

Global song divergence in barn swallows (*Hirundo rustica*): exploring the roles of genetic, geographical and climatic distance in sympatry and allopatry

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Divergence in acoustic signals plays an important role in the production and maintenance of biodiversity in numerous taxa. In this study, we assess patterns of acoustic divergence in geographically isolated and sympatric subspecies of barn swallows (*Hirundo rustica*), including analyses of whether song differentiation varies with geographical isolation, genetic distance and climatic distance. We provide the first description of geographical variation in song among five of six currently recognized barn swallow subspecies. Temporal traits describing terminal trills were the most distinct song traits among subspecies, adding to growing evidence that trills are important in speciation among many birds, insects and fish. Across a ~6000 km transect of Russia, acoustic distance was predicted by genetic and geographical distance, but not climatic distance. We also found no reproductive character displacement of song traits in a contact zone between *H. r. rustica* and *H. r. tytleri*. Based on patterns discovered in this study, we infer an important role of sexual selection, genetic and/or cultural drift in the gradual build-up of acoustic divergence, which is accelerated in small populations.

ADDITIONAL KEYWORDS: acoustic adaptation – acoustic divergence – barn swallow – birdsong – ecological selection – *Hirundo rustica* – neutral drift – reproductive character displacement – sexual selection – speciation.

INTRODUCTION

A wide diversity of animals uses acoustic signals to mediate competition and mate choice within species. As a result of their long-range communication capabilities and evolutionary lability, acoustic signals have been proposed to play an important role in the formation of pre-zygotic reproductive barriers and may facilitate or even drive the process of speciation (Blair, 1958; Jones,

1997; Slabbekoorn & Smith, 2002; Price, 2008; Wilkins, Seddon & Safran, 2013). Acoustic signals evolve through changes to the physiological, developmental or neurological bases of vocal production and learning (Podos, Huber & Taft, 2004; Podos & Warren, 2007; Catchpole & Slater, 2008; Wilkins *et al.*, 2013). For vocal learning species, such as oscine passerines, parrots, hummingbirds and a variety of mammals, additional signal variation can arise from copying errors, improvisation and mimicry (reviewed by Tyack 2016). Among birds in particular, a vast literature

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has attempted to quantify the contributions of genes, culture, development and the environment to the causes and consequences of acoustic diversity within and among populations (reviewed by Baker, 2001; Marler & Slabbekoorn, 2004; Podos *et al.*, 2004; Podos & Warren, 2007; Catchpole & Slater, 2008; Price, 2008).

As summarized in Table 1, there are numerous case studies demonstrating influences of different forms of sexual and ecological selection as well as genetic and cultural drift on the evolution of acoustic diversity. For example, a large number of ‘acoustic adaptation’ studies show that longer, slower-paced, lower-pitched signals encompassing a narrower frequency range are found in denser, closed habitats compared with open habitats (Morton, 1975; Wiley & Richards, 1978; Patten, Rotenberry & Zuk, 2004; Tubaro & Lijtmaer, 2006; Derryberry, 2009; Kirschel *et al.*, 2009a; Tobias *et al.*, 2010; Smith *et al.*, 2013; Rek & Kwiatkowska, 2016). However, overall support for acoustic adaptation is weak (Blumstein & Turner, 2005; Boncoraglio & Saino, 2007; Ey & Fischer, 2009), and several studies have failed to detect strong effects of habitat on song divergence (Sosa-López, Mennill & Navarro-Sigüenza, 2013; Mason & Burns, 2015; Tietze *et al.*, 2015; Graham *et al.*, 2017). Likewise, numerous studies have documented changes in vocal characteristics in response to anthropogenic noise (Slabbekoorn & Peet, 2003; Patricelli & Blickley, 2006; Mockford & Marshall, 2009; Francis, Ortega, & Cruz, 2011), although it is currently unclear to what degree such changes are evolutionary vs. plastic responses (Gross, Pasinelli & Kunc, 2010; Zollinger *et al.*, 2017). Other studies show important effects of sexual selection (Searcy & Andersson, 1986; Ryan & Rand, 1990; Irwin, 2000; Fitzpatrick & Gray, 2001; Price & Lanyon, 2004; Mendelson & Shaw, 2005; Rodríguez, Ramaswamy & Coccoft, 2006; Boul *et al.*, 2007; Seddon, Merrill & Tobias, 2008; Sullivan-Beckers & Coccoft, 2010; Grace & Shaw, 2012; Greig, Price & Pruett-Jones, 2013; Dreher & Pröhl, 2014; Gordinho *et al.*, 2015; Wei *et al.*, 2017) and genetic and/or cultural drift (Pröhl *et al.*, 2006, 2007; Irwin, Thimban & Irwin, 2008; Amézquita *et al.*, 2009; Campbell *et al.*, 2010; Sosa-López *et al.*, 2013; Lee *et al.*, 2016) on acoustic signal divergence. However, as the processes underlying these patterns have typically not all been explored within one taxon (Wilkins *et al.*, 2013), we currently lack a general understanding of the relative contribution of these processes to overall patterns of acoustic divergence.

Another principal gap in our understanding of signal evolution is how acoustic divergence accrued in allopatry should respond when species or populations come into secondary contact. Reproductive character displacement describes the process wherein acoustic signal characteristics shift when two genetically divergent populations come into secondary contact

(Grant, 1972). This phenotypic shift in mating signals may result from ecological selection to minimize agonistic interspecies interactions (Grether *et al.*, 2009) or from reinforcement to minimize the production of costly hybrids (Blair, 1955; Hoskin *et al.*, 2005; Kirschel, Blumstein & Smith, 2009b; Gerhardt, 2013). Generally, researchers expect reproductive character displacement to result in increased divergence in populations that live in sympatry with a related heterospecific; however, there are very few clear examples of reproductive character displacement in acoustic traits (Gerhardt, 2013), and researchers have sometimes documented convergent character displacement or no change in signals upon secondary contact (Table 1). Much of this variation may relate to the genetic heritability of the traits under consideration, as modelling suggests that learning can impede the process of reinforcement in certain conditions (Olofsson, Frame & Servedio, 2011). Thus, for any given system we currently have little ability to predict which processes should drive patterns of acoustic divergence in allopatry and whether signals will diverge, converge or remain unchanged upon secondary contact. The aim of the present study was to address these issues in a recently diverged, widely distributed species radiation (Dor *et al.*, 2010; del Hoyo & Elliott, 2014), which is uncommonly tractable for exploring the relative roles of geographical isolation, genetic divergence and climate differences (as a measure of ecological selection) on acoustic signal evolution at multiple spatial scales.

Few bird species have ranges as extensive as the barn swallow (*Hirundo rustica*), which has adapted to live alongside humans, and nests almost exclusively in association with human constructions. Barn swallows breed at elevations ranging from sea level to 3000 m, from 18 to 71°N latitude (BirdLife & NatureServe, 2017; del Hoyo & Elliott, 2014) and in a recently established Argentinian population (Billerman *et al.*, 2011). There are six commonly recognized subspecies of barn swallow (Dor *et al.*, 2010), and targets of sexual selection are known to be highly distinct across them (Scordato & Safran, 2014; Wilkins *et al.*, 2016; Safran *et al.*, 2016b; Romano *et al.*, 2017). Prior work has shown sexual selection favouring the following characteristics: long tail streamers (the outermost tail feathers) in the European subspecies *H. r. rustica* (Møller, 1988; reviewed by Scordato & Safran, 2014); dark ventral plumage (but not long tail streamers) in the North American subspecies *H. r. erythrogaster* (Safran & McGraw, 2004; Safran, 2005; Neuman, Safran & Lovette, 2007; Eikenaar *et al.*, 2011; Safran *et al.*, 2016b); a combination of dark ventral colour and long tail streamers in an Israeli population of *H. r. transitiva* (Vortman *et al.*, 2011, 2013); and darker throat colour in a Japanese population of *H. r. gutturalis* (Hasegawa *et al.*, 2010). Despite this long

Table 1. Predictions, rationale and evidence for drivers of acoustic signal divergence

Divergence mechanism	Acoustic distance associated with	Tested here?	Rationale	Exceptions and caveats	Examples
Genetic drift	Genetic and geographical distance; variation in population size	Yes	As a result of isolation by distance, populations breeding further apart have more distinct genomes and therefore more distinct phenotypes (to the degree that song production or learning is genetically controlled); smaller populations are expected to evolve faster	Large differences in population size across population pairs may confound the relationship predicted by isolation by distance	Birds (Irwin <i>et al.</i> , 2008; Sosa-López <i>et al.</i> , 2013), frogs (Prohl <i>et al.</i> , 2006, 2007; Amézquita <i>et al.</i> , 2009; Lee <i>et al.</i> , 2016) and singing mice (Campbell <i>et al.</i> , 2010)
Cultural drift	Geographical, not necessarily genetic distance; observed meme turnover among populations	Indirectly	For vocal learning species, memetic differences accrue randomly with distance, whereas postdispersal learning in unrelated individuals erases the signal–genotype link	Given that cultural drift can show an identical pattern to genetic drift among vocal learning species, monitoring meme frequencies among populations over time provides the most direct evidence	Inferred: bats (Sun <i>et al.</i> , 2013; Lin <i>et al.</i> , 2015; Xie <i>et al.</i> , 2017) and birds (Irwin <i>et al.</i> , 2008; González & Ornelas, 2014) Observed: birds (Ince, Slater & Weismann, 1980; Payne <i>et al.</i> , 1981; Nelson, Hallberg & Soha, 2004; Byers, Belinsky & Bentley, 2010) and whales (Deecke, Ford & Spong, 2000; Garland <i>et al.</i> , 2011)
Ecological selection	Habitat vegetation density, elevation, latitude, ambient noise profile or climatic variables, such as temperature, humidity and seasonality	Partly	Selection may act directly on acoustic signals (through acoustic adaptation) to maximize propagation in different environments, or pleiotropically through adaptive changes to morphological traits associated with sound production.	Acoustic adaptation and pleiotropy may often include sexually selected elements if, for instance, signal aspects corresponding to high propagation distance or degree of morphological adaptation are also preferred by females	Acoustic adaptation: bats (Snell-Rood, 2012; Sun <i>et al.</i> , 2013; Mutumi, Jacobs & Winker, 2016), birds (Morton, 1975; Ryan & Brenowitz, 1985; Patten <i>et al.</i> , 2004; Nicholls <i>et al.</i> , 2006; Ruegg <i>et al.</i> , 2006; Snell-Rood & Badyaev, 2008; Botero <i>et al.</i> , 2009; Kirschel <i>et al.</i> , 2009a; Tobias <i>et al.</i> , 2010; Weir & Wheatcroft, 2011;

Table 1. *Continued*

Divergence mechanism	Acoustic distance associated with	Tested here?	Rationale	Exceptions and caveats	Examples
			Here, we consider the broad relationship between body size and vocal frequency a constraint, in the absence of an explicit connection to ecological adaptation		Medina & Francis, 2012; Greig <i>et al.</i> , 2013; Smith <i>et al.</i> , 2013; Singh & Price, 2015; Rek & Kwiatkowska, 2016; Wei <i>et al.</i> , 2017) and frogs (Ryan, Perrill & Wilczynski, 1992; Vargas-Salinas & Amézquita, 2013) Pleiotropy: bats (Kingston & Rossiter, 2004) and birds (Podos, 2001; Podos, Dybbøe & Jensen, 2013; Krishnan & Tamma, 2016)
Non-ecological sexual selection models: Fisherian runaway; sexual conflict	Genetic, not necessarily geographical distance; measures of strength and/or direction of sexual selection	Indirectly	Random shifts in targets or strength of sexual selection lead to clock-like accrual of acoustic differences as populations diverge; correlations with measures of selection are necessary to separate from drift	Early in speciation, strong divergence in signals may correlate with divergence only at selected trait loci and show no relationship with neutral genetic markers	See ‘Sexual selection models with links to ecology’ below. Studies are generally not explicit about a particular sexual selection model, and most show concurrent relationships between acoustic signals and ecological variables such as latitude, climate or condition
Sexual selection models with links to ecology (ecological + sexual selection): direct benefits, Hamilton–Zuk, handicap and good genes	Measures of condition and reproductive performance; acoustic divergence not explicitly predicted to relate to geographical or genetic distance	No	Under these models of sexual selection, we expect acoustic divergence to be correlated with reproductive success and ecological differences (such as climate, latitude, migration distance, predator/parasite abundance, etc.) related to signal honesty, rather than geographical or genetic distance	It may be very difficult in practice to separate influences of ecology and sexual selection without explicit experiments designed to do so	Birds (Searcy & Andersson, 1986; Irwin, 2000; Price & Lanyon, 2004; Seddon <i>et al.</i> , 2008; Greig <i>et al.</i> , 2013; Gordinho <i>et al.</i> , 2015; Wei <i>et al.</i> , 2017), frogs (Ryan & Rand, 1990; Boul <i>et al.</i> , 2007;

Table 1. *Continued*

Divergence mechanism	Acoustic distance associated with	Tested here?	Rationale	Exceptions and caveats	Examples
					Dreher & Pröhl, 2014) and insects (Fitzpatrick & Gray, 2001; Mendelson & Shaw, 2005; Rodríguez <i>et al.</i> , 2006; Sullivan-Beckers & Cocroft, 2010; Grace & Shaw, 2012)
Reproductive character displacement (ecological + sexual selection)	Geographical context (sympatry vs. allopatry) with another taxon	Yes	To reduce costs of interspecific competition or maladaptive hybridization, acoustic signals may converge or diverge in sympatry with a particular taxon, compared with allopatry. Usually considered between recently diverged populations upon secondary contact, but has wider applicability	Character displacement may not occur if signals are already distinct enough or if receivers are selected to become more discriminating (Amézquita <i>et al.</i> , 2011; Pasch <i>et al.</i> , 2017). Also, patterns of reproductive character displacement will depend on the costs of hybridization	Convergence: birds (Secondi <i>et al.</i> , 2003; Haavie <i>et al.</i> , 2004; Qvarnström <i>et al.</i> , 2006; Tobias & Seddon, 2009; Kenyon, Toews & Irwin, 2011) Divergence: birds (Haavie <i>et al.</i> , 2004; Kirschel <i>et al.</i> , 2009b; Halfwerk <i>et al.</i> , 2016; Hamao <i>et al.</i> , 2016), frogs (Blair, 1955; Höbel & Gerhardt, 2003; Hoskin <i>et al.</i> , 2005; Lemmon, 2009) and insects (Marshall & Cooley, 2000; Jang & Gerhardt, 2006); review (Gerhardt, 2013)

Listed examples are intended to be rigorous but not comprehensive.

history of sexual selection studies, song characteristics have been studied in detail only within the European (*H. r. rustica*: Galeotti *et al.*, 1997, 2001; Saino *et al.*, 1997; Garamszegi *et al.*, 2005, 2006) and North American (*H. r. erythrogaster*: Wilkins *et al.*, 2015) subspecies, and we currently do not know the extent to which song has diverged across barn swallow lineages. Moreover, extremely rapid phenotypic divergence of the barn swallow subspecies complex is thought to have occurred as populations expanded out of Africa (see phylogeographical hypothesis in Fig. 1) within the last 100 000 years (Zink *et al.*, 2006; Dor *et al.*, 2010), making it an ideal system for investigating patterns of signal divergence during the early stages of speciation.

The aims of this study were as follows: (1) to provide the first description of geographical variation in barn swallow song, including an analysis of which song

components are most divergent between subspecies; (2) to assess whether character displacement occurs where subspecies come into contact; and (3) to assess whether geographical variation in song is primarily correlated with genetic, geographical or climatic distance between populations. We provide inferences with respect to theoretical predictions (Table 1) about the processes underlying the observed patterns of song divergence.

MATERIAL AND METHODS

ETHICAL NOTE

All methods were approved by the University of Colorado Institutional Animal Care and Use Committee (Protocols 07-07-SAF-01 and 1004.01).

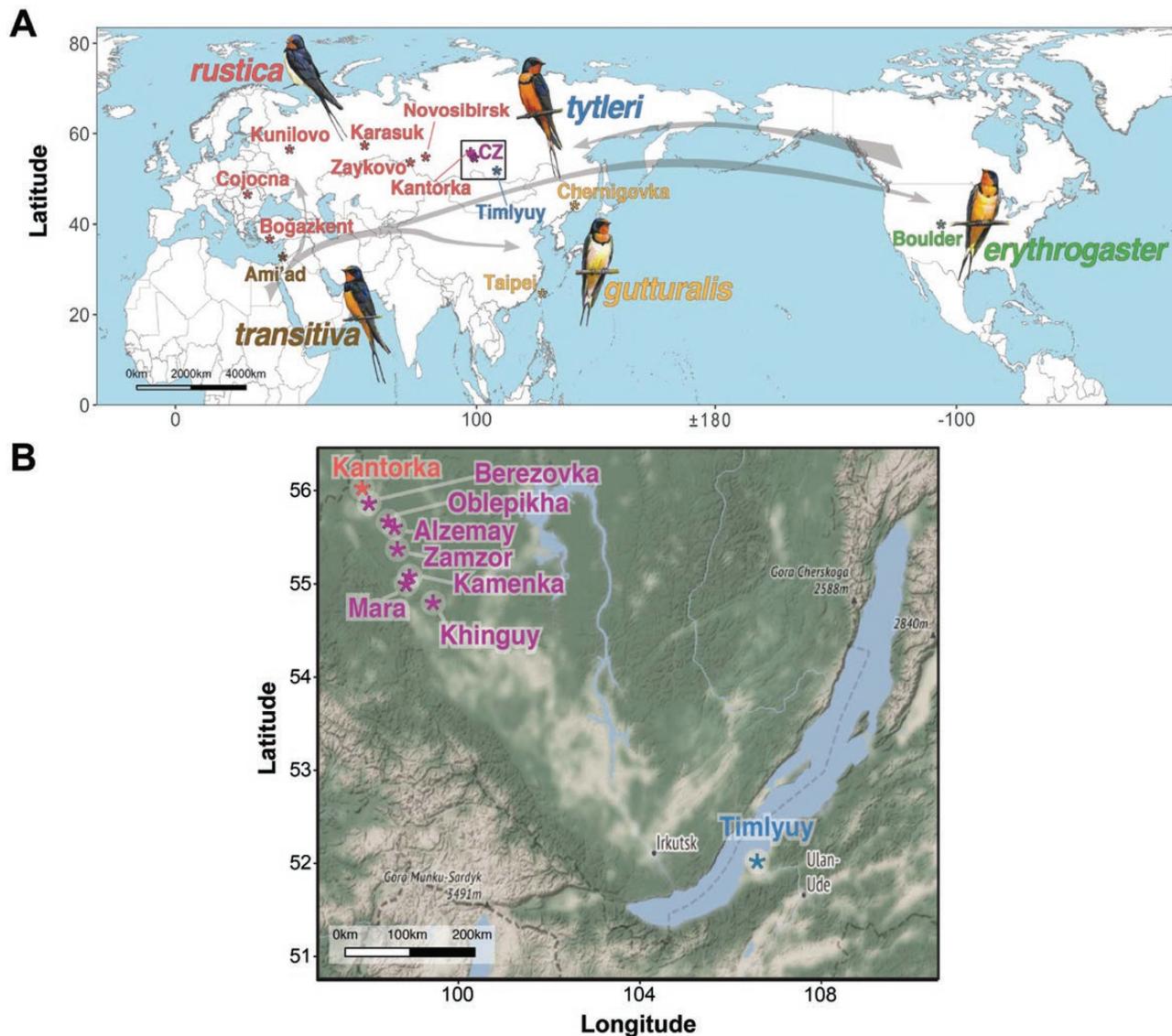


Figure 1. Maps of sampling sites. A, worldwide sites (Table S1). The stacked asterisks and label 'CZ' refer to the Russian contact zone between *Hirundo rustica rustica* and *H. r. tytleri*, near Lake Baikal. Grey arrows indicate the hypothesized biogeographical history of barn swallows, with an African origin and relatively recent back-colonization of Northern Asia by individuals from North America. B, an inset of the contact zone (sites Berezovka to Khinguy) and the flanking pure subspecies sites of Kantorka (*H. r. rustica*) and Timlyuy (*H. r. tytleri*). In both panels, site label colours reflect subspecies, as follows: green, *erythrogastrer*; red, *rustica*; brown, *transitiva*; blue, *tytleri*; purple, *rustica-tytleri* contact zone; and yellow, *gutturalis*.

SONG RECORDINGS

A total of 1700 songs were analysed for 180 male barn swallows (range 3–26; mean \pm SD 9.44 ± 4.95 songs per individual). Sampling included five of the six subspecies, recorded at 19 sites within six countries (Fig. 1; see also Supporting Information, Table S1; *H. r. savignii*, which is found in a narrow region of Egypt along the Nile, was not recorded owing to political unrest during the sampling period, and we are unaware of high-quality archival recordings for this subspecies). Recordings were made during

the period of high song activity between 05.00 and 13.00 h over the following dates: USA, 15 May–19 July 2009, 6–31 May 2011 and 1 May–21 August 2012; Turkey, 20 April–4 July 2010; Israel, 7–9 May 2010; Romania, 19–22 June 2010; Taiwan, 3–8 June 2011; and Russia, 21 May–24 July 2013. We used the year with the highest quality recordings for the two Colorado males recorded in multiple years. All songs were recorded by M.R.W. in 16-bit WAV format, with 48 kHz sampling rate using a Marantz PMD 660 or 661 digital recorder, paired with an Audiotechnica

AT815B shotgun microphone, Sennheiser MKH 20 and Telinga parabola, or Sennheiser ME62/k6 microphone and Telinga parabola. Ringed males were identified by unique combinations of permanent ink marker (Sharpie) colours applied to white dots on rectrices and a colour ring, whereas unringed males were differentiated by physical characteristics and distance between singing territories. Song samples included 12 areas with the following taxa: one *H. r. erythrogaster*, one *H. r. transitiva*, six *H. r. rustica*, two *H. r. gutturalis*, one *H. r. tytleri* and a contact zone between *H. r. tytleri* and *H. r. rustica* (Fig. 1).

SONG ANALYSIS

Individual songs were extracted using Syrinx-PC (J. Burt, Burtsoft Consulting, Portland, OR). Barn swallow songs are composed of a warbling series of syllables, followed by a harsh trill, termed the ‘rattle’ (Galeotti *et al.* 1997; Fig. 2). Although males sometimes utter isolated phrases of syllables, for this analysis we considered only complete songs, with syllables ≤ 0.2 s apart, which contained a rattle (and a terminal syllable after the rattle in some populations). Song variables (Table 2) were chosen to measure a large portion of song variation, including frequency and temporal parameters. We also measured Wiener entropy (the ratio of geometric to arithmetic mean of the power spectrum) of the warble to characterize tonality. This measure ranges from zero, representing

a pure tone, to one, representing random noise. To analyse song features, we first down-sampled WAV files to 22 kHz and applied a 1 kHz high-pass filter to remove low-frequency noise. We then segmented the warble and rattle elements of songs manually using the waveform to identify the beginning and end of elements, and then extracted acoustic measures using the Automatic Parameter Measurement tool in Avisoft SASLab Pro version 5.2 (www.avisoft.com; Avisoft Bioacoustics Glienicke, Germany). Where necessary, we used the on-screen eraser tool to delete overlapping signals or noise. We discarded songs where overlap was too severe or signal-to-noise ratio too low. These manual erasures did not bias our frequency measures because we used maximal rather than mean frequency measures. Rattle pulse number was counted manually from spectrograms. For frequency analyses, we measured the rattle and the ‘central rattle’ (i.e. disregarding the first and last pulse). This was necessary because in some barn swallow populations, the frequency of bounding pulses is much lower than the main pulse train.

We generated spectrograms for automatic parameter measurement in Avisoft (fast Fourier transformation = 512, frame = 100%, window = Hamming, overlap = 93.75%), aiming for a balance of frequency and temporal resolution (43 Hz and 1.45 ms, respectively). As reported by Wilkins *et al.* (2015), and unlike previous barn swallow song studies (Galeotti *et al.*, 1997; Garamszegi *et al.*, 2005,

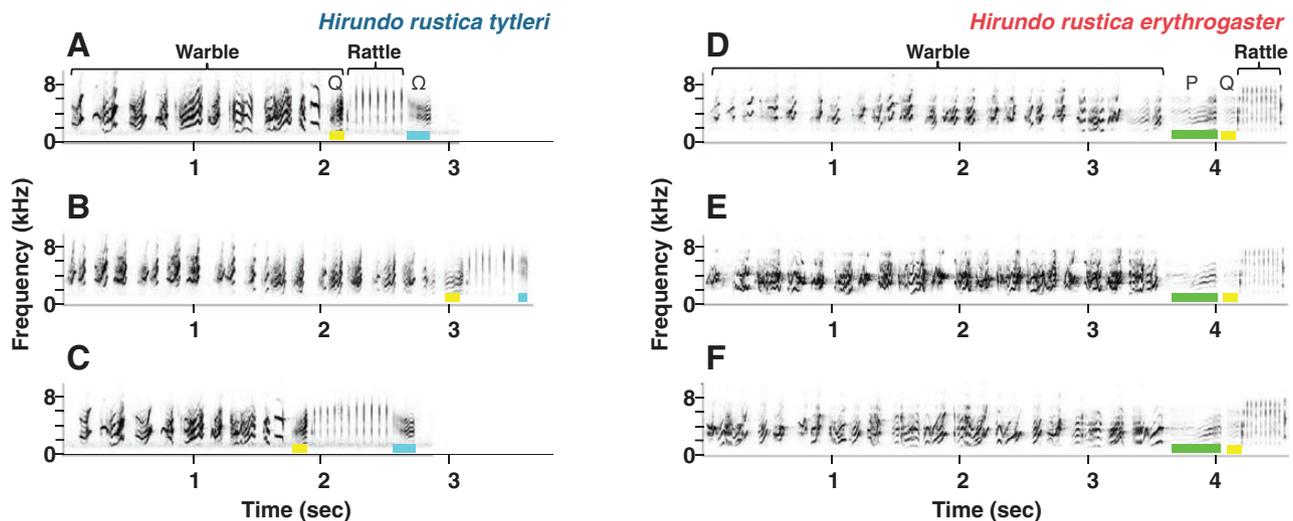


Figure 2. Intra-individual song variation for two subspecies. A–C, spectrograms of three songs from a single male *Hirundo rustica tytleri* from Timlyuy, Russia. D–F, spectrograms of three songs from a single male *H. r. erythrogaster* from Boulder, CO, USA. The ‘Ω-’ and ‘P-syllables’ (blue and green underscores) highlight discrete song components that distinguish these two subspecies. Although individual song renditions vary in syllable number and composition, distinctive subspecies song elements (‘Ω-’ and ‘P-syllables’) are rarely dropped from complete songs containing a rattle (but see main text for quantitative description). All five subspecies’ songs (including the two shown here) contain warbles, rattles and ‘Q-syllables’ (yellow underscores).

Table 2. Measured song traits and their correlations with the four linear discriminants maximizing song variation among subspecies

Category	Trait	Description	LD1	LD2	LD3	LD4
Time domain	Pulses	Pulse number, i.e. the number of pulses in the rattle	-0.10	-0.40	0.50	0.11
	WL	Warble length, i.e. distance between peak of first syllable and peak of last syllable before P (in seconds)	-0.33	-0.19	0.55	-0.30
	RL	Rattle length, i.e. distance between the first and last pulses in the terminal trill (in seconds)	0.50	-0.35	0.42	0.07
	RTmp	Rattle tempo, i.e. number of rattle pulses/rattle length (in herz)	-0.94	0.05	-0.13	0.11
Frequency domain	PF W	Peak frequency of the warble, i.e. frequency at the maximal amplitude in the warble (in herz)	0.45	-0.01	0.07	-0.50
	PF R	Peak frequency of the rattle, i.e. frequency at the maximal amplitude in the rattle (in herz)	0.23	-0.38	0.05	0.09
	PF CR	Peak frequency of the central rattle, i.e. frequency at the maximal amplitude in the rattle, excluding the first and last pulse (in herz)	0.14	-0.65	-0.17	0.18
	BW R	Frequency bandwidth of the rattle, i.e. song frequency bandwidth above a -10 dB threshold, relative to peak frequency, averaged across the entire rattle (in herz)	-0.21	-0.11	-0.62	-0.63
	WE W	Wiener entropy of the warble, i.e. ratio of the geometric mean to the arithmetic mean of the warble spectrum (0 = pure tone; 1 = random noise)	0.03	-0.66	-0.18	-0.30
		Proportion of between-group variance explained	0.78	0.11	0.09	0.01
	Cumulative variance explained	0.78	0.90	0.99	1.00	

Trait loadings ≥ 0.5 are in bold.

2006), we did not separate songs and analyses by ‘type A’ and ‘type B’ songs, because this distinction was not clear across all populations. Given that we did not attempt to classify syllable types or assess repertoire, we set a threshold of three complete songs per individual for estimating song parameters, as this was shown to be sufficient in previous rarefaction analysis (Wilkins *et al.*, 2015 Supporting Information, Fig. S2). Differences in sampling date may confound observed differences by sampling location, given that song length and sequence diversity may vary according to laying date (Galeotti *et al.*, 2001). However, asynchronous breeding and multiple broods within populations (Møller, 1994a) make this unlikely to be a systematic issue. Additionally, our inclusion of six *H. r. rustica* populations that were sampled in different years (2010 and 2013), locations (Romania and Russia) and parts of the breeding season (range 21 May–1 July) effectively controls for this confounding issue. Moreover, we have not found a significant effect of lay date on rattle length and tempo within Colorado *H. r. erythrogaster* (M.R.W.,

unpublished data). We therefore did not control for sampling date in our analysis.

For this study, subspecies distinctions follow previous work (Zink *et al.*, 2006; Dor *et al.*, 2010; Safran *et al.*, 2016a). Our sampling sites, subspecies designations and the hypothesized biogeographical history of the species complex are shown in Figure 1. Within the contact zone between *H. r. rustica* and *H. r. tytleri*, subspecies identity of singers was inferred by visually assessing morphology (*H. r. tytleri* have dark, rusty ventral plumage, in stark contrast to the pale plumage of nominate *H. r. rustica*; see cartoons in Fig. 1). *Hirundo r. tytleri* are genetically more closely related to North American *H. r. erythrogaster* than to sympatric *H. r. rustica* (Dor *et al.*, 2010; Safran *et al.*, 2016a) and have pronounced morphological divergence. Moreover, restriction-assisted digest sequencing (RAD-Seq) population genetic analyses show that there is very little ongoing hybridization between this subspecies pair; furthermore, differences in ventral plumage colour are associated with genetic

assignment to subspecies in this population (Scordato *et al.*, 2017). However, because we do not have song and genotype data for the same individuals in the contact zone, we cannot assess how genetic admixture affects song structure and syntax.

CLIMATIC DISTANCE

Given that we did not directly measure ecological variables or sound propagation at all study sites, we instead gathered nine climatic variables (annual mean temperature, isothermality, seasonality, maximal and minimal temperature, annual precipitation, precipitation of the wettest and driest months, and precipitation seasonality) for each study site from the Bioclim dataset of the WorldClim database (Hijmans *et al.*, 2005), at a resolution of 2.5' (~4.6 km²). These variables describe aspects of the breeding habitat likely to have direct influences on sound propagation or indirect effects through the density and structure of vegetation (Morton, 1975; Wiley & Richards, 1978; Snell-Rood, 2012). For example, frequencies < 8 kHz show a linear decrease in attenuation as humidity increases (Wiley & Richards, 1978), and there is a well-studied but overall weak pattern of longer duration, lower frequency vocalizations occurring in closed habitat, where these signals exhibit reduced attenuation compared with high-frequency, highly modulated signals (Morton, 1975; Boncoraglio & Saino, 2007; Ey & Fischer, 2009). Climatic effects of temperature and humidity on sound absorption have also been shown to explain variation in wood warbler and bat vocalizations (Snell-Rood, 2012). Moreover, many of the climate variables chosen here have previously been shown to affect acoustic spectral characteristics, such as syllable frequency and duration (Ruegg *et al.*, 2006; Laiolo, 2012; Xing *et al.*, 2017).

We performed principal component analysis on the nine bioclimatic variables with the *psych* R package (Revelle, 2017), using varimax rotation to extract two factors describing 87.8% of the variation in climate (Supporting Information, Table S2). Principal component 1 (PC1) values indicate diel and annual temperature variability, whereas principal component 2 (PC2) describes the intensity and seasonality of annual precipitation. We calculated the Euclidian distance for these two variables across all populations as our measure of climatic distance between pairs of populations.

GENETIC DISTANCE

We determined genetic distance between populations by calculating pairwise F_{ST} from genotype data described by Safran *et al.* (2016a) and Scordato *et al.* (2017). Briefly, we used RAD-Seq (Parchman *et al.*, 2012)

to identify single nucleotide polymorphisms (SNPs) segregating between populations. With these data, we measured the genetic distance for two subsets of our dataset. For 'allopatric populations', i.e. those from all countries except for Russia, we used the pairwise F_{ST} values reported by Safran *et al.* (2016a), derived from 9493 SNPs, as measures of genetic distance between allopatric populations. In contrast, we measured genetic distance within the contact zone in Russia by first subdividing individuals into groups based on assignment probabilities generated in fastSTRUCTURE (Raj, Stephens & Pritchard, 2014) using 23 251 SNPs (Scordato *et al.*, 2017). Individuals that were assigned to one of the two 'parental' clusters (*rustica* or *tytleri*) with assignment probabilities > 70% (q score > 0.70) were included in the analysis. Individuals with lower assignment probabilities to a particular cluster were considered to be early-generation hybrids and were excluded from measures of genetic distance. We then calculated pairwise F_{ST} between all Russian populations, with parental individuals within the contact zone divided into two separate 'populations', contact zone *rustica* and contact zone *tytleri*. We calculated pairwise F_{ST} using Weir and Cockerham's method implemented in the R package *hierfstat* (Goudet, 2005).

DISCRIMINANT FUNCTION ANALYSIS

All statistical tests were performed using R v3.0.2 (R Core Team, 2017). Given that songs varied across populations in both continuous features (e.g. temporal and spectral measures of song elements; Table 2) and discrete features (presence or absence of specific syllables), we analysed these aspects of variation independently.

To identify the continuous song traits that were most important for distinguishing subspecies, we performed linear discriminant function analysis (DFA) on centred song variables using the *MASS* package (Venables & Ripley, 2013). This analysis creates $N - 1$ axes, which minimize misclassification of the N categories (our five subspecies), based on the continuous predictor variables (our nine song measures), with each subsequent axis (DF1, DF2, ...) explaining less of the variation among groups. Given that linear discriminant coefficients are not always easy to interpret, we instead report discriminant loadings, which are equivalent to principal component analysis loadings (i.e. correlations of trait measures with each set of discriminant scores). We used two methods to assess DFA accuracy. First, we trained the DFA using ten random individuals from the eight populations for which we had at least ten samples (Boulder, *H. r. erythrogaster*; Cojocna, *rustica*; Boğazkent, *rustica*; Ami'ad, *transitiva*; Kuniilovo, *rustica*; Zaykovo, *rustica*; Timlyuy, *tytleri*; and Taipei, *gutturalis*), and used the

remaining 100 samples to test assignment accuracy. All songs for a selected individual were included for each set (see Supporting Information, Table S3 for sample sizes of testing data by population). As the individuals or populations chosen for the training data can have a large effect on the resultant discriminant functions, and because typical DFA significance tests may inflate DFA performance (Mundry & Sommer, 2007), we also ran a nested permuted analysis (pDFA) on the 144 individuals in our eight reference populations with the highest sample sizes. Using a custom R script provided by Roger Mundry Max Planck Institute for Evolutionary Anthropology, we randomly sampled one population from each of the five subspecies (addressing the issue of having three *H. r. rustica* reference populations) and ten individuals from each population. Discriminant function analysis was performed on these 50 individuals and compared with a DFA on a sample with subspecies identity permuted randomly. This entire procedure was then iterated 10000 times. The significance of the DFA was calculated as the number of randomly permuted datasets that generated a higher proportion of accurately classified subjects than the original dataset, divided by the total number of datasets tested (Mundry & Sommer, 2007).

TESTING PREDICTORS OF SONG DIVERGENCE

To characterize broad patterns of overall divergence in continuous song measures, we calculated average DFA scores for each of the four linear discriminant axes (Table 2) within our 14 populations (collapsing the five contact zone sites into two populations of *rustica* and *tytleri*, as shown in Fig. 1A) and then calculated pairwise-population Euclidian trait distances (hereafter termed ‘song distance’). We used variation partitioning and redundancy analysis (Borcard, Legendre & Drapeau, 1992) to assess the relative contributions of genetic, geographical and climatic distance to population song distance, controlling for inherent autocorrelation among these variables. To do this, we partitioned song distance on the three predictor variables using the *varpart* function in the ‘vegan’ package (Oksanen *et al.*, 2018). We then performed redundancy analysis to assess the significance of these relationships with Monte Carlo permutation tests ($N = 999$ permutations) using the *rda* function.

Note that because genetic relatedness (F_{ST}) estimates for our Russian transect were derived from distinct RAD-Seq runs from the other countries, we performed separate analyses for ‘allopatric populations’ (Boulder, CO, USA; Cojocna, Romania; Boğazkent, Turkey; Ami’ad, Israel; and Taipei, Taiwan) and ‘Russian transect populations’ (Kunilovo, Zaykovo, Karasuk, Novosibirsk, Kantorka, contact zone *rustica*,

contact zone *tytleri*, Timlyuy and Chernigovka). These populations (listed in Table 3) differed in the density of sampling (mean \pm SD interpopulation distance in Russia, 2109.04 ± 1466.01 km; allopatric populations, 7065.46 ± 4175.23 km) and subspecies diversity (Russia, three subspecies; allopatric populations, four subspecies), allowing us to test for broad signal divergence patterns at continental and global scales.

RESULTS

SONG DIVERGENCE WITHIN AND AMONG SUBSPECIES

We found large amounts of intra-individual variation in the speed, length, number and composition of song syllables in all populations. Figure 2 shows representatives of two subspecies, *H. r. tytleri* and *H. r. erythrogaster*. Nevertheless, the shared basic song structure of barn swallow song is apparent across all 14 sampled populations (Fig. 3); all songs include a continuously varying series of warbled syllables, followed by a trilled pulse series (the rattle). This is consistent with previous descriptions of the European subspecies (Galeotti *et al.*, 1997; Garamszegi *et al.*, 2006). All sampled *H. r. rustica* and *H. r. erythrogaster* populations also produce a long, highly harmonic to noisy, rising frequency element termed the ‘P-syllable’ (green underscores in Figs 2, 3), followed by a short ‘Q-syllable’ (yellow underscores), immediately before the trill (*sensu* Galeotti *et al.*, 1997). The P-syllable is distinguishable by its penultimate position in the warble, by its upward sweep in amplitude and frequency, and by generally being the longest syllable in the song. Males in all populations appear to produce a short Q-syllable immediately before the rattle, although the amplitude and length of this element varies markedly. In contrast, the P-syllable is not universally conserved, and populations of *H. r. transitiva*, *H. r. tytleri* and *H. r. gutturalis* do not produce components easily identifiable as P-syllables. Some populations produced previously unreported additional syllables after the rattle, as can be seen for Zaykovo *rustica*, both *tytleri* samples and Taipei *gutturalis* (blue underscores in Figs 2, 3). We refer to these as ‘ Ω -syllables’, which ranged from pure tonal notes (Zaykovo) to highly harmonic (both *tytleri* samples) to a compound syllable combining a tonal and harmonic note (Taipei).

Populations and subspecies ranged in the flexibility of Ω -syllable production within and among individuals. For example, *H. r. gutturalis* in Taipei, Taiwan exhibited high within- and low among-individual variation in Ω -syllable production; all of the ten sampled males produced Ω -syllables, although they varied in the proportion of songs that included terminal syllables (21.4–72.7%). In contrast, none of the *H. r. gutturalis* males in Chernigovka, Russia

Table 3. Population pairwise distances in song, geography, genetics and climate

Song distance	Geographical distance	Genetic distance	Climatic distance	Dataset
1.310	1257	0.037	2.116	Allopatric
1.868	9131	0.053	0.982	Allopatric
1.875	1831	0.044	2.502	Allopatric
1.916	8419	0.059	4.517	Allopatric
2.010	8576	0.067	3.609	Allopatric
2.125	592	0.031	0.637	Allopatric
2.855	10382	0.046	1.248	Allopatric
2.986	11311	0.046	4.208	Allopatric
2.990	8194	0.065	5.149	Allopatric
3.612	10962	0.054	1.537	Allopatric
0.701	610	0.002	0.088	Russia
0.876	3693	0.004	1.205	Russia
0.982	1364	0.003	0.532	Russia
0.986	2530	0.002	0.708	Russia
1.300	2126	0.005	0.306	Russia
1.505	1502	0.001	0.496	Russia
1.850	2224	0.004	0.730	Russia
2.023	1039	0.002	0.212	Russia
2.270	2777	0.007	0.528	Russia
2.477	361	0.002	0.181	Russia
2.492	3586	0.003	0.801	Russia
2.690	1013	0.003	0.703	Russia
2.781	1304	0.001	0.095	Russia
2.865	1276	0.014	0.032	Russia
2.952	130	0.000	0.457	Russia
3.086	946	0.009	0.274	Russia
3.829	0	0.019	0.000	Russia
4.045	610	0.020	0.088	Russia
4.294	6292	0.050	0.720	Russia
4.409	1364	0.026	0.532	Russia
4.525	3693	0.029	1.205	Russia
4.570	1928	0.027	0.496	Russia
4.689	4871	0.050	0.387	Russia
4.801	4304	0.030	1.189	Russia
4.854	3676	0.073	0.384	Russia
4.896	2751	0.049	0.406	Russia
4.905	4018	0.046	0.363	Russia
4.979	1013	0.028	0.703	Russia
5.084	2666	0.038	0.564	Russia
5.393	1593	0.047	0.674	Russia
5.431	2224	0.028	0.730	Russia
5.691	2830	0.029	0.703	Russia
6.492	130	0.020	0.457	Russia
6.744	723	0.026	0.413	Russia
8.093	2666	0.019	0.564	Russia
8.473	2092	0.024	0.586	Russia

Rows are sorted by dataset, then song distance. As genetic distance estimates are not comparable across datasets, allopatric–Russian pairwise comparisons are not reported. Song distances are Euclidian distances between the four discriminant function scores, averaged for population. Geographical distances were estimated in kilometres from longitude–latitude coordinates with the haversine method. Genetic distances are the average genome-wide F_{ST} , and climatic distance is the Euclidian distance for the two climate principal components. The greatest song and genetic divergence measures are both found in the Russian dataset, between Timlyuy *tyleri* and Chernigovka *gutturalis* and between Novosibirsk *rustica* and Chernigovka *gutturalis*, respectively.

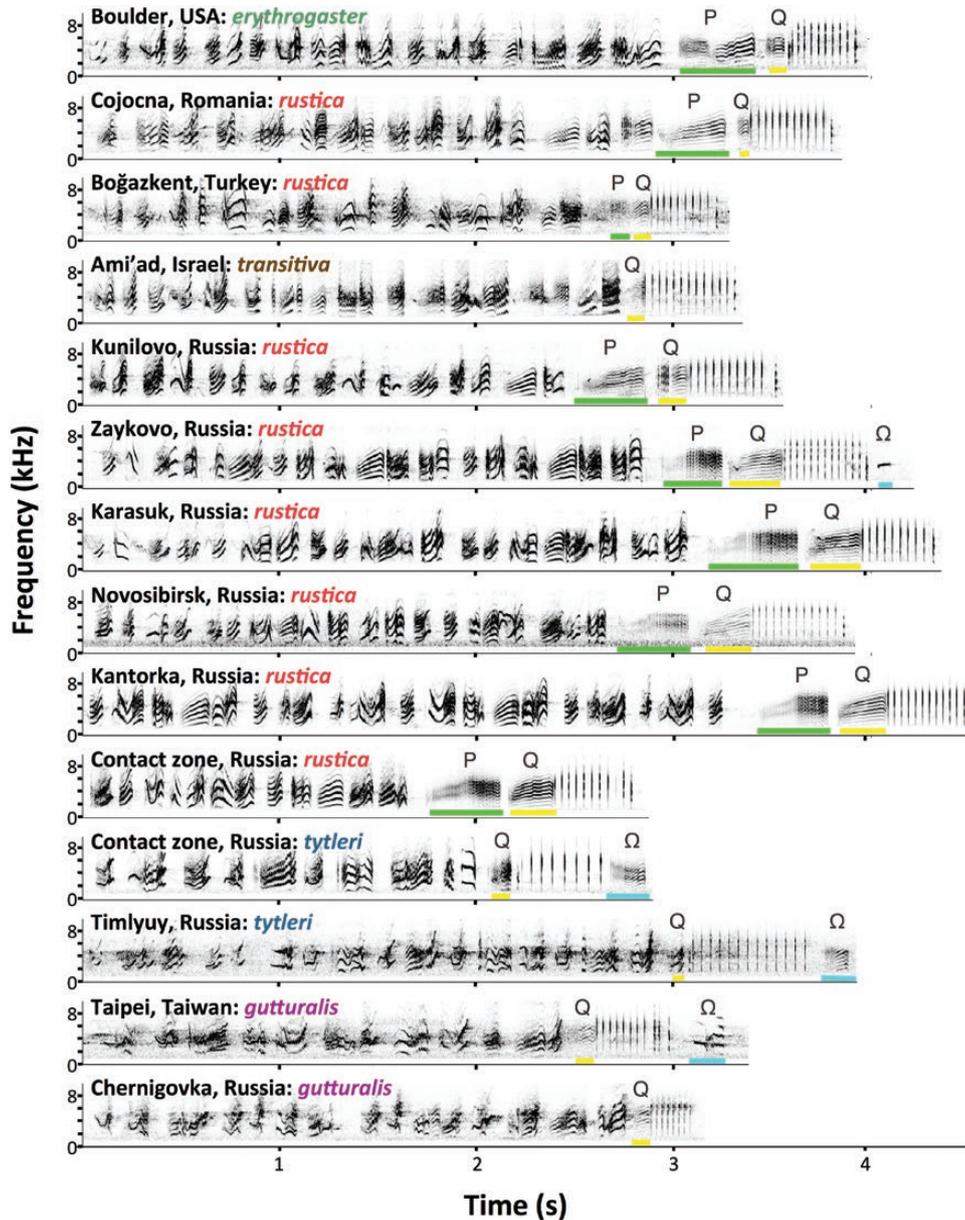


Figure 3. Song spectrograms from a single male from each study area. Spectrograms are arranged from west (top) to east (bottom). Songs were chosen based on high signal-to-noise ratios, and the most similar length and tonality across populations, although there is considerable within-individual variation (see Fig. 2). Inferred subspecies is noted after country designations. As a result of the much smaller geographical scale for sampling, villages within the contact zone (Berezovka to Khinguy) were combined for analysis, and only one representative song is shown for each subspecies from this region. Green and yellow underscores denote ‘P-’ and ‘Q-syllables’, following Galeotti *et al.* (1997). Cyan underscores indicate terminal syllables (termed ‘Ω-syllables’ here).

produced Ω-syllables, although this sample consisted of only 13 songs from four males. Still, if we assume conservatively that any given male has the lowest observed rate of Ω-syllable production found in Taipei (21.4%), the chance of not detecting an Ω-syllable in Chernigovka in 13 songs is $(1 - 0.214)^{13} = 0.044$, i.e. negligible. Thus, all *H. r. gutturalis* males in Taipei

produce terminal syllables, but not in every song, whereas in Chernigovka, male *H. r. gutturalis* do not ever produce these syllables.

In general, *H. r. rustica* did not produce Ω-syllables, with the exception of a population in Zaykovo, Russia. In this population, males exhibited low within- but high among-individual variation in Ω-syllable production.

Four of 15 males (26.7%) produced Ω -syllables in 34 of their collective 38 songs (89.5%), whereas the other 11 males never produced Ω -syllables in their collective 72 songs. Thus, in this population of the European subspecies, slightly more than a quarter of males sang Ω -syllables, and males that sang this note did so for almost all songs.

There was low within- and among-individual variation in Ω -syllable production in *H. r. tytleri* populations. In the allopatric *H. r. tytleri* population in Timlyuy, Russia, 93 of 98 total recorded songs (94.9%) ended with an Ω -syllable. All 12 recorded males in this population produced Ω -syllables, with 77.8–100% of each male's songs containing these terminal syllables. Ω -syllables thus seem to be a relatively fixed and unique attribute of the *H. r. tytleri* subspecies' song. In the contact zone, all nine males identified as *H. r. tytleri* produced terminal Ω -syllables in 83.3–100% of their songs. No pale-breasted (*H. r. rustica*) male produced an Ω -syllable, and no dark-breasted (*H. r. tytleri*) male produced a recognizable P-syllable. We tentatively interpret this result to mean that unique song attributes of each subspecies are maintained in the contact zone. However, we do not have individually matched genotypic and song data and cannot directly assess how genetic admixture influences song in the contact zone.

DISCRIMINANT FUNCTION ANALYSIS

Linear discriminant function analysis using ten random individuals from our eight well-sampled populations (training data) extracted four functions describing the major axes of song differentiation among the five barn swallow subspecies considered. Overall, the model was significant (MANOVA: Pillai = 1.61, $F_{\text{approx}} = 5.24$, d.f. = (4,75), $P = 2.85 \times 10^{-16}$). The first linear discriminant explained 78% of the among-subspecies variation, while the second and third explained 11 and 9%, and the fourth explained 1% (Table 2). Linear discriminant 1 (LD1) showed a strong negative correlation (−0.94) with rattle tempo and a positive correlation (0.50) with rattle length, demonstrating that differences in the speed and length of rattle pulses are the most important structural features for distinguishing between subspecies' songs, aside from the discrete, syntactical differences described above. Linear discriminant 2 (LD2) showed strong negative correlations with the peak frequency of the central rattle (−0.65) and Wiener entropy (−0.66). There is clear separation of *H. r. tytleri* from the other subspecies based on LD1 (Fig. 4A), as this subspecies has the slowest rattles, whereas *H. r. erythrogaster* and *H. r. gutturalis* have the fastest. On the y -axis, LD2 partly separates *H. r. erythrogaster* and *H. r. gutturalis*, indicating that the latter subspecies

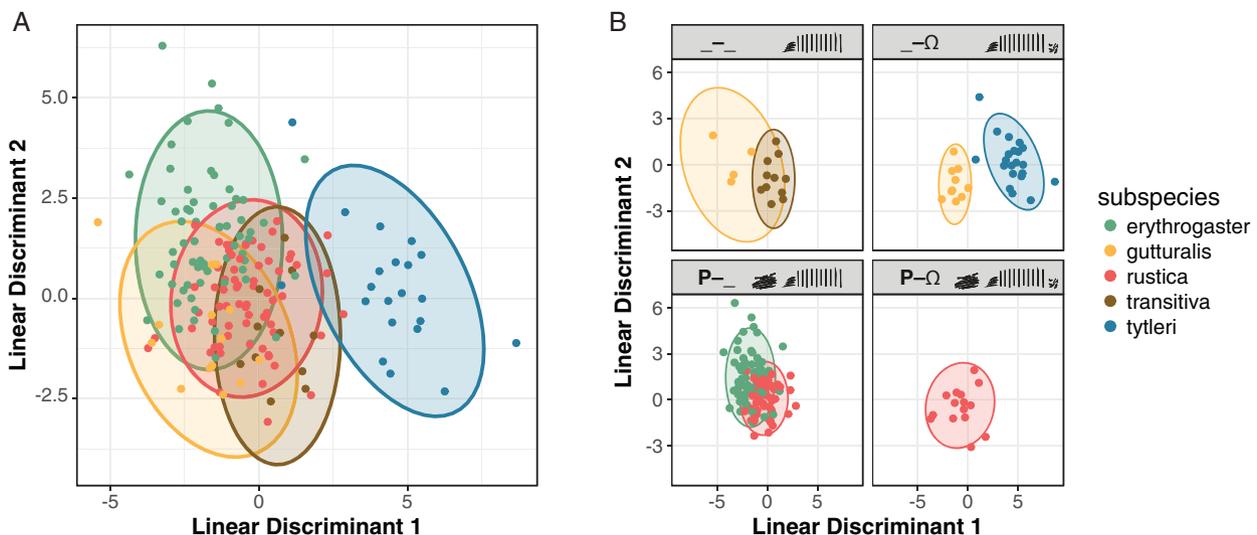


Figure 4. Subspecies difference in songs. A, the x - and y -axes indicate 180 individual scores for the first and second linear discriminants, describing differences in the nine song metrics considered. Colours reflect subspecies assignment, based on sampling location and phenotype; 95% confidence ellipses are shown for each subspecies. Higher DF1 scores primarily indicate slower rattles; higher DF2 scores indicate more tonal songs, with lower frequency rattles. Thus, *tytleri* songs were the most distinct, with much shorter, slower rattles. In B, populations are separated according to whether some individuals produced P- or Ω -syllables. Example spectrograms of terminal song sequences are shown for each category. The only subspecies populations that overlap in panel B (*erythrogaster*–*rustica* and *transitiva*–*gutturalis*) are separated by thousands of miles; thus, subspecies breeding in geographical proximity have distinct fine structural or syntactical song differences.

has higher central rattle frequencies and scratchier warbles. However, there is a substantial overlap between all the subspecies, except *H. r. tytleri*. Indeed, although posterior classification of the training data was 78.8% accurate on average (range, 50–100%), it was only 62.0% accurate for the testing subset (range, 0–100%; see Supporting Information, Table S3 for full DFA performance results). Our much more rigorous pDFA analysis of the eight well-sampled populations indicated that, on average, for the 10 000 iterations, 84.9% of individuals were correctly classified to subspecies, whereas 78.5% were correctly classified in the randomized dataset (permutation test: $P = 0.165$), indicating non-significant subspecies classification based on continuous song features.

Our analyses of spectral song characteristics alone thus did not distinguish subspecies well, largely owing to wide variation in frequency and temporal parameters within populations. However, when we accounted for discrete differences in song, based on the presence or absence of P-syllables before and Ω -syllables after the rattle, we were able to discriminate clearly between subspecies with overlapping geographical ranges; that is, although *H. r. rustica* overlaps with several subspecies in Fig. 4A, it is separated from *H. r. gutturalis* and *H. r. transitiva* by syntactical differences (Fig. 4B) and geographically separated from *H. r. erythrogaster* by a minimum of 8712 km (Table 3).

EFFECT OF SUBSPECIES SYMPATRY ON SONG DIVERGENCE

We did not find evidence of reproductive character displacement in continuous or discrete song components in the contact zone between *H. r. rustica* and *H. r. tytleri*. In fact, the two lowest song distances measured in the present study were between *tytleri* populations in Timlyuy and the contact zone (0.70) and between *rustica* populations in Kunilovo and the contact zone (0.88; Table 3). Figure 5 illustrates trait variation across the Russian populations for the four traits loading highest on the first two discriminant functions, which explained 91% of subspecies distinctiveness in our nine continuous song traits. Most of this song variation is attributable to differences in rattle tempo, which showed a correlation of -0.93 with LD1. Figure 5A demonstrates the significant divergence among the three Russian subspecies in rattle tempo (different letters above boxplots indicate significantly different means, based on Tukey's honestly significant difference tests). *Hirundo r. gutturalis* had the fastest rattles of any Russian subspecies and *H. r. tytleri* the slowest. Furthermore, *H. r. rustica* and *H. r. tytleri* were not different in the contact zone compared with allopatric populations of their own subspecies. Owing to the negative relationship between rattle tempo and length,

rattle length showed the reverse pattern, with longer rattles for *tytleri* and the shortest for *gutturalis*. Yet, for rattle length, subspecies distinctions were not as clear as for tempo (Fig. 5B). Peak frequency of the central rattle and warble Wiener entropy showed substantial variation within populations, and these traits did not show significant interpopulation or intersubspecies differences within the Russian transect (Fig. 5C, D). See Supporting Information (Fig. S1) for pairwise differences for all populations and traits.

PREDICTORS OF SONG DIVERGENCE

Pairwise acoustic, geographical, genetic and climatic differences are reported in Table 3 and illustrated in Figure 6. Figure 6B demonstrates a strong positive relationship between song and genetic distance for the Russian dataset. The broad trend is asymptotic at a song distance of approximately five; however, several pairwise song distances including *tytleri* populations are far off the cubic spline describing this relationship, highlighting the highly divergent nature of this subspecies' song. Patterns appear weaker for the other variables and the allopatric dataset. These conclusions are reinforced by our variation partitioning and redundancy analysis. As shown in Figure 6D, among allopatric population samples (USA, *erythrogaster*; Romania, *rustica*; Turkey, *rustica*; Israel, *transitiva*; and Taiwan, *gutturalis*), geographical distance, accounting for genetic and climatic distance, explained the most variation in song distance, although it was marginally non-significant [43.6% variation explained (i.e. $R^2_{\text{adj}} = 0.436$), $F_{1,6} = 6.314$, $P = 0.067$]. The individual fractions explained by genetic (-5.9% , $F_{1,6} = 0.452$, $P = 0.558$) and climatic distance (-10.6% , $F_{1,6} = 0.028$, $P = 0.867$) were even lower, and 76% of variation in song distance was unexplained. Note that some adjusted R^2 values are negative owing to the poor explanatory power of some variables.

For the more densely sampled Russian transect [from Kunilovo to contact zone (*rustica*), contact zone to Timlyuy (*tytleri*), and Chernigovka (*gutturalis*)] geographical distance, conditioned on genetic and climatic distance, predicted a small but significant amount of variation in song distance (Fig. 5E; 8.95% variation explained, $F_{1,33} = 6.29$, $P = 0.021$). In contrast, genetic distance, conditioned on geographical distance, explained 48.3% of the variation in song distance ($F_{1,33} = 29.5$, $P = 0.001$). Climatic distance, conditioned on geographical and genetic distance, explained a meager 3.33% ($F = 2.97$, $P = 0.098$), and there was 55.8% residual variation. The distinction between these datasets may be explained, in part, by the much greater variation in song distance between Russian populations (range, 0.86, 8.74) compared with the allopatric set (range, 1.25, 3.62).

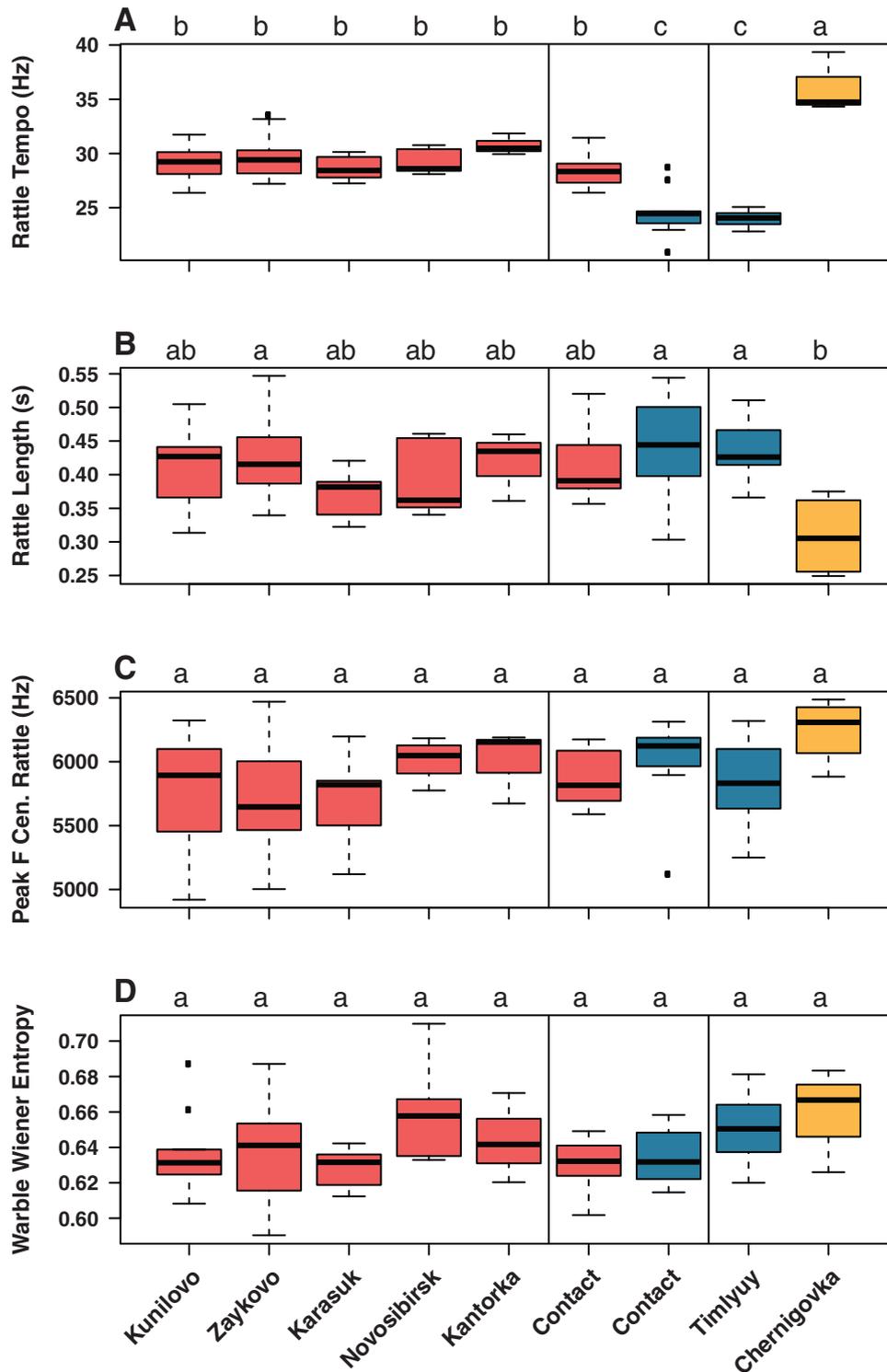


Figure 5. Variation across the Russian transect in the four song traits that loaded highly on the first two linear discriminants. A–D, differences in rattle length (A), warble length (B), rattle tempo (C) and Wiener entropy of the warble (D). Red, *H. r. rustica*; blue, *H. r. tytlerei*; and yellow, *H. r. gutturalis*. The vertical lines bound populations sampled in the contact zone. Different letters above boxplots denote significant difference using Tukey's honestly significant difference tests to account for multiple comparisons.

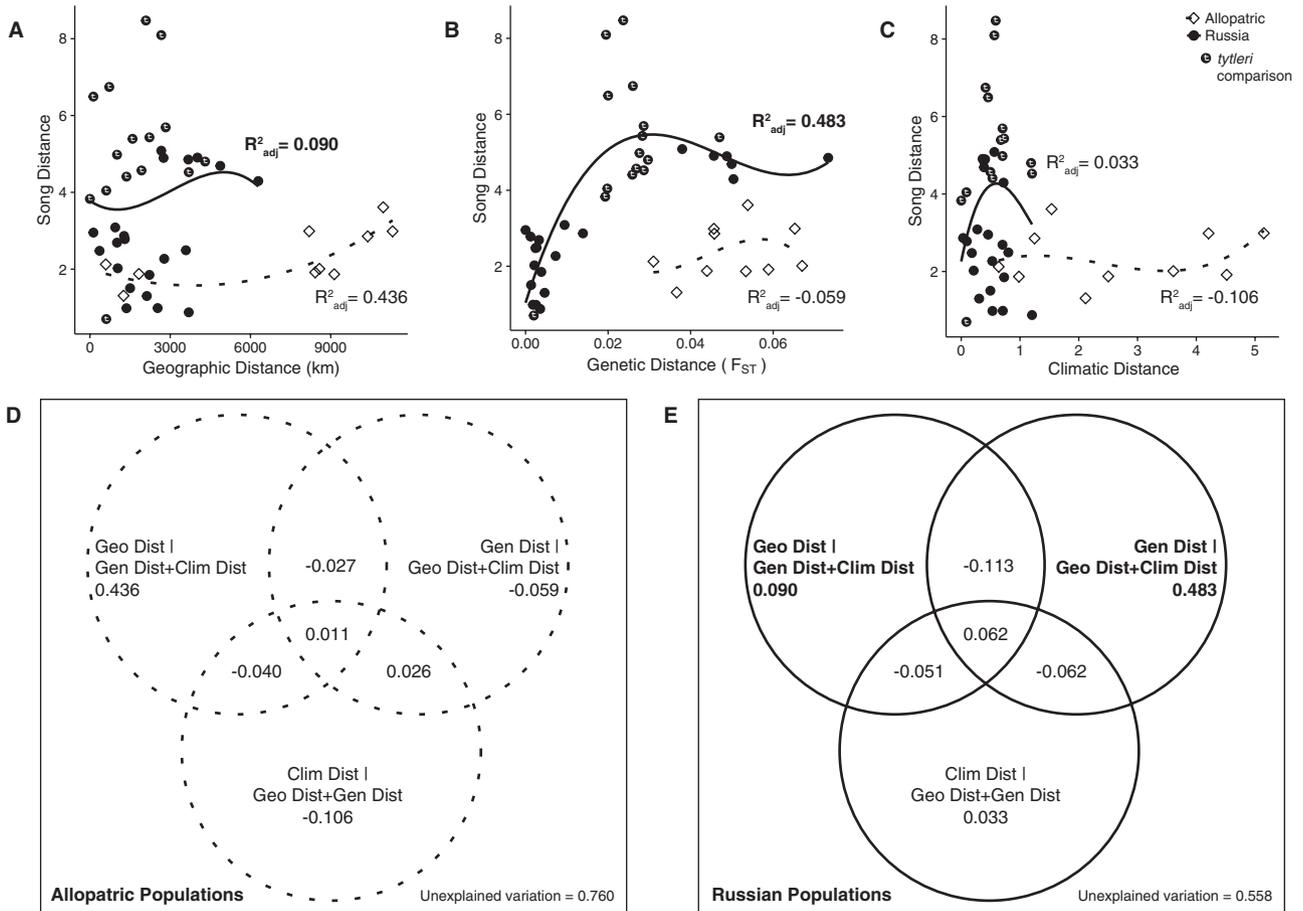


Figure 6. Relationships between population pairwise song distance and geographical (A), genetic (B) and climatic (C) distance. Open diamonds represent comparisons for the allopatric dataset, whereas closed circles represent the Russian transect populations and closed circles with a 't' indicate comparisons involving at least one *H. r. tytlteri* population. Cubic splines demonstrate patterns for each dataset. The minimal geographical distance is 0 km in the Russian *H. r. rustica*–*H. r. tytlteri* contact zone, and the maximal distance is 11 300 km, between Boulder, CO, USA and Taipei, Taiwan. D, E, variation partitioning plots show the extent to which geographical distance (in kilometres), genetic distance (mean genome-wide F_{ST}) and climatic distance explain song distance (Euclidean distance between discriminant function scores) for the allopatric (D) and Russian (E) transect populations. Note that partition labels follow the convention: X1 | X2, indicating the variation in song distance explained by X1, conditioned on X2. No predictor conditioned on genetic and climatic distance, explained significant variation in song divergence among allopatric populations. In contrast, for the Russian dataset, genetic distance, conditioned on geographical and climatic distance, explained a large and significant portion of song divergence, as did geographical distance to a much lesser extent.

DISCUSSION

We provide the first evidence of song divergence among barn swallow subspecies, adding an acoustic modality to our growing knowledge of phenotypic differentiation across this recent and rapid subspecies radiation. After accounting for significant within-individual song variation, we found that temporal traits, especially the rate of terminal trills (rattles), were the most important continuous traits for distinguishing subspecies, whereas measures of frequency and tonality varied more within

than among subspecies. Although our DFA showed substantial overlap among subspecies, when combined with discrete syntactical differences, the first two linear discriminants reliably distinguished between pairs of subspecies that overlap geographically. In contrast, some pairs of subspecies that are separated by thousands of kilometres overlapped substantially in these song attributes (e.g. *H. r. rustica* and *H. r. erythrogaster* in Fig. 4B). We also showed that at the coarsest global scale, geographical distance explained a small (and marginally non-significant) amount of variation in continuous song

trait divergence, whereas climatic and genetic distance explained effectively no variation in song. At a slightly finer continental scale, where populations are likely to interact with homo- and heterotypic individuals, genetic distance was a strong predictor of continuous song trait divergence, whereas geographical distance contributed some explanatory value, and climatic distance did not. These results imply a role for genetic drift, cultural drift and/or non-ecologically based models of sexual selection in barn swallow song divergence (see Table 1).

SONG DIVERGENCE WITHIN AND AMONG SUBSPECIES

None of the spectral measures included in our study loaded strongly in discriminant function analysis, as these traits varied widely within and across populations. This overlapping frequency range may represent species physiological limits of singers or optimal tuning to receivers (Ryan & Wilczynski, 1988; Endler, 1992). However, divergence in certain song elements, such as the P-syllable and rattle, might be driven by sexual selection. Previous work in European *H. r. rustica* indicates that several acoustic traits, including rattle tempo, peak frequency of the P-syllable, and repertoire size were associated with pairing date (Garamszegi *et al.*, 2006). Males in an Italian population were also shown to increase rattle length and peak frequency in response to the number of competitors, and rattle length is positively correlated with testosterone levels (Galeotti *et al.*, 1997). Furthermore, among North American *H. r. erythrogaster*, rattle tempo was positively associated with paternity success and negatively related to nearest competitor distance (Wilkins *et al.*, 2015), indicating the potential role of this trait in both competition and mate choice. Although detailed song studies have not taken place in other subspecies populations, these findings are consistent with a role of sexual selection in driving trill divergence among subspecies. This result is also consistent with findings in other recently diverged taxa, such as crickets (Gray & Cade, 2000; Mendelson & Shaw, 2005), antbirds (Seddon & Tobias, 2007), wrens (Toews & Irwin, 2008) and cichlids (Amorim *et al.*, 2008), which are most easily distinguished by temporal differences in acoustic traits.

As frequency characteristics are often tied to body mass and the size of acoustic signalling structures (Ryan & Brenowitz, 1985; Gillooly & Ophir, 2010), whereas the rate or length of acoustic signal production is probably subject to fewer constraints, variation in temporal traits might be a common distinguishing feature between recently diverged populations with similar ecologies. Nonetheless, all acoustic signals are bounded by energetic, physiological or developmental constraints (Gil & Gahr, 2002; Wilkins *et al.*, 2013). The mechanism of barn swallow rattle production is unknown and is distinct from the well-known trade-off between trill rate

and frequency bandwidth in many bird species (Podos, 2001; Wilson *et al.*, 2014). Unlike Darwin's finches and numerous other birds whose trills are constrained by the trade-off between beak size and velocity (Herrel *et al.*, 2009), barn swallows keep their beaks open during trill production (M.R.W., personal observation). Instead, a negative correlation between trill rate and trill length (Wilkins *et al.*, 2015) suggests a performance trade-off that might bound the evolution of this trait across populations, with some populations favouring long and others fast rattles. This type of trade-off is similar to the call rate–duration trade-off in treefrogs (Wells & Taigen, 1986; Reichert & Gerhardt, 2012). Comparative studies of evolutionary rates for different types of acoustic traits across subspecies, species and higher taxonomic levels are necessary to shed light on the relative lability of frequency, amplitude or temporal acoustic features during vocal evolution and speciation.

EFFECT OF SUBSPECIES SYMPATRY ON SONG DIVERGENCE

The key exceptions to the above pattern are comparisons involving *H. r. tytleri* populations (filled circles with a 't' in Fig. 6). The song of *H. r. tytleri* is the most distinct of any subspecies, and the top seven song distances in our study involve comparison with a *tytleri* population (Table 3). One potential explanation for this divergence is rapid evolution in a small founder population (Lynch & Baker, 1994). Previous phylogenetic work suggests that the *tytleri* subspecies arose from *erythrogaster* through subsequent colonization of northeastern Asia (Zink *et al.*, 2006; Dor *et al.*, 2010; Fig. 1A). Patterns of mitochondrial evolution suggest a recent founder population for *tytleri* within the last ~25 000 years, which has subsequently maintained a small population size with low genetic diversity (Zink *et al.*, 2006). It is thus likely that genetic patterns have evolved primarily through drift, making it very plausible that the pronounced acoustic divergence in this subspecies derives from founder effects and rapid genetic and cultural drift of small populations, as observed in other bird species (Lynch & Baker, 1994; Baker, Baker & Baker, 2001; Parker, Hauber & Brunton, 2010). Alternatively, or possibly in addition, pronounced divergence in the *tytleri* subspecies from the other four sampled subspecies might result from reproductive character displacement with the more closely related *gutturalis*. It is noteworthy that by far the highest song distances in Table 3 are between *tytleri* populations and Chernigovka *gutturalis* populations in eastern Russia. Unfortunately, we were unable to sample songs in the expansive > 500 km *tytleri*–*gutturalis* contact zone (Scordato *et al.*, 2017), and further song and genetic sampling within and outside this zone is necessary to assess how hybridization levels affect divergence in song and the response to song differences.

Thus, although there is some evidence for accelerated signal divergence through stochastic processes in a small founder population, we cannot rule out reproductive character displacement resulting from cascading effects of reinforcement between *tytleri* song and *gutturalis* song as a possible driver of acoustic divergence. We can, however, eliminate reproductive character displacement in the *rustica*–*tytleri* contact zone. In contrast to previous work showing evolution of increased signal similarity (Seddon & Tobias, 2010; Tobias *et al.*, 2014) or dissimilarity (Marshall & Cooley, 2000; Kirschel *et al.*, 2009b) upon secondary contact (reviewed by Gerhardt, 2013), we failed to show any evidence of change in continuous or discrete song traits resulting from subspecies sympatry. Given that *H. r. tytleri* greatly outnumbered *H. r. rustica* in villages where they both occurred (and were behaviourally dominant, based on local accounts), we might have expected *rustica* males to shift song parameters towards *tytleri* for mate attraction if homotypic mates are in short supply, or away from *tytleri* to minimize aggression. Such asymmetric patterns of character displacement have been shown in several bird taxa (Haavie *et al.*, 2004; Halfwerk *et al.*, 2016; Hamao, Sugita & Nishiumi, 2016). Instead, the lack of any song trait changes in the contact zone relative to flanking allopatric populations, together with evidence of very limited ongoing hybridization (Scordato *et al.*, 2017), suggests that there has been no selection for increased divergence. Indeed, *H. r. tytleri* was the most distinct subspecies in continuous acoustic space (Fig. 4A) and has two discrete song differences from contact zone *H. r. rustica* (Fig. 4B). One explanation for a lack of acoustic character displacement might be low levels of ecological competition, if aerial insects and nesting habitats are plentiful, and that mating signals are already perceived as distinct. Alternatively, sympatric populations might have shifted receiver perception of heterotypic song (i.e. the window of recognition), rather than the songs themselves (Amézquita *et al.*, 2011; Pasch, Bolker & Phelps, 2013; Hudson & Price, 2014). Further study of subspecies interactions and responses to playbacks are required to distinguish these possibilities.

PREDICTORS OF SONG DIVERGENCE

There are primarily four processes driving divergence in acoustic signals (genetic drift, cultural drift, ecological selection and sexual selection), which may interact to varying degrees (Wilkins *et al.*, 2013). Table 1 summarizes the predictions, rationale and current evidence for each of these processes as primary drivers of acoustic divergence. For example, genetic and cultural drift should lead to a linear accumulation of acoustic differences with geographical and/or genetic

distance; that is, because of the effect of isolation by distance, populations in close geographical proximity are expected to be more genetically similar and to have more similar signals (Wright, 1943; Slatkin, 1993). As a result, acoustic divergence primarily through genetic drift predicts positive relationships between acoustic, geographical and genetic distance (Irwin *et al.*, 2008; Campbell *et al.*, 2010). Although cultural drift will often produce the same relationship, species with postdispersal learning (i.e. in which immigrants can learn non-local songs and breed successfully after natal dispersal), will show an association between acoustic and geographical distance but not between acoustic and genetic distance (Podos & Warren, 2007; Sun *et al.*, 2013; González & Ornelas, 2014). Additionally, some indirect benefits models of sexual selection, with minimal or no dependence on the environment for signal content (i.e. Fisherian runaway and sexy sons hypotheses), may also produce a linear accrual of signal distance with genetic distance; that is, stochastic shifts in the strength, direction or targets of sexual selection may lead to the clock-like accumulation of sexual signal differences with genetic (but not necessarily geographical) distance (Irwin *et al.*, 2008; Martin & Mendelson, 2012; Winger & Bates, 2015). However, in general, it is challenging to differentiate the effects of these drivers of acoustic divergence, and non-ecological forms of sexual selection are likely to interact extensively with cultural and genetic drift to result in gradual evolution of signal differences and reproductive isolation (Irwin *et al.*, 2008; Uyeda *et al.*, 2009). In support of this, recent research shows a linear reduction in playback response to more divergent song stimuli, even after controlling for genetic relatedness (Sosa-López, Martínez Gómez & Mennill, 2016; Lipshutz *et al.*, 2017).

Our results provide different clues about the drivers of acoustic signal divergence at different spatial scales. Notably, song divergence levels in both continuous and discrete traits were significantly lower among allopatric populations compared with the Russian transect populations. A potential, yet untested, explanation is that low signal divergence among geographically distant populations might stem from a lack of biotic interactions, allowing for convergence by chance or evolutionary conservation of song characteristics. In contrast, structural song divergence in Russian populations shows a weak relationship with geographical distance and a strong association with genetic divergence, indicating a link between the processes affecting population gene flow and song divergence. Given our knowledge of sexual selection for certain song attributes, it is likely that genetic and cultural drift interact with random shifts in the strength and direction of sexual selection to build up acoustic and genetic differences gradually. However, signal divergence seems to asymptote (Fig. 6B) and

might even diminish as populations become more geographically and genetically distant.

In line with the overall weak pattern of acoustic adaptation, we failed to find any relationship between song distance and climatic distance. This, of course, does not rule out acoustic adaptation in barn swallow song, as our measures of acoustic environment were indirect, and we did not perform signal propagation tests. However, this is consistent with the idea that this form of selection may primarily be important for a subset of taxa moving into new environments with distinct signal attenuation properties (Wilkins *et al.*, 2013); that is, many taxonomic groups inhabit only one type of environment or encounter a broad range of climates between breeding and wintering grounds (such as in barn swallows) and may not show strong effects of ecology on signal characteristics of different populations (e.g. Graham *et al.*, 2017).

SONG LEARNING

A major limitation of the present study is our inability to distinguish between culturally and genetically inherited song characteristics. As with most wild bird species, we currently know almost nothing about the heritability of song components in barn swallows. We do know that barn swallows have high offspring natal dispersal (Møller, 1994a, b), and there is evidence that male songs change with age (Galeotti *et al.*, 2001; Garamszegi *et al.*, 2005), which may imply postdispersal learning. If males born into one genetic and cultural background can learn new songs and breed in a population with a different genetic background, this should reduce the predicted link between song divergence and genetic distance. However, for the Russian transect, we recovered a strong relationship between genetic and acoustic distance, accounting for climate and geographical distance, which we interpret as gradual, concurrent divergence of genes and song in allopatry. Future studies should aim to quantify heritability of song traits, characterize the ontogeny of song development more rigorously and attempt to correlate genotypes with acoustic phenotypes in order to gain a better understanding of the relative importance of genetic and cultural evolution in the observed patterns.

CONCLUSIONS

This study adds to accumulating evidence for a role of stochastic processes in the gradual build-up of acoustic divergence and associated reproductive isolation (Pröhl *et al.*, 2006, 2007; Rugg *et al.*, 2006; Irwin *et al.*, 2008; Campbell *et al.*, 2010; González, Ornelas & Gutiérrez-Rodríguez, 2011; Sosa-López *et al.*, 2013, 2016; Lipshutz *et al.*, 2017). Recent theoretical (Mendelson, Martin & Flaxman, 2014)

and empirical (Martin & Mendelson, 2012; Winger & Bates, 2015) work suggests that phenotypic divergence via sexual selection may accrue in a clock-like manner through a mutation-order process (Mani & Clarke, 1990), in which populations experiencing similar ecological selection diverge through random fixation of selectively equivalent but incompatible alleles (i.e. song characteristics or preferences). Thus, for taxa in which sexual selection is thought to be the dominant driver of acoustic divergence, such as numerous species of birds (Irwin, 2000; Price & Lanyon, 2004; Seddon *et al.*, 2008), arthropods (Fitzpatrick & Gray, 2001; Mendelson & Shaw, 2005; Rodríguez *et al.*, 2006; Sullivan-Beckers & Cocroft, 2010) and frogs (Boul *et al.*, 2007), we might expect the same positive linear association between acoustic and genetic divergence. However, this remains to be tested for the majority of species, as the roles of ecological selection, sexual selection and drift on signal evolution have not typically been investigated within the same systems (Wilkins *et al.*, 2013). As ours is among very few studies (e.g. Lipshutz *et al.*, 2017; Kenyon *et al.*, 2017) to estimate the association between acoustic and genetic divergence using high-throughput sequencing, it should be informative to revisit previous studies that failed to detect this relationship using a small number of mitochondrial or microsatellite markers. For example, does a strong association between acoustic and ecological distance but not between acoustic and genetic distance in some birds and bats (Nicholls *et al.*, 2006; Rugg *et al.*, 2006; Sun *et al.*, 2013) result from low genetic coverage, rapid ecological speciation and consequent acoustic adaptation; a reduced influence of song on reproductive isolation; or phenotypic plasticity/postdispersal learning? Also, as noted by Kenyon *et al.* (2017), observed relationships between acoustic and genetic distance might depend on the geographical scale of analysis (e.g. if gene flow within a hybrid zone washes out a broader pattern of isolation with distance in allopatry). Answering these questions will be essential to strengthen our understanding of the prevailing forces driving the evolution of communication systems. Although we highlight a role for stochastic processes for song evolution in barn swallows, deterministic ecological processes clearly dominate the evolution of some song parameters for some species (Boncoraglio & Saino, 2007; Ey & Fischer, 2009; Mason & Burns, 2015). Identifying the particular evolutionary contingencies, natural histories or selection pressures that lead to the dominance of one evolutionary process in signal evolution should be a major goal. In addition, future work, leveraging recent advances in automated spectral analysis (Ranjard & Ross, 2008; Große Ruse *et al.*, 2016) and network motif approaches (Weiss *et al.*, 2014) to analyse finer scale syllabic complexity, could provide further biologically

relevant insight into the complex dynamics of song evolution in this system.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Differences across populations for the nine continuous song traits. Significant differences following Tukey's HSD *post hoc* tests are indicated by different letters at the top of each plot. Different colours indicate subspecies, as follows: green, *Hirundo rustica erythrogaster*; red, *H. r. rustica*; brown, *H. r. transitiva*; blue, *H. r. tytleri*; and yellow, *H. r. gutturalis*. All traits differ significantly across at least two populations, although there is broad overlap across subspecies in most parameters; particularly, peak frequency of the central rattle. In contrast, rattle tempo shows pronounced differentiation across subspecies and is the only trait to show a significant difference between contact zone *H. r. tytleri* and *H. r. rustica* after controlling for multiple comparisons.

Table S1. Summary of sampled populations. Means (SE) are included for each song parameter. Note that the shaded grey locations were collapsed into 'contact zone *rustica*' and 'contact zone *tytleri*' for analysis. Letters 'r' and 't' indicate the number of *rustica* and *tytleri* individuals sampled in this region, respectively.

Table S2. Climate variable loadings on principal component analysis used to calculate climatic distance. Eigenvalues and loadings are calculated following Varimax rotation. BIO# for each variable corresponds to bioclim codings at www.worldclim.org/bioclim.

Table S3. Discriminant function analysis sampling details and accuracy. Ten random individuals were selected for the training set from the eight well-sampled populations. The remaining individuals from all populations were used for the testing set. All song recordings were used for selected individuals in each set.

SHARED DATA

All song recordings, individual song measurements, and averaged song measurements are available at Figshare: <https://doi.org/10.6084/m9.figshare.5866926.v1>.