


Fig. 4 Average pairwise genetic distance (F_{ST}) is strongly correlated with phenotypic distance among pairwise comparisons of eight barn swallow populations, consistent with a pattern of IBA. Table 6 shows results of Mantel tests between F_{ST} and these four phenotypic measures; all are significantly associated with the exception of throat colour.

influence of wing length on its own accounted for 42% of pairwise genomic distance among the populations in our sample (Fig. 8 and Table 7). When the matrix containing pairwise distance in throat colour is conditioned upon the matrices containing pairwise differences in geographic distance and wing length, the influence of throat colour on its own explained 5% of pairwise genomic distance among the populations in our sample. Finally, when conditioned upon the wing and colour matrices, the effect of geographic distance on its own did not explain additional variation in genomic distance among the populations in our sample (Fig. 8 and Table 7). Interestingly, the only place where the geographic distance matrix explained a significant amount of variation in genomic divergence is when both geographic distance and wing length were considered side by side and conditioned upon their correlation with the colour matrix (Fig. 8 and Table 7).

Discussion

Geographic distance and phenotypic distance are strongly correlated among our study populations. Thus, we applied two complementary methods of variance partitioning – multiple matrix regression, and constrained redundancy analysis – which enabled us to analyse the relative contributions of correlated matrices

(geographic distance and phenotypic distance) to genome-wide divergence. Overall, our results demonstrate clear evidence that both IBA and IBD contribute to genome-wide divergence among these closely related populations of barn swallows. When spatial autocorrelation between phenotype and geographic distance are accounted for, our results suggest that divergence in an ecological trait (wing length) and a sexual signalling trait (throat colour) play a larger role in population genetic divergence than does geographic distance.

Surprisingly, we found no evidence that elevation and temperature differences were influential to genome-wide divergence. Although these features of the environment, which are relevant for obligate aerial insectivores, did vary spatially, they were neither associated with geographic distance nor genome-wide divergence among the populations in our sample. In a further investigation, we also found no evidence that maximal temperatures or precipitation patterns at each location differed as a function of geographic distance or were associated with genome-wide divergence (R.J. Safran, unpublished data). Given the span of our sampling locations, ranging from Israel to North America, these results either suggest that barn swallows occupy fairly similar environments with respect to elevation and temperature or are not particularly sensitive to these ecological variables. The latter explanation seems

Table 6 Results of Mantel tests. Tests of isolation by adaptation and phenotypic-genetic distance correlations using traits related to mate selection (colour, tail length) and migratory behaviour (wing length) and environmental traits including elevation and various metrics of temperature during the breeding season. To control for multiple testing, we have indicated the confidence intervals around the Mantel test coefficient

Variable	Association with genome-wide F_{ST}			Association with geographic distance		
	Mantel r	CI	P value	Mantel r	CI	P value
Wing length	0.85	0.79–0.91	0.002	0.60	0.34–0.76	0.010
Tail length	0.67	0.60–0.81	0.012	0.59	0.38–0.85	0.013
Throat colour	0.29	–0.08 to 0.66	0.091	0.48	0.27–0.78	0.030
Breast colour	0.34	0.16–0.53	0.023	0.46	0.24–0.74	0.031
Vent colour	0.40	0.14–0.59	0.037	0.30	–0.11 to 0.54	0.094
Elevation	–0.04	–0.27 to 0.38	0.593	0.17	–0.07 to 0.35	0.202
Min breeding temp	0.07	–0.18 to 0.40	0.348	–0.01	–0.28 to 0.38	0.483
Mean breeding temp	0.20	–0.18 to 0.64	0.145	0.13	–0.14 to 0.54	0.274
Var breeding temp	0.07	–0.11 to 0.29	0.346	–0.05	–0.30 to 0.27	0.560

P -values highlighted in boldface are statistically significant at $P < 0.05$.

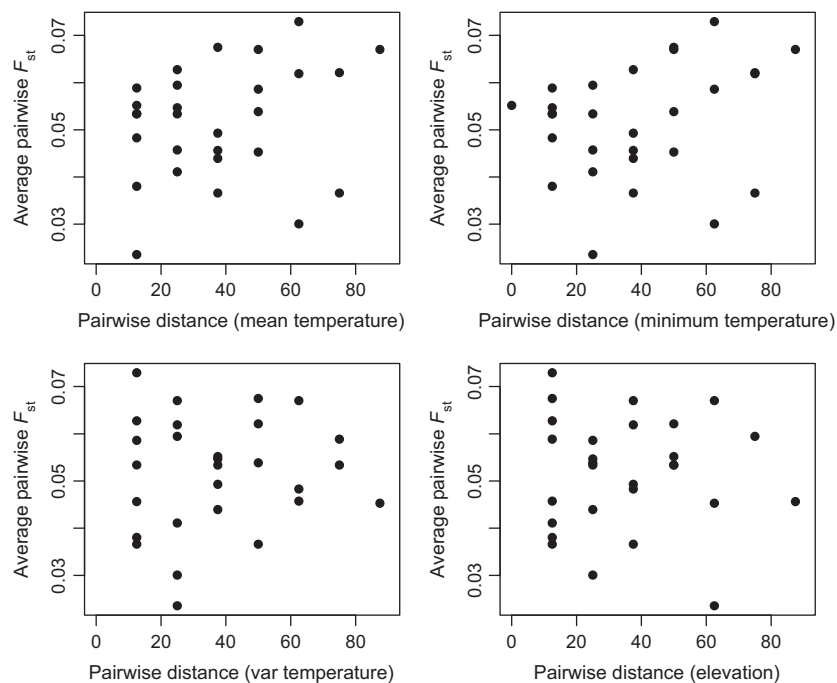


Fig. 5 Average pairwise genetic distance (F_{ST}) as a function of environmental distance among pairwise comparisons of eight barn swallow populations. Table 6 shows results of Mantel tests between F_{ST} and environmental distance; that there are no statistically significant associations between genetic distance and these environmental variables.

most likely as these populations are very cosmopolitan in distribution during both the breeding and nonbreeding season and during long migratory trips where they likely inhabit a wide range of environments.

Population genomic structure and phenotypic divergence

Our data demonstrate genetic similarity of individuals within sampling localities, with greater differences

between populations that are separated by large geographic distances, and genome-wide clustering that generally corresponds to named subspecies. Principal component analysis shows that the most genetically differentiated populations along PC1 correspond to a highly supported east–west split in the current phylogenetic hypothesis for barn swallows (Zink *et al.* 2006; Dor *et al.* 2010). Populations in Asia (*H.r. gutturalis*) and North America (*H.r. erythrogaster*) are more closely related to one another than either are to populations in

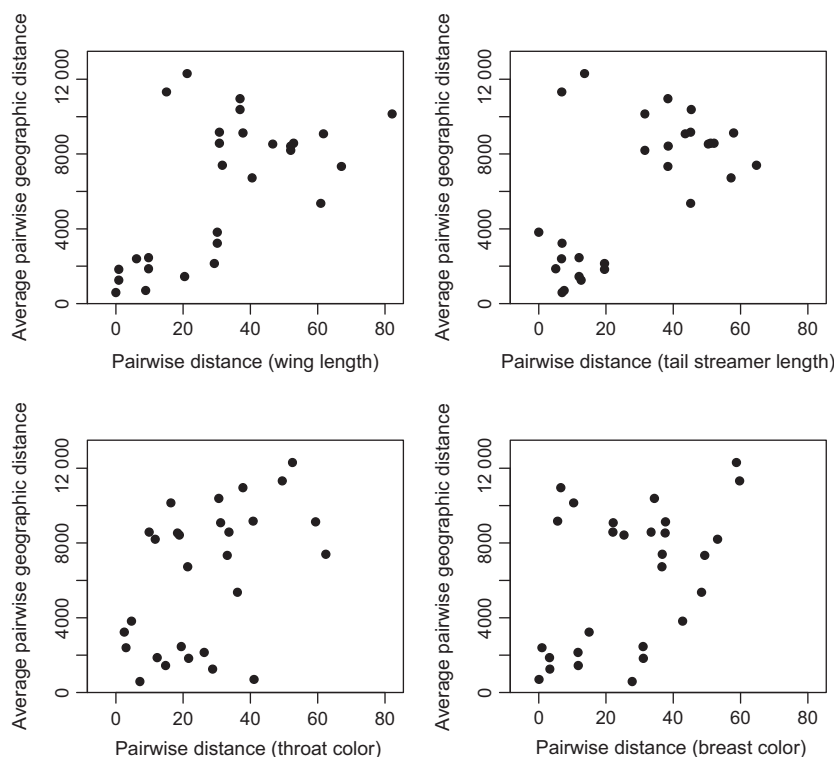


Fig. 6 Average pairwise geographic distance as a function of phenotypic distance among pairwise comparisons of eight barn swallow populations indicates spatial autocorrelation between morphological and geographic distance. Table 6 shows results of Mantel tests between geographic distance and phenotype distance; all of these associations are statistically significant.

Europe (*H.r. rustica*) and the Middle East (*H.r. transitiva*). PC2 further separates *H.r. gutturalis* (TW) from two samples from the North American *H.r. erythrogaster* populations (IA and CO), and the UK population from other mainland populations of *H.r. rustica* (TR, CR and RM). Samples from the Israeli subspecies (IL) are clustered closely with other mainland European populations of barn swallows, consistent with an unresolved relationship between this subspecies and *H.r. rustica* in the current phylogeny (Dor *et al.* 2010). Despite being relatively genetically similar, individuals from Israel and the continental European populations are fairly divergent in phenotype, particularly with respect to ventral colour.

Despite shallow genomic divergence, phenotypic differentiation is apparent in all aspects of morphology we analysed in this study, ranging from traits related to body size and flight (wing and tail length) to ventral colour. Phenotypic variation despite shallow genomic divergence appears common among many taxa, including cichlid fishes (e.g. Wagner *et al.* 2012) and particularly in birds (e.g. Parchman *et al.* 2006; Poelstra *et al.* 2014; Rodrigues *et al.* 2014; Mason & Taylor 2015), where it is often the case that a few genes are implicated in morphological variation against a fairly homogenous genomic background (e.g. Poelstra *et al.*

2014; Kardos *et al.* 2016). In other words, although a larger number of genes may be involved in generating plumage coloration, studies to date have suggested that a large proportion of segregating trait variation is due to variation in a small number of genetic loci. Collectively, these studies suggest an important role of divergent selection on signalling traits in population differentiation (Wagner *et al.* 2012; Poelstra *et al.* 2014), which might play a particularly important role during the earliest stages of speciation (Kraaijeveld *et al.* 2010). Finer-scale genomic analyses in a broader geographic context will enable us to test whether phenotypic differentiation is a barrier to gene flow when different subspecies of barn swallows are in secondary contact.

Isolation by adaptation, controlling for isolation by distance

Because trait divergence is correlated with geographic distance among populations, we applied two complementary statistical methods to tease apart the relative significance of geographic and phenotypic distances in explaining genome-wide divergence. Both sets of analyses reveal that wing length and, to a lesser extent, throat colour, are most strongly associated with genome-wide divergence, when geographic distance among

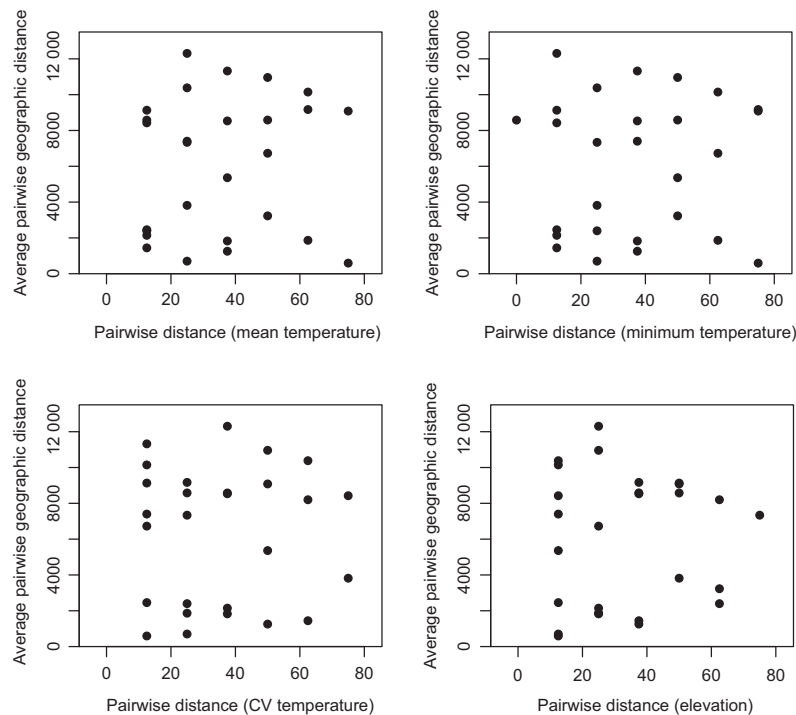


Fig. 7 Average pairwise geographic distance as a function of environmental distance among pairwise comparisons of eight barn swallow populations. Table 6 shows results of Mantel tests between geographic distance and environmental distance; none of these associations are statistically significant.

populations is accounted for statistically (Fig. 8). The variance partitioning model enables us to directly quantify the influence of correlations among geographic distance, wing length and throat colour and to partition the contribution of each of these variables towards differences in genome-wide divergence (Fig. 8 and Table 6). Thus, our study reveals an important role of phenotypic divergence and supports a model of IBA for explaining genomic differentiation of geographically isolated populations, bearing in mind that phenotypic divergence is also strongly correlated with geographic distance, and a model of IBD, among populations (Fig. 8).

IBD is one of the most common patterns in population genetic data (e.g. Jenkins *et al.* 2010). Evidence is accumulating to suggest that IBA (which includes models of isolation by environment, or IBE) is also well supported in a variety of empirical systems among closely related populations (e.g. Lee & Mitchell-Olds 2011; Ede-laar *et al.* 2012; Lasky *et al.* 2012; Shafer & Wolf 2013; Morgans *et al.* 2014; Wang & Bradburd 2014). A recent meta-analysis further revealed that the effect of geographic distance or spatial autocorrelation (between phenotypes, environmental variables and geographic distance) is critical to control for in statistical tests of IBA but has rarely been done in previous studies (Shafer & Wolf 2013). Simulation studies reveal that a

failure to account for spatial autocorrelation can lead to biased results (Shafer & Wolf 2013). In our own study, separate analyses of IBA and IBD each revealed significant effects on genome-wide divergence. Analyses that explicitly considered the correlations of phenotypic and geographic distances were critical to separating out the relative significance of each factor. Variance partitioning (Legendre and Fortin 2010) and multiple matrix regression (Wang 2013) are excellent methods for dealing with spatial autocorrelation, analysing the relative significance of IBA and IBD, and are particularly important alternatives to partial Mantel tests (Bradburd *et al.* 2013; Wang 2013; Wang *et al.* 2013), which are subject to false-positive results (Diniz-Filho *et al.* 2013).

Barn swallows and divergent selection

Our results support an important role of IBA in genome-wide divergence. In particular, differences in wing length, the most divergent phenotypic trait among populations, explained a significant amount of variation in among-population genomic divergence; throat colour is also associated with genomic divergence, but to a lesser extent. From these results and our understanding of the function of these traits, we infer an influence of both natural selection and sexual selection in genomic divergence among these eight populations of barn swallows.

Table 7 Quantitative tests of variance partitioning (Fig. 7): RDA analyses of the effects of selection and geographic distance on genome-wide divergence. WING = pairwise differences in wing length, COLOUR = pairwise differences in throat colour, DIST = pairwise differences in geographic distance. Note that the matrix title before the symbol '|' indicates the variance due to that matrix that contributed to genome-wide divergence, conditioned upon the matrices listed after the '|' symbol

Model	F	P	Adjusted R-squared
WING, COLOUR, DIST	WING _{1,24} = 78.36 COLOUR _{1,24} = 7.03 DIST _{1,24} = 0.03	<0.001 0.010 0.878	0.73
WING COLOUR + DIST	WING _{1,24} = 44.17	<0.001	0.42
COLOUR WING + DIST	COLOUR _{1,24} = 6.03	0.018	0.05
DIST WING + COLOUR	DIST _{1,24} = 0.03	0.878	0.00
WING + COLOUR DIST	WING _{1,24} = 38.66 COLOUR _{1,24} = 6.03	<0.001 0.021	0.40
DIST + COLOUR WING	DIST _{1,24} = 1.04 COLOUR _{1,24} = 6.03	0.321 0.024	0.05
DIST + WING COLOUR	DIST _{1,24} = 32.15 WING _{1,24} = 44.17	<0.001 <0.001	0.70

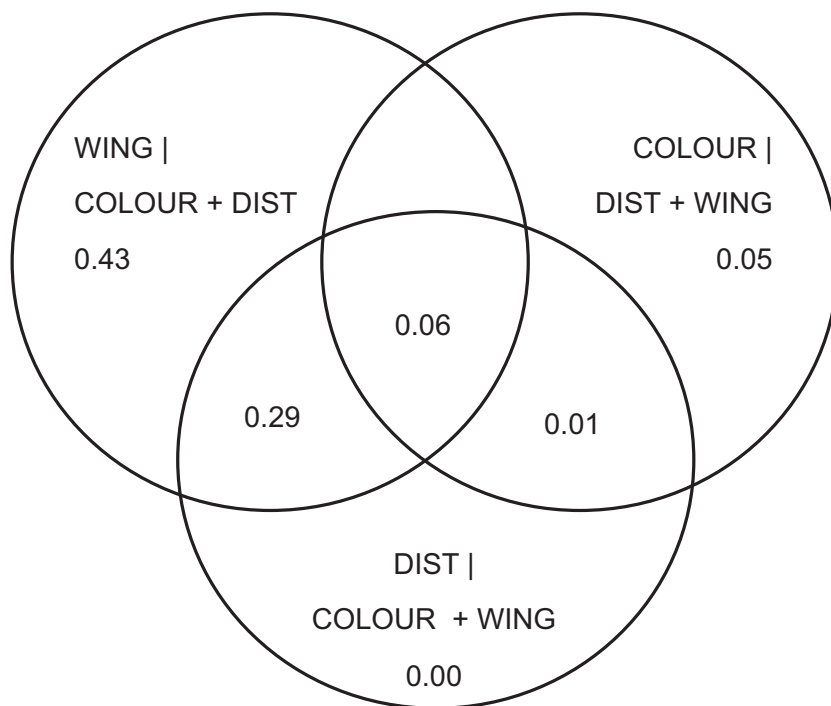


Fig. 8 Variance partitioning plot to represent the extent to which natural selection (WING), sexual selection (COLOUR) and geographic distance (DIST) explain variance in pairwise, genome-wide differences in F_{ST} . Note that the matrix title before the symbol '|' indicates the variance due to that matrix that contributed to genome-wide divergence, conditioned upon the matrices listed after the '|' symbol.

These results are intuitive for several reasons. First, wing length and shape are traits associated with migratory behaviour (Marchetti *et al.* 1995; Lockwood *et al.* 1998). Barn swallows vary in migratory distance, and there is evidence of migratory divides throughout their range (e.g. Irwin & Irwin 2005; von Rönin *et al.* 2016).

All four representatives of the *H.r. rustica* subspecies (UK, Czech Republic, Romania and Turkey) have the longest wing lengths among populations sampled, whereas individuals from distantly related Taiwan (*H.r. gutturalis*) have the shortest. Interestingly, the sole nonmigratory population in our study, Israel, has

intermediate wing lengths that overlap in variation with individuals from Turkey and Romania and is genomically indistinguishable from *H. r. rustica*, based on pairwise F_{ST} . In other migratory bird populations, variation in migratory behaviour can influence both the evolution of wing shape and genomic differentiation (e.g. Ruegg 2007, 2008; Rolshausen *et al.* 2009; Delmore *et al.* 2012, 2015; Delmore & Irwin 2014; von Rönne *et al.* 2016). Thus, variation in migratory routes and behaviours (e.g. timing of arrival to breeding grounds, which may affect mate selection) is proposed as a potentially influential contributor to population divergence (Irwin & Irwin 2005; Rolshausen *et al.* 2009). Further study is required to better understand the role of migratory behaviour in divergence among barn swallow populations, as well as the extent to which these seasonal movement patterns influence trait evolution.

Our analyses of IBA also revealed that population-level differences in colour contribute to genomic differentiation. Plumage colour in various ventral regions (throat, breast, vent) varies both within and among closely related populations of barn swallows (Scordato & Safran 2014). Both throat coloration and breast coloration are variable among the populations in this study, but in different ways. For example, males in our North American populations (Colorado, New York) have the darkest breast colour, yet the lightest throat colour compared with other populations. Ventral coloration has been the focus of several correlational and experimental studies in barn swallows. Together, these studies indicate that melanin-based ventral colour varies among populations (Scordato & Safran 2014), is heritable (Hubbard *et al.* 2015) and relates to social mate selection and paternity allocation (Safran & McGraw 2004; Vortman *et al.* 2011). Thus, patterns of ventral colour differentiation are likely under divergent sexual selection, although this hypothesis requires further testing.

Patterns of IBA may be underlain by divergent selection on traits that directly impede gene flow, or through the build up of an association between genome-wide divergence and trait divergence in allopatric populations. Whereas models of IBA have typically been applied to scenarios where gene flow is possible between populations, most of the eight populations in our study are geographically isolated from one another, with the exception of those within continental Europe (Czech Republic and Romania). It is possible that gene flow occurs among populations sampled from western and central Europe and Israel, although a formal analysis is needed to make direct inferences about recent historical or ongoing gene flow in these regions. Nevertheless, divergence in the absence of gene flow is an important aspect of avian diversification (e.g. Price 2008), and thus it is highly relevant to ask questions

about associations between phenotypic and genomic divergence in order to better understand patterns and consequences of trait evolution in isolated yet closely related populations. Still, ongoing studies of IBA in locations where divergent populations come into sympatry will be critical for inferring whether trait divergence that may have evolved in isolation imposes a barrier to gene flow.

Acknowledgements

We thank C. Alex Buerkle for collaboration on all aspects of this project and the Safran, Kane and Flaxman laboratory groups at the University of Colorado for feedback and advice. We thank Matthew Evans for collaboration on barn swallows in the UK. Funding for this project was provided by the National Science Foundation (DEB-CAREER-1149942) to R.J.S. M.R.W. was supported by an NSF graduate research fellowship and P.N. by a Royal Society of London University Research Fellowship. Work in the Czech Republic was funded by the Ministry of Education, Youth and Sports of the Czech Republic (project LH14045). All field and laboratory protocols were compliant with University of Colorado, and Federal animal care and import/export protocols. We are grateful for the excellent reviews provided by Dr. Anna Santure and three anonymous reviewers.

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R.J.S. designed the study, R.J.S., M.R.W, J.K.H, B.R.J., T.A., H.K., Y.V., P.P., S.S. and S.C., collected data in the field, N.C and E.S.C.S. completed the draft assembly of the genome, B.R.J. conducted most of the laboratory work, R.J.S., E.S.C.S., S.M.F. and T.P. analysed the data with input from P.N., R.J.S. wrote the manuscript with input from E.S.C.S., P.N, T.P and N.K. All authors gave final approval for publication.

Data accessibility

Genome assembly: NCBI: SUBID: SUB1553927, BioProject: PRJNA323498, BioSample: SAMN05180083. Genomic, Phenotype and environmental data and protocols: <http://dx.doi.org/10.5061/dryad.17hr9>.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Statistical summary of population genetic structure based on principal component axes one (PC1), and two (PC2) [Panel A], three (PC3), and four (PC4) [Panel B] based on genotype probabilities.