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Genomic variation across two barn swallow hybrid zones reveals traits associated with divergence in sympatry and allopatry

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Abstract

Hybrid zones are geographic regions where isolating barriers between divergent populations are challenged by admixture. Identifying factors that facilitate or inhibit hybridization in sympatry can illuminate the processes that maintain those reproductive barriers. We analysed patterns of hybridization and phenotypic variation across two newly discovered hybrid zones between three subspecies of barn swallow (*Hirundo rustica*). These subspecies differ in ventral coloration and wing length, traits that are targets of sexual and natural selection, respectively, and are associated with genome-wide differentiation in allopatry. We tested the hypothesis that the degree of divergence in these traits is associated with the extent of hybridization in secondary contact. We applied measures of population structure based on >23,000 SNPs to confirm that named subspecies correspond to distinct genomic clusters, and assessed coincidence between geographic clines for ancestry and phenotype. Although gene flow was ongoing across both hybrid zones and pairwise F_{ST} between subspecies was extremely low, we found striking differences in the extent of hybridization. In the more phenotypically differentiated subspecies pair, clines for ancestry, wing length and ventral coloration were steep and coincident, suggestive of strong isolation and, potentially, selection associated with phenotype. In the less phenotypically differentiated pair, gene flow and phenotypic variation occurred over a wide geographic span, indicative of weaker isolation. Traits associated with genome-wide differentiation in allopatry may thus also contribute to isolation in sympatry. We discuss potentially important additional roles for evolutionary history and ecology in shaping variation in the extent hybridization between closely related pairs of subspecies.

KEYWORDS
barn swallow, geographic clines, hybrid zone, population genomics, reproductive isolation, speciation

1 | INTRODUCTION

Species ranges expand and contract over time, often bringing isolated populations into secondary contact. When these divergent populations regain sympatry, several outcomes are possible. First, members of previously isolated lineages can hybridize freely (i.e., there is no reproductive isolation between taxa). This results in extensive gene flow; over time, phenotypes and genotypes will

eventually become homogenous (Abbott et al., 2013; Gompert & Buerkle, 2016). Second, individuals from divergent populations can remain completely reproductively isolated (no gene flow, Coyne & Orr, 2004). Both genomes and phenotypes remain differentiated in this scenario. Third, the two divergent groups can exhibit a moderate amount of interbreeding (incomplete reproductive isolation) and form a hybrid zone (Barton & Hewitt, 1985). This third scenario is most relevant for understanding the evolutionary and ecological processes that contribute to reproductive isolation, because reproductive barriers that formed in allopatry are challenged by hybridization in sympatry (reviewed in Harrison & Larson, 2016; Payseur & Rieseberg, 2016). In cases of incomplete reproductive isolation, identifying associations between phenotypic and genetic differentiation across a hybrid zone can provide insights into the factors that may facilitate or impede gene flow among divergent populations.

Classic hybrid zone models predict that genotypes will vary clinally across a geographic region of contact. Clines can be maintained by several different processes, including divergent selection along an ecological gradient (Endler, 1977), superiority of hybrids within the region of contact (Moore, 1977; Moore & Price, 1993) or a balance between migration of parental individuals into, and selection against hybrids within, the contact zone ("tension zone model," Barton & Hewitt, 1985). In both ecological gradients and tension zones, steep clines are interpreted to indicate strong selection and reduced gene flow, whereas wide clines suggest weaker selection and greater gene flow (Barton & Gale, 1993; Barton & Hewitt, 1985; Gompert, Parchman, & Buerkle, 2012; Larson, White, Ross, & Harrison, 2014). Particularly in cases of recent secondary contact, phenotypic clines can mirror genotypic clines. Traits that exhibit steep clines are therefore often inferred to be under selection and involved in reproductive isolation, with the strength of selection related to cline width (Alcaide et al., 2014; Brumfield, Jernigan, McDonald, & Braun, 2001; Gay, Crochet, Bell, & Lenormand, 2008; Jiggins & Mallet, 2000). This interpretation is bolstered by observations that traits exhibiting steep clines across hybrid zones are often involved in local adaptation (e.g., Culumber, Shepard, Coleman, Rosenthal, & Tobler, 2012; Culumber et al., 2011; Mullen & Hoekstra, 2008; Taylor et al., 2014; Walsh, Rowe, Olsen, Shriver, & Kovach, 2016) and that individuals with intermediate phenotypes are frequently maladapted to their environments (e.g., Delmore & Irwin, 2014; Hanson, Moore, Taylor, Barrett, & Hendry, 2016; Huber, De Leon, Hendry, Bermingham, & Podos, 2007), are unattractive as mates (e.g., Naisbit, Jiggins, & Mallet, 2001; Rundle, Nagel, Boughman, & Schluter, 2000; Saether et al., 2007) or both (e.g., Jiggins, Estrada, & Rodrigues, 2004). Furthermore, a particular trait is more likely to contribute to reproductive isolation if its cline is steeper than the average ancestry cline, as this suggests that the loci associated with that trait introgress across the hybrid zone at lower rates than the genome-wide average (Baldassarre, White, Karubian, & Webster, 2014; Gay et al., 2008; Walsh, Shriver, Olsen, & Kovach, 2016; Wu, 2001). Comparing the location and width of phenotypic and genotypic clines is thus informative about the roles of different traits in contributing to isolating barriers.

Genome scans for differentiated "outlier" loci can further identify genomic regions with lower rates of introgression and clarify the architecture of reproductive isolation. For example, many differentiated loci spread throughout the genome suggest isolation may involve many genes (Parchman et al., 2013; Seehausen et al., 2014). However, patterns of variation in outliers must be interpreted with caution, as different evolutionary and intragenomic processes (e.g., variation in selection, linkage disequilibrium and recombination rates) and different contexts of speciation (e.g., with or without gene flow) can result in similar patterns of genome-wide divergence and heterogeneous differentiation (e.g., Butlin 2005; Cruickshank & Hahn, 2014; Lindtke & Buerkle, 2015; Nachman & Payseur, 2012; Noor & Bennett, 2009). Interpretation of both cline analyses and outlier scans is therefore facilitated in recently diverged systems for which information on biogeographic and demographic history is available (Payseur & Rieseberg, 2016). One such system is the barn swallow (*Hirundo rustica*) species complex. We take advantage of this lineage to examine patterns of hybridization across two contact zones between three barn swallow subspecies. We specifically test the hypothesis that divergence in traits predictive of genome-wide differentiation among allopatric populations is also associated with the extent of hybridization in secondary contact.

Barn swallows comprise six morphologically variable subspecies that breed throughout the Northern Hemisphere and are divided into two mitochondrial DNA clades (Figure 1). The African, Middle Eastern and European subspecies form one clade, and the Asian, North American and Siberian subspecies a second (Zink, Pavlova, Rohwer, & Drovetski, 2006). Analysis of both mitochondrial and nuclear loci shows that the North American subspecies is derived from a trans-Beringian colonization event by the ancestors of Asian *gutturalis* approximately ~25,000 years ago (Zink et al., 2006; Dor, Safran, Sheldon, Winkler, & Lovette, 2010; Figure 1). The Siberian subspecies, *H. r. tytleri*, is believed to have arisen from a post-Pleistocene recolonization event, wherein North American birds back-colonized central Siberia (~10,000 years ago, Zink et al., 2006; Dor et al., 2010). This has resulted in a unique extant distribution, wherein the colonizing *tytleri* subspecies now shares range boundaries in Siberia with European *rustica* to the west (to which it is comparatively distantly related) and Asian *gutturalis* to the east (to which it is more closely related). Despite recent divergence, there is substantial phenotypic variation among subspecies: European *rustica* is large-bodied with white ventral coloration, Asian *gutturalis* is small with white- to-tan ventral colour, and Siberian *tytleri* has dark brown ventral colour and intermediate body size (Scordato & Safran, 2014; Figures 1 and 2a).

A recent study of four of the six barn swallow subspecies showed that wing length and ventral colour are associated with genome-wide differentiation when controlling for geographic distance between populations (Safran, Scordato, et al., 2016). These results suggest a role for morphological divergence in driving or maintaining population genetic differentiation across the species complex; however, this study examined only allopatric populations. The overarching goal of the current study was to determine

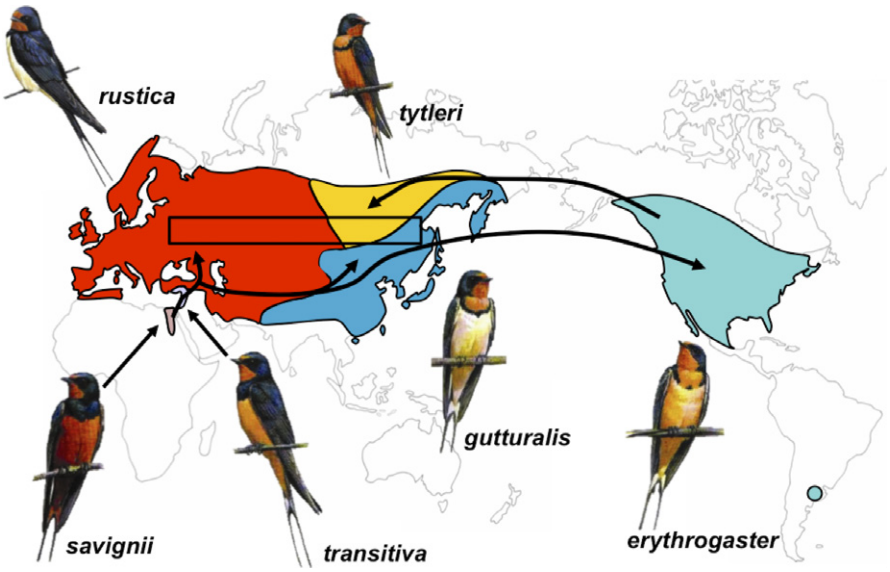

FIGURE 1 Range map of the barn swallow species complex. Cartoons highlight differences in plumage coloration between subspecies. Arrows show hypothesized biogeographic history, with an African origin for the group. Rectangle shows the sampling area of this study. Red = *rustica*; gold = *tytleri*; blue = *gutturalis*. Cartoons reprinted with permission from artist Hilary Burn [Colour figure can be viewed at wileyonlinelibrary.com]

whether greater differences in ventral coloration and/or wing length are also associated with reduced hybridization in sympatry, which would further support a role for these traits in contributing to the evolution and maintenance of reproductive isolation. To address this question, we sampled intensively along a west-to-east transect, bisecting the breeding ranges of *rustica*, *tytleri* and *gutturalis* as they replace each other longitudinally across Russia (Figures 1 and 2a). Our specific goals were as follows: (i) to determine whether the three named subspecies correspond to distinct genomic clusters, and assess evidence for ongoing hybridization between clusters; (ii) to determine whether divergence in traits correlated with differentiation in allopatry is associated with the extent of hybridization in sympatry; (iii) to compare the genomic architecture of divergence in the two contact zones by identifying differentiated loci in genomic regions potentially subject to spatially heterogeneous selection and assessing the distribution of these loci throughout the genome; and (iv) to determine whether differentiated loci are associated with divergent morphological traits. We infer stronger reproductive isolation and reduced gene flow in the presence of few hybrid individuals (both F1 and backcross) and weaker isolation and more gene flow in regions with more hybrids. If phenotypic differences are associated with reproductive isolation or reductions in hybridization, we expect steeper morphological and genomic ancestry clines between the more phenotypically differentiated populations, and wider clines for both morphological traits and genomic ancestry between phenotypically similar populations.

2 | METHODS

2.1 | Sampling

Barn swallows are long-distance migrants that breed throughout the Palaearctic and overwinter in sub-Saharan Africa, southern Asia and South America. They nest exclusively in human structures situated near open landscapes, and their distribution is therefore patchy rather than continuous, with villages containing nest sites separated by tracts of unsuitable nesting habitat. During the summer (May–July 2013), we sampled an 8,400 km west-to-east transect through the Russian breeding ranges of *rustica*, *tytleri* and *gutturalis* (Figures 1 and 2a). For sites within the putative parental subspecies ranges, we sampled ~30 birds per site at intervals of several hundred kilometres. In the contact zones, we sampled as densely as possible, typically at 5- to 50-km intervals. Locations of contact zones in the field were inferred from differences in ventral coloration (Figure 2). We sampled 538 birds; see Table S1 for locations and sample sizes.

Within villages, we captured swallows in mist nets and gave each bird an individually numbered aluminium ring (Russian Bird Ringing Center). We collected a small (25–50 μl) blood sample via brachial venipuncture and stored samples in lysis buffer treated with 2% SDS. Standardized photographs, mass (g), wing length (nearest mm), tail length (nearest mm), tarsus length (nearest 0.1 mm) and bill dimensions (nearest 0.1 mm) were collected from each bird. We measured each trait three times and averaged the measurements. We also collected 10–15 feathers from each of four body regions (throat, breast, belly and vent) for quantification of ventral colour. Ventral colour of barn swallows does not reflect in the UV spectrum (Safran & McGraw, 2004). We therefore used a UV-VIS spectrometer to objectively measure feather colour along three axes of the visual spectrum: hue, chroma and brightness (Safran, McGraw, Wilkins, Hubbard, & Marling, 2010); see supporting information for details of colour analysis.

2.2 | Genotyping by sequencing and SNP calling

Genomic DNA was extracted from blood samples using DNeasy blood and tissue kits (Qiagen). Genotyping-by-sequencing (GBS) libraries for Illumina sequencing were prepared following Parchman et al. (2012) using the restriction enzymes Mse1 and EcoRI. A unique barcode was

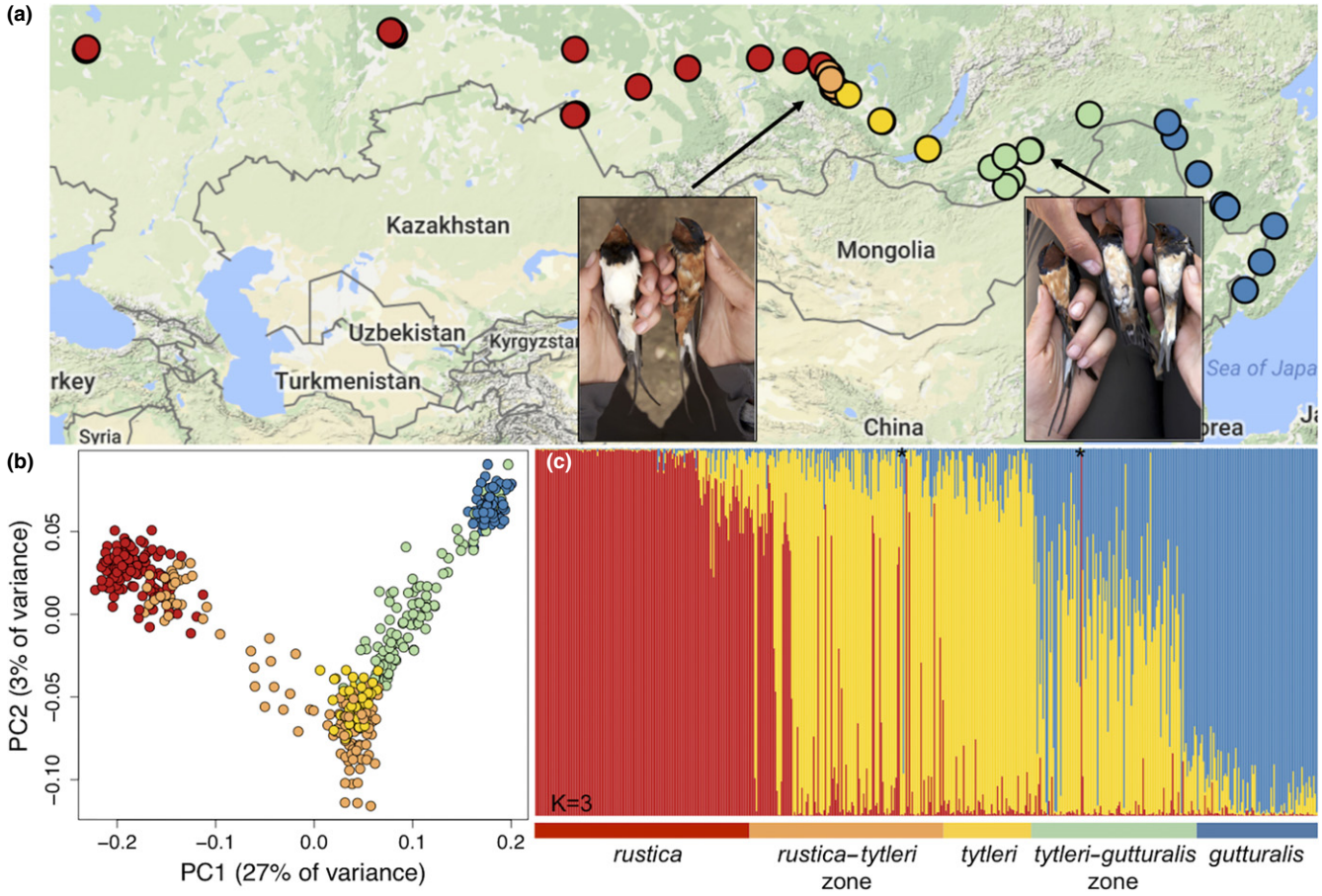

FIGURE 2 Sampling transect and population structure analysis of the three barn swallow subspecies. (a) Sampling locations, with inset photographs of representative *rustica* and *tytleri* individuals (left photograph) and *tytleri*, hybrid and *gutturalis* individuals (right photograph). (b) PCA of the genome-wide covariance matrix. Points are coloured by geographic origin of each sample. Three clusters correspond to the three subspecies, while admixed individuals fall between the three clusters. (c) $K = 3$ FASTSTRUCTURE plot shows three clusters corresponding to the three subspecies, as well as individuals with intermediate assignment probabilities. Individuals are ordered according to sampling longitude, west (left) to east (right). Bars below the plot indicate geographic region of each sample. Black stars denote one *gutturalis* individual captured in the *rustica*–*tytleri* contact zone and one *rustica* captured in the *tytleri*–*gutturalis* zone. Colour codes for geographic regions are as follows: red = *rustica*; orange = *rustica*–*tytleri* hybrid zone; gold = *tytleri*; green = *tytleri*–*gutturalis* hybrid zone; blue = *gutturalis* [Colour figure can be viewed at wileyonlinelibrary.com]

ligated to the fragment libraries from each individual prior to PCR. Libraries were size-selected for a 350- to 400-bp region and sequencing of 100-bp single-end reads was performed on an Illumina HiSeq 2500 at the University of Texas, Austin Genomic Sequencing and Analysis Facility. We ran each of the 538 samples on four replicate lanes. We filtered contaminant sequences, trimmed barcodes, removed low-coverage individuals ($n = 5$), aligned reads to the draft of the barn swallow genome and identified single nucleotide polymorphisms (SNPs) as described in Safran, Scordato, et al. (2016). We required a median read depth of 7 reads at a locus, and 80% of individuals to have data at that locus, for it to be included in further analyses. We used a minor allele frequency cut-off of 5%, as this subsets the data to those loci most relevant for population-level comparisons (Gompert, Lucas, et al., 2012). We also included only one SNP per 100 bp to remove tightly linked loci, resulting in a data set of 23,251 SNPs derived from 533 individuals. See supporting information for additional sequencing and genotyping details.

2.3 | Analysis

2.3.1 | Population structure: do named subspecies correspond to distinct genomic clusters?

To determine whether named subspecies correspond to distinct genetic clusters, we ran a principal components analysis (PCA) on the mean-centred genetic covariance matrix of all individuals in the data set ($n = 533$) using the R function PRCOMP. We further examined patterns of genomic clustering using the variational Bayesian approach implemented in the program FASTSTRUCTURE (Raj, Stephens, & Pritchard, 2014). We ran the model with a flat ("simple") prior for values of K from 1 to 15, with 1,000,000 iterations of each K and 10 reps per K. We used the chooseK.py script to choose the best value of K, which for FASTSTRUCTURE is proposed to be the minimum number of model components that explain 99.99% of the admixture in the sample (Raj et al., 2014). The FASTSTRUCTURE results consistently

showed $K = 3$ to be the most likely number of populations (Figures 2 and S1). Both FASTSTRUCTURE and PCA found three distinct clusters of individuals corresponding geographically to the three named subspecies, as well as individuals with intermediate genotypes that we interpret to be hybrids.

2.3.2 | Assignment of individuals to hybrid classes: is there ongoing gene flow between subspecies?

Although there were individuals with intermediate ancestry in each hybrid zone (Figure 2), analyses of ancestry proportions cannot differentiate extant from historic admixture. To determine whether gene flow is ongoing (presence of F1 and backcross individuals) or historic (no F1 hybrids, only later-generation backcrosses), we assigned individuals in each of the two contact zones to hybrid classes. First, we conservatively designated birds with $q > 0.98$ in the $K = 3$ FASTSTRUCTURE model as parental individuals for each subspecies. We identified ancestry-informative markers by calculating Weir and Cockerhams's F_{ST} for each SNP between pairs of subspecies (*rustica–tytleri* and *tytleri–gutturalis*) in the R package HIERFSTAT (Goudet, 2005). There were no diagnostic loci ($F_{ST} = 1$) segregating between either pair of subspecies, so we defined ancestry-informative markers as those loci with $F_{ST} > 0.6$ between a subspecies pair.

We used the R package INTROGRESS (Gompert & Buerkle, 2009) to calculate maximum-likelihood estimates of hybrid index (i.e., the proportion of alleles inherited from each parent) for each individual across informative loci. This method does not require fixed differences between parental types and makes few assumptions about marker neutrality and linkage (Walsh, Shriver, et al., 2016). We then calculated each individual's average heterozygosity across informative loci. To broadly assess evidence for ongoing interbreeding and gene flow, we then compared each individual's hybrid index to its average heterozygosity (c.f. Milne & Abbott, 2008; Hamilton, Lexer, & Aitken, 2013; Larson et al., 2014; Bouchemousse, Liautard-Haag, Bierne, & Viard, 2016; Walsh, Shriver, et al., 2016). This method differentiates F1 hybrids from later-generation hybrids and backcrosses, as F1 individuals have high average heterozygosity at differentiated loci (expectation of heterozygosity = 1 if loci are fixed between parentals), whereas later-generation hybrids and backcrosses will have lower average heterozygosities. Assignment to hybrid classes is inexact when using nondiagnostic markers with missing data, and is particularly difficult for later-generation hybrids (Buerkle, 2005). We therefore broadly grouped individuals following Milne and Abbott (2008) and Larson et al. (2014). Individuals with hybrid indices ranging from 0.02 to 0.25 or 0.75 to 0.98 were considered backcrosses to one or the other parental type. Individuals with hybrid indices >0.25 and <0.75 and heterozygosity ≥ 0.80 were classified as F1 hybrids. Individuals with these intermediate hybrid indices but lower heterozygosity (<0.80) were considered later-generation hybrids. Although these classifications are broad, they are suitable for our goal of determining whether there is ongoing gene flow (both F1 hybrids and backcrosses) in the two contact zones.

2.3.3 | Phenotypic clustering: is phenotypic variation associated with genomic differentiation?

We used linear discriminant analysis (LDA) and PCA to test correspondence between phenotype and genotype and determine whether greater phenotypic differences are associated with reduced hybridization. First, we trained a linear discriminant model on the phenotypes of parental individuals, defined as birds with $q > 0.98$. We ran the training model using 50% of the parental individuals, and then asked the model to classify the remaining 50% to a cluster based on seven body size variables and 12 colour variables. Assignment error rates were calculated based on 1,000 model repetitions. Next, to determine which traits were diagnostic of subspecies assignment, we ran two PCAs: one on colour traits and one on body size traits. We then repeated the training model procedure using the subset of traits that loaded most highly on the first two PCs in each group ($n = 6$ traits), and examined cluster assignment probabilities.

2.3.4 | Geographic cline analysis: does the geographic extent of hybridization covary with phenotype?

We used geographic cline analysis to describe patterns of phenotypic and genomic variation across the hybrid zones and assess concordance between clines in phenotypic traits and ancestry. We fit sigmoidal geographic clines (Szymura & Barton, 1986, 1991) to the traits that best predicted cluster membership in the PCA and LDA models (bill depth, breast chroma, breast brightness, throat chroma, throat brightness and wing length). We also fit clines to the ancestry proportions (q values) for each contact zone from the $K = 3$ FASTSTRUCTURE model. In the *rustica–tytleri* contact zone, we used the probability that an individual was assigned to the *rustica* cluster as its measure of ancestry; thus, parental *rustica* had $q = 1$ and parental *tytleri* had $q = 0$ (Fig. S1). In the *tytleri–gutturalis* hybrid zone, we fit a cline to the probability of assignment to the *gutturalis* cluster; thus, parental *tytleri* had $q = 0$ and parental *gutturalis* had $q = 1$ (Fig. S1). We excluded any individual that had $q > 0.10$ to a third cluster ($n = 4$); these are individuals with poor assignment probabilities or evidence of three-way hybrid ancestry. This approach allowed us to examine (i) the width of clines for each trait and ancestry proportion, and (ii) concordance between phenotypic and genotypic clines.

We fit cline models using the R package HZAR (Derryberry, Derryberry, Maley, & Brumfield, 2014) by first calculating the mean and variance for each trait and ancestry proportion in each population, and then using cline equations (Szymura & Barton, 1986, 1991) to describe changes in frequency of phenotypes and ancestry along our sampling transects. Because sampling was linear from west to east, we calculated distances (km) between populations using the Haversine great circle distance, with zero fixed in Moscow (the westernmost sampling point). We fit 10 different cline models for each trait in each hybrid zone. Each model estimated the centre (c, km from Moscow) and width (w, 1/maximum slope) of the cline. The centre of the cline is the location where the direction of selection acting on

a trait switches, and the cline width is reflective of overall strength of selection (Barton & Hewitt, 1985). In the first set of models, we fit five different exponential tails (none, both tails, right, left and mirrored) that indicate the distance from the cline centre to the tail (δ) and the tail slope (τ), and estimated the mean and variance as free parameters. In the second set of models, we fit the same tails, but fixed the mean and variance based on trait values from the terminal populations in each parental pair. In all models, we restricted the MCMC space to the geographic distance sampled $\pm$ 100 km to increase the efficiency of the algorithm. We initialized each model (10^5 generations of burn-in and 10^6 chains) and then ran three MCMC chains (chain length = 10^6), checking for convergence of each chain. These three independent chains were concatenated for each model, model fits were compared using AICc (corrected for small sample sizes, Cavanaugh, 1997), and the maximum-likelihood parameters were extracted for the best-fitting model. We assessed concordance between clines using $\pm$ 2-log-likelihood confidence intervals from the best cline model for each trait. We considered cline centres with nonoverlapping confidence intervals to occur in different geographic locations. We applied neutral diffusion equations (Barton & Gale, 1993; Endler, 1977) to determine whether clines were narrower than expected under a model with no selection or reproductive isolation.

2.3.5 | Identification of F_{ST} outlier loci: what is the genomic architecture of differentiation?

To identify genomic regions that may be associated with population differentiation, we scanned for outlier loci. Outlier scans may generate false positives if assumptions about demographic history and range expansions are violated (Lotterhos & Whitlock, 2015). We therefore used two robust methods of outlier detection: outFLANK, an F_{ST}-based approach that has a low rate of false positives for two-refuge demographic histories (Whitlock & Lotterhos, 2015), and PCADAPT (Luu, Bazin, & Blum, 2016), a principal components-based method, which has similarly low rates of false-positive detection and does not require a priori population assignment. OUTFLANK builds on the Lewontin and Krakauer (1973) method, but accounts for nonindependence among sampled populations by generating an F_{ST} distribution from many loci and then fitting a χ^2 model to the centre of this distribution. This produces a null distribution that excludes loci potentially under diversifying (high F_{ST}) or balancing (low F_{ST}) selection. The null distribution is used to test for the outlier loci in the whole data set. The PCADAPT method requires the user to choose K principal components, typically based on inspection of a scree plot, in which K is the number of PCs with eigenvalues that depart from a straight line (Luu et al., 2016). The program then computes a test statistic based on Mahalanobis distance and controls for inflation of test statistics and false discovery rate.

To differentiate loci that may reside in or near genomic regions contributing to reproductive isolation from loci that may be divergent as a result of other processes (and thus less relevant to reproductive isolation), we ran the outlier methods on different subsets of our data. We grouped individuals into two categories for each pair of subspecies: parental individuals in sympatry (defined as within 50 km of each other) and parental individuals in allopatry (defined as >500 km apart). We then compared the number and identity of outlier loci between groups, as well as their distribution throughout the genome.

2.3.6 | Association mapping: are differentiated loci associated with divergent traits?

We tested for associations between phenotypic traits and individual loci using the mixed linear effects model implemented in TASSEL (Bradbury et al., 2007). We used TASSEL's kinship function to generate a relatedness matrix among all individuals. We included the relatedness matrix and ancestry proportion as covariates in the model to control for population structure. We ran association tests separately for each of the two hybrid zones for the same six traits used in the geographic cline analysis. *p*-values were adjusted for false discovery rate using *fdrtool* (Strimmer, 2008). We compared the identity of SNPs significantly associated with morphological traits to SNPs identified in the outlier analyses.

3 | RESULTS

3.1 | Population structure: do named subspecies correspond to distinct genomic clusters?

Average genome-wide F_{ST} calculated in HIERFSTAT was 0.018 between *rustica* and *tytleri*, 0.017 between *tytleri* and *gutturalis* and 0.026 between allopatric *rustica* and *gutturalis*. These extremely low pairwise F_{ST} values are consistent with previously observed shallow differentiation in the barn swallow complex (Safran, Scordato, et al., 2016). Nonetheless, PCA and FASTSTRUCTURE identified three distinct population clusters corresponding to the three phenotypic groups observed in the field (Figure 2). The first PC axis explained 27% of the genomic covariance and separated the three subspecies (Figure 2b). Likewise, clusters in the $K = 3$ FASTSTRUCTURE model corresponded to the geographic regions of *rustica*, *tytleri* and *gutturalis* (Figure 2c). Both contact zones contained sympatric parental individuals, indicating the continued opportunity for parental forms to interbreed, a prerequisite for ongoing hybridization.

PCA and FASTSTRUCTURE also identified individuals with admixed genotypes between the *rustica*–*tytleri* and *tytleri*–*gutturalis* subspecies pairs. Although we found no evidence for hybrids between allopatric *rustica* and *gutturalis* (Figures 1 and 2), we did capture one *rustica* individual in the *tytleri*–*gutturalis* contact zone, and one *gutturalis* in the *rustica*–*tytleri* zone (asterisks on Figure 2c). Moreover, although we sampled the entire longitudinal range of *tytleri* and identified 61 individuals with >0.98 assignment probability to the *tytleri* cluster, every site within this range also contained individuals that were up to 20% admixed (Fig. S2). Gene flow from neighbouring subspecies therefore occurs throughout the *tytleri* range and is possible between the two allopatric forms (*rustica* and *gutturalis*) through occasional migration events or via backcrossing through the range of *tytleri*.

3.2 | Assignment to hybrid classes: is there ongoing gene flow between subspecies?

Consistent with low genome-wide differentiation, we identified few loci that were informative for assigning hybrid classes: there were 46 loci with $F_{ST} > 0.6$ between parental *rustica* and *tytleri*, and six loci between *tytleri* and *gutturalis* in our data set of 23,251 SNPs (Fig. S3). Comparison of individual hybrid indices and average heterozygosity using these differentiated loci revealed F1s, later-generation hybrids and backcrossed individuals in both contact zones, confirming ongoing gene flow between both subspecies pairs (Figure 3). However, there were qualitative differences in hybrid composition among the two zones: there were proportionally many more backcrossed individuals than early-generation hybrids in the *rustica*–*tytleri* zone (Figure 3a), whereas F1s and later-generation hybrids comprised >50% of admixed individuals in the *tytleri*–*gutturalis* zone (Figure 3b). These results are consistent with population clustering (Figures 2, S2), and show more hybridization, and consequently weaker reproductive isolation, between *tytleri* and *gutturalis* than between *rustica* and *tytleri*.

3.3 | Phenotype assignment: is phenotypic variation associated with genomic differentiation?

Assignment of individuals with parental genotypes to the correct subspecies cluster based on 19 traits was 98% accurate, indicating clear correspondence between phenotype and genome-wide variation. Most incorrect assignments occurred between *tytleri* and *gutturalis*, consistent with more similar ventral colour between this pair (Figures 1 and 2, Table S2). Cluster assignment using the six traits with the highest PC loadings in PCAs of body size and colour (wing length, breast chroma, breast brightness, throat chroma, throat brightness and bill depth) recovered the three parental clusters with 93% assignment accuracy.

We used the model trained on these six diagnostic traits in parental individuals to classify hybrids into one of the three parental clusters based on phenotype. We then plotted the first two linear discriminants (LDs) from this analysis against the first two principal components (PCs) from the PCA of the genome-wide covariance matrix. This enabled us to identify mismatches between phenotypic cluster assignment and genotype and test the hypothesis that phenotypic differences are associated with genomic differentiation (Figure 4). Genomic PC1 separated individuals of predominantly *rustica* ancestry from *tytleri/gutturalis* individuals in phenotypic (LD1) space, whereas individuals of *tytleri* vs. *gutturalis* ancestry were not well separated (Figure 4a). Phenotypic LD2 also separated *rustica*-like individuals along genomic PC1, and further separated *tytleri* and *gutturalis* (Figure 4c). Genomic PC2, which explains only 3% of genome-wide covariance, did not correspond to phenotypic variation, as evident from the disconnects between cluster assignment and PC score (Figure 4b, d). The subspecies pair that exhibited less hybridization (*rustica* and *tytleri*) was also more phenotypically differentiated when examining both parentals and admixed individuals.

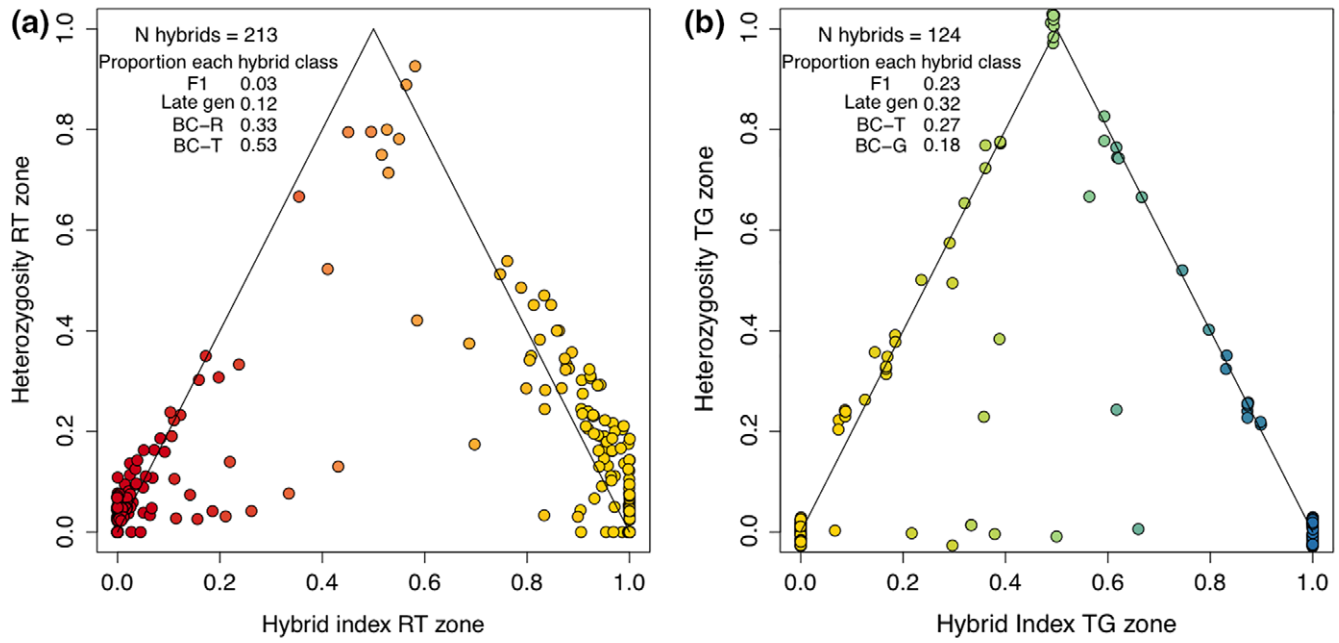

FIGURE 3 Distribution of hybrid classes in each hybrid zone. Plots show genome-wide heterozygosity plotted against hybrid index. There are more backcrosses and fewer early-generation hybrids in the (a) *rustica*–*tytleri* zone compared to the (b) *tytleri*–*gutturalis* zone. Individuals with an intermediate hybrid index and high heterozygosity are considered to be F1 hybrids; those with intermediate hybrid index with lower heterozygosity are later-generation hybrids; and individuals with hybrid index >0.02 and <0.25 or >0.75 and <0.98 are backcrosses. The proportion of hybrid individuals assigned to each hybrid class is given on the plots. BC-R = *rustica* backcross, BC-T = *tytleri* backcross, BC-G = *gutturalis* backcross. Points are coloured by hybrid index using the same scheme as Figure 2 and are jittered slightly for visibility [Colour figure can be viewed at wileyonlinelibrary.com]

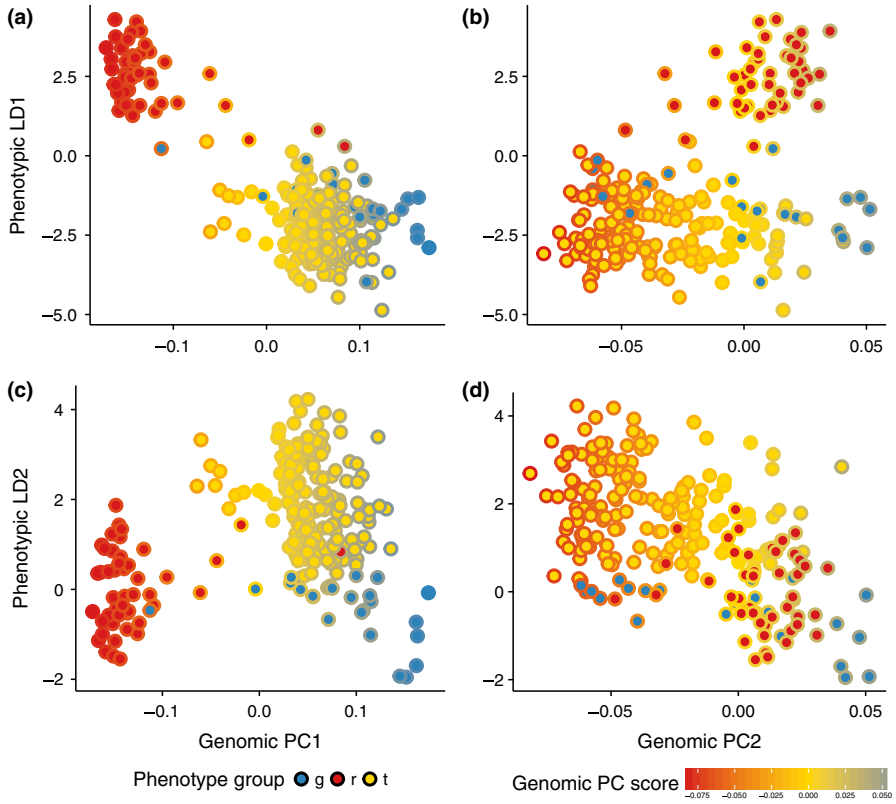

FIGURE 4 Correspondence between phenotypic classification and genomic differentiation. Plots show the first two linear discriminants (LDs) from analysis of phenotypic traits plotted against PC1 and PC2 from the PCA of the genome-wide covariance matrix. The centre colour of points indicates phenotypic cluster assignment (red = *rustica*; gold = *tytleri*; blue = *gutturalis*). The outer border of points is coloured by genomic PC (i.e., the *x*-axis value), using the same colour scheme. Greater mismatch in colour between centre and border of points indicates a mismatch between phenotypic classification and genotype. Note that while predominantly *rustica* individuals are generally classified accurately, individuals with genomic PC1 scores intermediate between *tytleri* and *gutturalis* were usually phenotypically classified as *tytleri* [Colour figure can be viewed at wileyonlinelibrary.com]

The linear discriminant analysis revealed asymmetric phenotypic variation among *tytleri–gutturalis* hybrids. When we subset the data to genomically intermediate individuals (ancestry proportion of 0.4–0.6 *gutturalis*) and examined phenotypic cluster assignment, individuals with the same genomic ancestry were significantly more likely to be classified as *tytleri* than *gutturalis* based on phenotype ($n = 29$, $\chi^2 = 18.6$, d.f. = 2, $p < 0.001$). There were only five individuals with similarly intermediate ancestry in the *rustica–tytleri* contact zone, but three of these were classified as *rustica* and two as *tytleri*. Hybrids between *tytleri* and *gutturalis* thus appear phenotypically similar to *tytleri*.

3.4 | Geographic clines: does the geographic extent of hybridization covary with phenotype?

We found strikingly different patterns of concordance for phenotypic and genomic ancestry clines between hybrid zones. In the *rustica–tytleri* zone, the clines for wing length, breast chroma, breast brightness and throat chroma were concordant with each other and with ancestry (Table 1, Figures 5a and S4). Phenotypic clines were steep and narrow (<105 km), and the best-fit cline models included no exponential tails, indicating no introgression of these traits outside the contact zone. The cline model for ancestry fit two exponential tails, indicating some genomic introgression in both directions outside the contact zone. Nonetheless, this cline was also <100 km wide (Table 1). A model of neutral expansion predicts that the width of a cline in the absence of reproductive isolation should be approximately proportional to the root mean square dispersal distance per generation and the number of generations since secondary contact (Barton & Gale, 1993; Endler, 1977). Estimates of natal dispersal distance for barn swallows are variable and range from 14 km (Paradis, Baillie, Sutherland, & Gregory, 1998) to several hundred kilometres (Møller, 1994; R.J. Safran, unpublished data). Assuming a conservative natal dispersal distance of 14 km and a generation time of one year (Zink et al., 2006), the *rustica–tytleri* hybrid zone would have to be less than one year old to produce the observed 92-km ancestry cline in the absence of reproductive isolation. A more realistic 100 km natal dispersal distance (c.f. Møller, 1994) also yields a hybrid zone age of less than one year.

Bill depth and throat brightness varied little among populations, and cline model fits were poor (Table 1, Fig. S4, Table S2). There was a reasonable cline fit to throat chroma, but also little variation among populations, and the cline was consequently very narrow (<2 km, Table 1, Fig. S3, Table S2). Together, our results

TABLE 1 Best-fit cline models for morphological traits and genomic ancestry in each hybrid zone. Centre and width are given for each cline ±2 log-likelihood credible intervals. Traits with centres that coincide with ancestry are boldfaced. Clines were a poor fit to bill depth and throat brightness in both zones

Contact zone	Trait in model	Tails	Centre (km from Moscow)	Width (km)
Rustica–tytleri	Bill depth	None	947.27 (−30–1498.13)	4.59 (0–2144.1)
	Breast brightness	None	**3655.47** (3639.63–3665.76)	104.9 (83.55–146.62)
	Breast chroma	None	**3647.42** (3635.93–3656.43)	94.3 (69.13–132.25)
	RT ancestry	Both	**3639.86** (3626.62–3646.74)	91.99 (37.66–119.88)
	Throat brightness	NA	3759.45 (3737.54–4303.38)	0.68 (0–42.34)
	Throat chroma	None	**3686.53** (3645.97–3690.74)	1.81 (0.01–6.04)
	Wing length	None	**3644.4** (3608.53–3660.05)	16.98 (0.98–185.76)
Tytleri–gutturalis	Bill depth	None	5364.84 (4759.43–6064.34)	1752.45 (211.81–2999.42)
	Breast brightness	None	5065.66 (4844.73–5362.74)	1364.19 (740.51–2697.23)
	Breast chroma	None	5056.06 (4900.82–5248.08)	953.2 (565.75–1708.4)
	Throat brightness	NA	4000.24 (4000.08–4512.9)	0.26 (0.01–2811.7)
	Throat chroma	None	5904.07 (4059.65–6999.95)	4.76 (0.01–357.34)
	TG ancestry	Right	**4793.43 (4767.62–4815.77)**	432.47 (362.17–533.33)
	Wing length	Left	**4819.95 (4792.69–4835.2)**	140.96 (54.79–330.23)

demonstrate that wing length and ventral coloration are the traits most closely associated with transitions in genomic ancestry between *rustica* and *tytleri* (Figure 5a).

In contrast to the *rustica–tytleri* hybrid zone, the clines for wing length, breast chroma and breast brightness in the *tytleri–gutturalis* zone were very wide, with broad 95% credible intervals due to high phenotypic variance within and between populations (Table 1, Figure 5B). There were no differences in throat chroma, throat brightness or bill depth across this hybrid zone (Table 1, Fig. S5). The centres of the ancestry and wing length clines overlapped and were thus not considered significantly different. Although the *tytleri–gutturalis* ancestry cline was much wider than the *rustica–tytleri* cline (Table 1), indicating weaker reproductive isolation, complete neutral diffusion across this contact zone is unlikely. A 14 km natal dispersal distance and an ancestry cline of 432 km (Table 1) give a fairly young hybrid zone age of ~950 years, whereas the more realistic dispersal distance of 100 km yields a zone age of just 19 years. The cline for wing length was much narrower than the ancestry cline (Table 1), suggesting weak isolation between *tytleri* and *gutturalis* may be associated with variation in body size. By contrast, the cline centres for breast chroma and brightness were displaced approximately 270 km to the east of the ancestry cline, with nonoverlapping credible intervals (Table 1), which may reflect differential introgression of ventral coloration into the *gutturalis* range. This is consistent with the phenotypic clustering analysis, wherein *tytleri–gutturalis* hybrids were phenotypically more similar to parental *tytleri*.

3.5 | Detection of outlier loci: what is the genomic architecture of differentiation?

Analyses in OUTFLANK and PCADAPT identified different numbers of outliers in different subsets of individuals (parental individuals in sympatry vs. allopatry, *rustica–tytleri* vs. *tytleri–gutturalis*, Table S3, Figure 6). The OUTFLANK method, which uses F_{ST} to identify outliers, found more divergent loci between allopatric than sympatric parental individuals. There were 36 loci differentiated only in sympatry between *rustica* and *tytleri* (Table S3); these may be candidates for contributing to reproductive isolation. No loci were differentiated in sympatry between *tytleri* and *gutturalis* (Table S3). Only six loci were identified as outliers in both hybrid zones, suggesting different genomic regions may contribute to divergence between subspecies.

The PCADAPT program requires a user-defined number of clusters (K), which can introduce subjectivity into the analysis. We therefore ran PCADAPT with two different sets of Ks: one set identified by inspecting scree plots (Luu et al., 2016) and a second set using the biologically intuitive $K = 2$ for comparisons between subspecies. K values obtained from scree plots ranged from 1 to 6, with larger values of K almost always identifying more outliers. We therefore conservatively used results from the $K = 2$ analysis. Even with a conservative K, PCADAPT identified more outliers in each comparison than OUTFLANK (Table S3).

There was limited overlap in the loci identified as outliers by both OUTFLANK and PCADAPT (Figure 6). Loci identified as outliers by both methods are more likely to be under selection than outlier loci identified by a single method (Lotterhos & Whitlock, 2015). Among shared outliers, there were more divergent loci in allopatric than in sympatric comparisons, and more divergent loci overall between *rustica* and *tytleri* than between *tytleri* and *gutturalis* (Figure 6).

We were able to place approximately 5,700 loci from each subset of individuals (sympatric and allopatric comparisons in the two hybrid zones) on chromosomes. Inspection of Manhattan plots showed that outlier loci were distributed widely across the genome rather than in colocalized clusters (Figure 7). As is common in birds, many outliers were on the Z chromosome. Relatively few outliers were identified overall; of 23,251 SNPs, the maximum number of

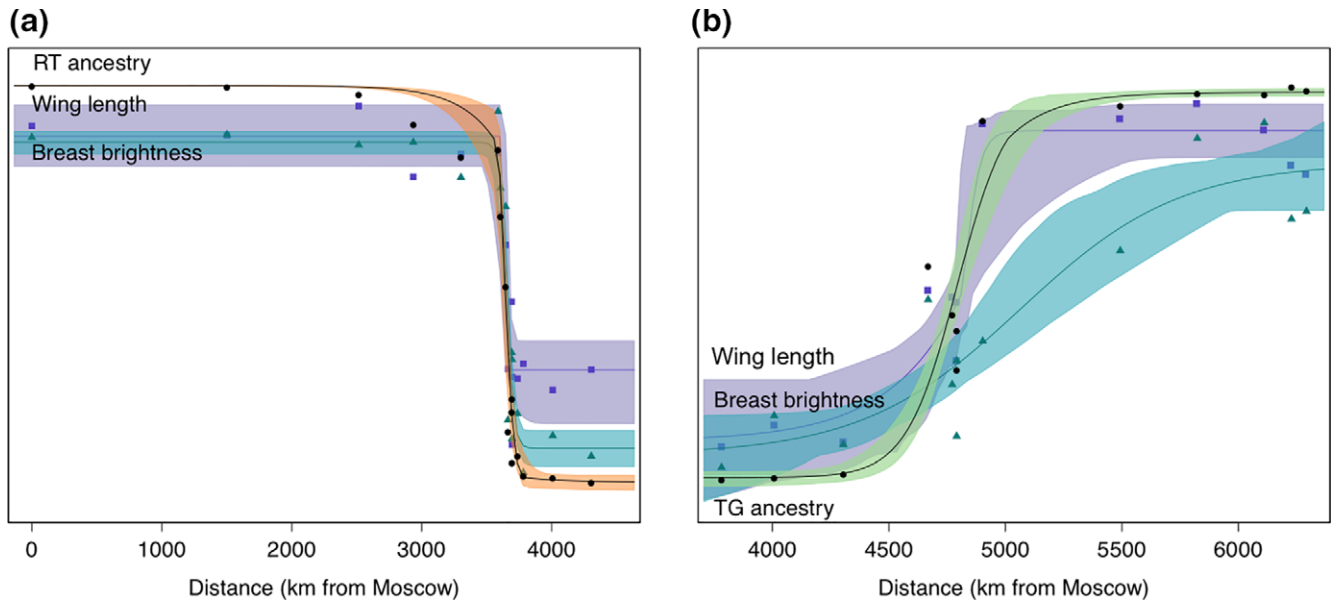

FIGURE 5 Geographic clines for wing length, breast brightness and ancestry. (a) *Rustica–tytleri* hybrid zone (orange cline) and (b) *tytleri–gutturalis* hybrid zone (green cline). Points show population means for each trait plotted against distance from Moscow. Solid lines are maximum-likelihood clines for the best-fit model of each trait. Shaded regions are ±2 log-likelihood credible intervals for each cline. Clines are narrow and coincident in the *rustica–tytleri* zone, but wider with poorer concordance in the *tytleri–gutturalis* zone [Colour figure can be viewed at wileyonlinelibrary.com]

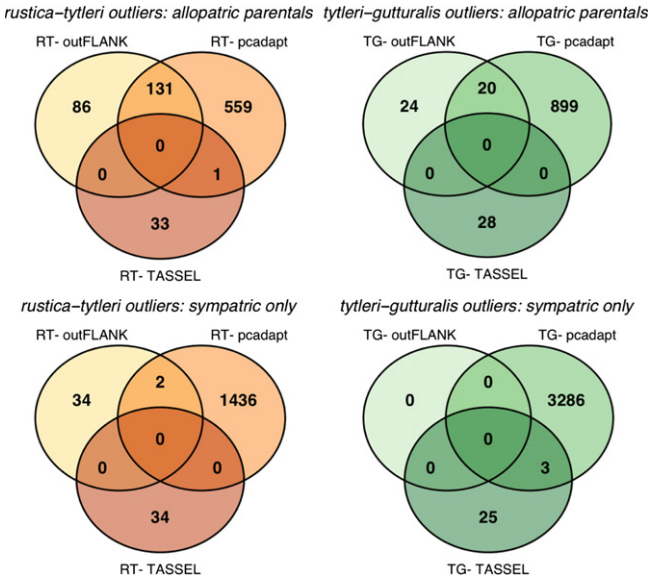

FIGURE 6 Overlap in identity of outlier loci identified by PCADAPT, OUTFLANK and TASSEL for each subset of individuals: allopatric parentals between *rustica–tytleri* and *tytleri–gutturalis* (top panels), and sympatric parentals (bottom panels). OUTFLANK consistently identified fewer outlier loci than PCADAPT. There was almost no overlap between loci significantly associated with any phenotypic trait in TASSEL and those identified as outliers in the genome scans [Colour figure can be viewed at wileyonlinelibrary.com]

outliers identified by both PCADAPT and OUTFLANK was 131, between allopatric *rustica* and *tytleri* (Table S3). These results are consistent with shallow genomic differentiation between all three subspecies.

3.6 | Association mapping: are differentiated loci associated with divergent traits?

We found few genomic regions associated with phenotypic traits. In the *rustica–tytleri* zone, throat brightness and wing length were significantly associated with regions of chromosomes 4A, 7 and 11 (Figure 8). We did not find the same genomic regions associated with these traits in the *tytleri–gutturalis* zone, although there were several regions of slightly elevated differentiation associated with throat brightness (Figure 8). We found no associations between individual loci and ventral coloration, despite this being the most obviously divergent trait between subspecies. The loci associated with divergent traits in TASSEL were also not the same loci identified as outliers by OUTFLANK or PCADAPT (Figure 6). There was little overlap in the chromosomal regions where divergent SNPs were located, with the exception of the Z chromosome (Figs 7 and 8). These results suggest that many loci spread throughout the genome contribute to differentiation between the three barn swallow subspecies.

4 | DISCUSSION

In this study, we examined two contact zones between three barn swallow (*Hirundo rustica*) subspecies that differ in wing length and plumage colour, phenotypic traits previously shown to be associated with genome-wide differentiation in allopatry (Safran, Scordato, et al., 2016; Safran, Vortman, et al., 2016). We asked whether divergence in these traits covaries with the extent of hybridization in sympatry, which could

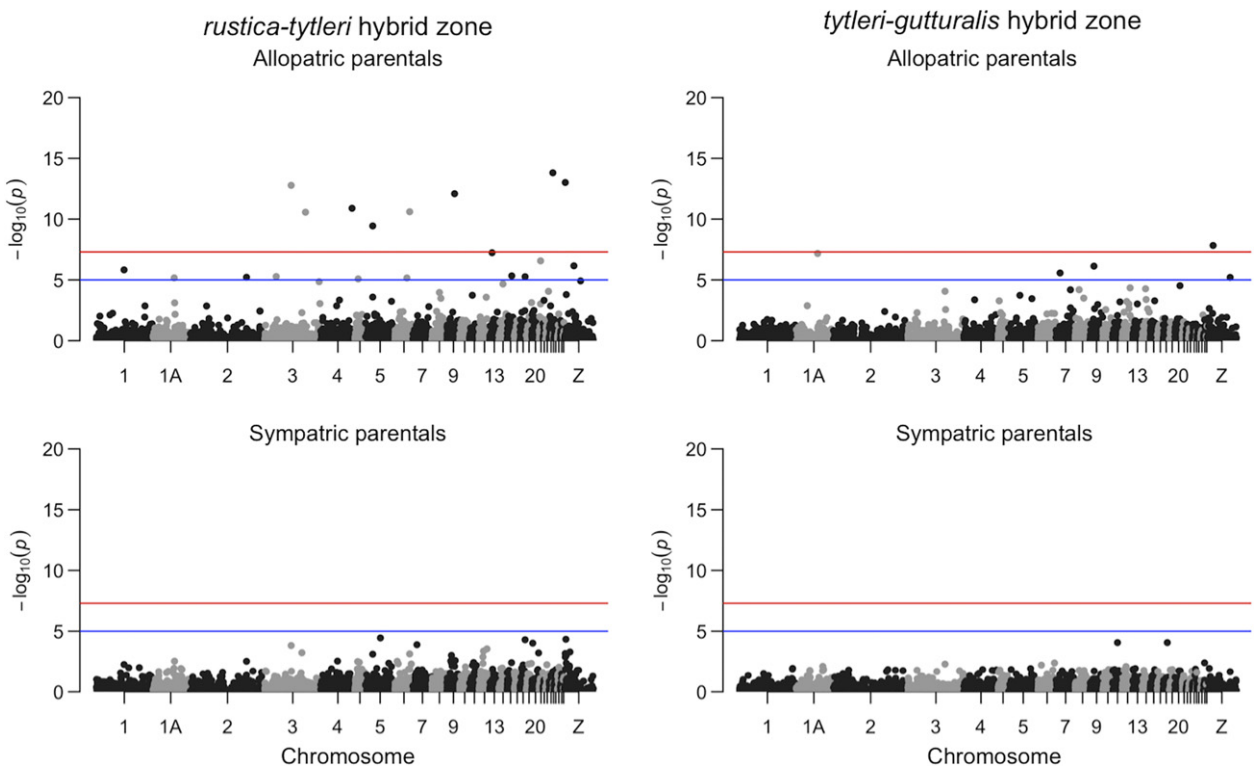

FIGURE 7 Manhattan plots showing chromosome positions of outlier loci identified by OUTFLANK. Plots show *rustica*–*tytleri* comparisons (left) and *tytleri*–*gutturalis* comparisons (right). Top panels show allopatric parental individuals only. Bottom panels are sympatric parentals. Outlier loci are spread throughout the genome in allopatric parental comparisons. There are few loci differentiated only in sympatry. Blue lines indicate the "suggestive" line at $p = -\log10(1e\text{-}05)$, and red lines indicate genome-wide significance at $-\log10(5e\text{-}8)$ [Colour figure can be viewed at wileyonlinelibrary.com]

indicate a role for phenotypic differentiation in the maintenance of subspecies boundaries. Although there was evidence for ongoing gene flow in both hybrid zones, the extent and geographic scale of hybridization differed dramatically, with the more phenotypically differentiated subspecies pair exhibiting less interbreeding. This result is more remarkable given our finding that genome-wide differentiation is both extremely shallow and generally equivalent between the two subspecies pairs. Divergence in wing length and plumage colour may thus indeed contribute to the substantial reduction in hybridization between *rustica* and *tytleri* compared to *tytleri* and *gutturalis*, although we also discuss potential roles for evolutionary history and ecological factors.

4.1 | Population structure: subspecies correspond to genomic clusters despite ongoing gene flow

Analyses of population structure recovered three genomic clusters corresponding to the named barn swallow subspecies, as well as intermediate individuals that could confidently be classified as hybrids. Hybridization occurred over a broad and continuous geographic span in the *tytleri*–*gutturalis* contact zone, but was very limited in the *rustica*–*tytleri* zone, despite denser geographic sampling in this region. We also found proportionally fewer F1s and more backcrosses in the narrow *rustica*–*tytleri* hybrid zone. Although assignment to hybrid classes is imperfect, the presence of F1s and backcrosses supports variable, but ongoing gene flow between parental forms in both hybrid zones. We also found admixture occurring throughout the entire longitudinal range of *tytleri*; that is, there were no populations comprising exclusively pure parental *tytleri*. The weak isolation and extensive gene flow observed between *tytleri* and *gutturalis* may thus eventually erode differentiation between these subspecies to the point that they are indistinguishable.

In animals, relatively few hybrid zones have been identified with clearly extant gene flow (but see, e.g., *Xiphophorous*, Culumber et al., 2011), either because F1s are absent and hybridization is historic (e.g., *Lycaeides*, Gompert et al., 2014), parentals are absent from the contact zone and hybrids breed with each other (e.g., *Mus*, Wang et al., 2011), there is little to no backcrossing of F1s to parentals, indicating strong postzygotic barriers (e.g., *Ficedula*, Kawakami et al., 2014), or "hybrid zones" are actually panmictic populations with variation only at a few loci underlying differentiated phenotypes (e.g., *Vermivora*, Toews et al., 2016). In barn swallows, we find evidence for ongoing gene flow as well as sympatry between parental individuals in both hybrid zones. These patterns of hybridization allow us to infer stronger reproductive isolation between *rustica* and *tytleri* than between *tytleri* and *gutturalis*. The geographic distribution of parentals and hybrids, coupled with recent divergence and ongoing hybridization, makes the barn swallow a powerful system for examining factors that

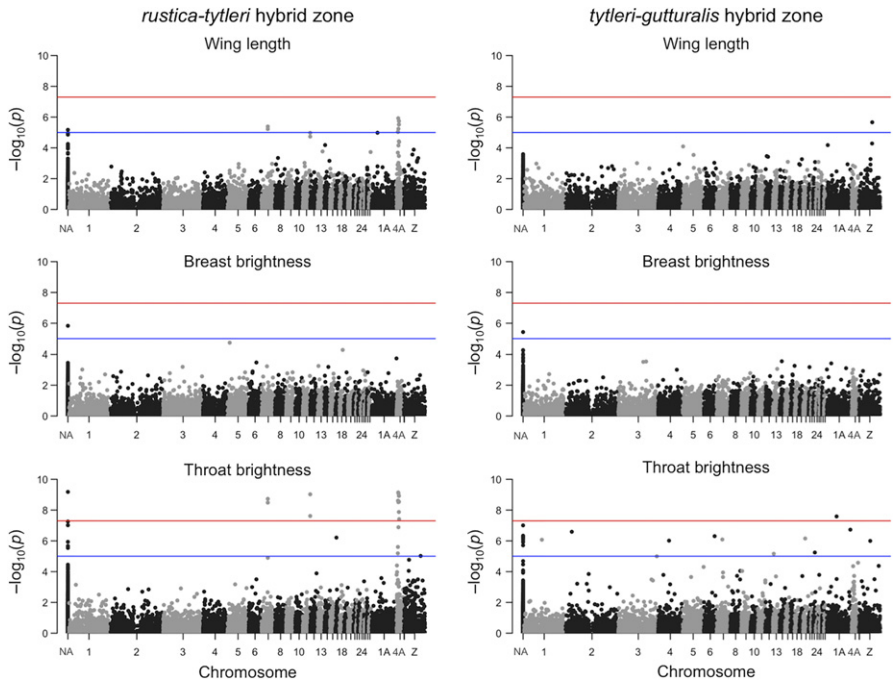

FIGURE 8 Association mapping showing loci associated with morphological traits. Plots show *rustica–tytleri* comparisons (left) and *tytleri–gutturalis* comparisons (right). Note regions of chromosomes 4A, 7 and 11 associated with wing length and throat brightness in the *rustica–tytleri* hybrid zone. Blue lines indicate the "suggestive" line at $p = -\log10(1e\text{-}05)$, and red lines show genome-wide significance at $-\log10(5e\text{-}8)$. NA are loci that could not be placed on chromosomes [Colour figure can be viewed at wileyonlinelibrary.com]

currently contribute to the maintenance or erosion of reproductive barriers.

4.2 | Phenotypic variation: traits associated with differentiation in allopatry correspond to extent of hybridization in sympatry

We used geographic cline and phenotypic clustering analyses to ask how traits associated with genomic differentiation in allopatry—specifically wing length and ventral coloration—covaried with ancestry in sympatry. We found narrow, coincident clines for breast coloration (chroma and brightness), wing length and ancestry in the *rustica–tytleri* contact zone, as well as phenotypically and genomically distinct clusters corresponding to these two subspecies. There was no introgression in either direction for phenotypic clines, and the cline for wing length was narrower than the ancestry cline. These patterns may reflect selection on wing length and breast colour, or on loci linked to these traits (Gay et al., 2008; Gompert, Lucas, Fordyce, Forister, & Nice, 2010; Larson et al., 2014), and we therefore infer a potential role for phenotypic divergence in maintaining isolation in secondary contact, as well as contributing to differentiation between subspecies (Safran, Scordato, et al., 2016). Simulations have shown that sharp phenotypic clines can emerge under models of secondary contact with neutral evolution if migration rates are low and sampling occurs relatively early in the contact history (Gompert & Buerkle, 2016). However, the observed clines are much narrower than would be expected under a model of neutral diffusion, even when considering a range of possible dispersal distances. Steep, concordant clines and the absence of a population density trough in the *rustica–tytleri* contact region thus suggest that zone width and location are likely maintained by selection against hybridization (Abbott et al., 2013; Bierne, Welch, Loire, Bonhomme, & David, 2011).

The genomic ancestry cline in the *tytleri–gutturalis* hybrid zone was much wider than in the *rustica–tytleri* zone, consistent with a broader geographic range of hybridization in this subspecies pair. Nonetheless, cline width was narrower than expected under a neutral diffusion model unless the contact zone is very young (~20 years), and there is thus likely some degree of reproductive isolation between *tytleri* and *gutturalis*. The wing length cline was narrower than the ancestry cline but with a coincident centre, indicating a possible role for variation in body size contributing to this weak isolation. By contrast, the cline centres for breast colour were displaced to the east of the ancestry cline, providing some evidence for introgression of darker plumage colour from *tytleri* into *gutturalis*. This interpretation is further supported by phenotypic clustering, which classified more genomic hybrids as belonging to *tytleri* than to *gutturalis* based on phenotype. Differential introgression of plumage ornaments is relatively common in birds (e.g., Baldassarre et al., 2014; Brumfield et al., 2001; Sardell & Uy, 2016; Stein & Uy, 2006) and often results from directional female preferences for certain colours. In North American barn swallows, from which *tytleri* are derived, females prefer darker males (Safran, Neuman, McGraw, & Lovette, 2005; Safran, Vortman, et al., 2016). It is therefore plausible that dark-coloured *tytleri* males have greater reproductive success than paler *gutturalis* in the contact zone, leading to

introgression of plumage colour loci. However, within-population variance in plumage colour was high in *gutturalis* and clines were broad (~1000 km) with wide confidence intervals. We are therefore cautious about inferring differential introgression from small differences in cline centre confidence intervals. Nonetheless, geographic clines and phenotypic clustering clearly show limited hybridization between *rustica* and *tytleri* associated with differences in wing length and breast colour, whereas these traits are less clearly associated with selection against hybridization in the *tytleri–gutturalis* hybrid zone.

4.3 | Loci underlying differentiation: reproductive isolation likely involves many genes

A major goal of outlier detection is to identify loci that may be under selection and thus contribute to local adaptation and/or reproductive isolation (Haasl & Payseur, 2016). A second goal is to assess the genomic architecture of divergence or the physical locations of divergent loci (Seehausen et al., 2014). However, it is important to consider the geographic context of comparisons when using outliers to make inferences about reproductive isolation. Loci that are differentiated between populations thousands of kilometres apart may not be relevant to isolation in sympatry (Harrison & Larson, 2016). For example, Moscow *rustica* are separated from *tytleri* by >5000 km, and loci that are differentiated only between these populations may be less likely to contribute to reproductive isolation than loci differentiated between sympatric *rustica* and *tytleri*. We therefore examined patterns of genome-wide differentiation in both sympatry and allopatry. We found few loci that were differentiated only in sympatry (none in the case of *tytleri–gutturalis*), and none of these loci were clearly colocalized on chromosomes. This pattern suggests we did not identify obvious genomic "islands" of differentiation that could contribute to reproductive isolation in sympatry (e.g., Feder, Egan, & Nosil, 2012; Flaxman, Feder, & Nosil, 2013). We also found little overlap in outlier loci between subspecies pairs, suggesting that the identity of divergent genomic regions differs between subspecies.

Genome scans can identify potential targets of selection without requiring additional information, such as environment or phenotype (Haasl & Payseur, 2016). However, identifying the loci responsible for phenotypic variation helps clarify the evolutionary and ecological processes that contribute to divergence and reproductive isolation. Finding these loci is facilitated in hybrid zones, where recombination makes it easier to pinpoint genomic regions underlying phenotypes, but more difficult to identify causal variants if there is high linkage disequilibrium (Buerkle & Lexer, 2008). We found regions of chromosomes 7, 11 and, in particular, 4A associated with wing length and throat colour in the *rustica–tytleri* zone. Synteny is highly conserved across birds, and chromosome 4A harbours regions of differentiation marked by low recombination in multiple species of flycatcher (Burri et al., 2015). Conserved differentiation on chromosome 4A across barn swallows and flycatchers coincides with theoretical predictions that loci contributing to differentiation will accumulate in low-recombination regions (Feder et al., 2012; Noor & Bennett, 2009; Ortiz-Barrientos, Engelstädter, & Rieseberg, 2016). In this scenario, it is challenging to determine whether phenotypic differences are causally associated with reductions in hybridization, or whether phenotypic differentiation is a by-product of reduced recombination and limited introgression. We cannot differentiate these alternatives with our current data.

It is also critical to consider marker density when making inferences about genome-wide differentiation and quantitative trait architecture (Wellenreuther & Hansson, 2016). We were able to place ~5,700 SNPs on chromosomes in each hybrid zone. Although this is a large number of markers in the context of analysing population structure, it is almost certainly too few to detect large-effect loci when divergence is shallow. For example, we did not identify differentiated loci associated with ventral coloration, the trait that exhibited the greatest variation between subspecies, which may be a consequence of low marker density. However, failure to identify loci associated with divergent traits could also result from correlations between phenotype and ancestry. Ancestry was included as a covariate in our TASSEL models, which means that a large proportion of phenotypic variance was explained by this term, leaving little remaining variance to be explained by individual loci. Future work with higher marker density may begin to resolve these alternatives. Nonetheless, outlier loci were scattered throughout the genome, which is suggestive of differentiation, and potentially reproductive isolation, involving many genes. These patterns are consistent with the evolutionary history of barn swallows, which most likely involved divergence in allopatry followed by secondary contact, rather than primary divergence with gene flow (Dor et al., 2010; Zink et al., 2006). In such a scenario, divergent loci are expected to be spread throughout the genome rather than clustered in "islands" (Flaxman et al., 2013).

4.4 | Reproductive isolation and speciation in barn swallows

We found clear differences in the extent of hybridization between barn swallow subspecies: hybridization was reduced, and there were more differentiated loci, between the more phenotypically divergent subspecies pair. However, we hesitate to infer a causal relationship between phenotypic differences and reproductive isolation because phenotype is confounded with evolutionary history. Despite indistinguishable genome-wide differentiation in our SNP data (likely a result of admixture following secondary contact in both hybrid zones), the more phenotypically divergent pair (*rustica–tytleri*) is more distantly related based on mtDNA (Dor et al., 2010; Zink et al., 2006). The differences we observed in patterns of hybridization therefore set up testable hypotheses for why the strength of isolation varies between these closely related subspecies. First, assortative mating by phenotype may represent a strong premating barrier, resulting in less interbreeding between the more phenotypically differentiated *rustica–tytleri* pair. Second, postzygotic isolation may be stronger between *rustica* and *tytleri* because greater evolutionary divergence has resulted in genetic incompatibilities that create unfit hybrids. Third, the ages of the contact zones

may differ. It is likely that all three subspecies have recently expanded their ranges as humans have built suitable nest structures throughout Siberia, but western Siberia, where the *rustica–tytleri* zone is located, has a longer history of human settlement compared to the eastern hybrid zone. Hybridization is most frequent in the early stages of contact, when there has not been sufficient time for previously allopatric groups to evolve new isolating barriers (Abbott et al., 2013); this may be the case for *tytleri* and *gutturalis*. Finally, hybridization may be more restricted between *rustica* and *tytleri* due to ecological factors, such as differences in nesting habitat, foraging niche, timing of breeding or migratory route (e.g., Bearhop et al., 2005; Bensch, Grahn, MüLler, Gay, & Åkesson, 2009; Rolshausen, Segelbacher, Hobson, & Schaefer, 2009). Indeed, clines for wing length, which is associated with migratory distance (von Rönn, Shafer, & Wolf, 2016), are steeper than the ancestry clines in both zones. The results of our study clearly show that reproductive isolation differs between subspecies, is likely underlain by many loci, and is at least partially explained by differences in phenotype. Future work will require observational and experimental approaches to disentangle the processes contributing to divergence in this system and shed light on why the strength of isolation varies so dramatically across this recently diverged species complex.

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DATA ACCESSIBILITY

GBS data are available on the NCBI short-read archive and are associated with NCBI BioProject PRJNA323498. Genotype estimates and metadata are available on Dryad at https://doi.org/10.5061/dryad.0g6k1.

AUTHOR CONTRIBUTIONS

E.S.C.S. and R.J.S. designed the study; E.S.C.S., M.R.W., G.S. and A.S.R. collected data in the field; E.S.C.S. analysed the data with input from N.C.K.; E.S.C.S. wrote the manuscript with input from R.J.S. and N.C.K.; all authors signed off on the final manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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