Call divergence is correlated with geographic and genetic distance in greenish warblers (*Phylloscopus trochiloides*): a strong role for stochasticity in signal evolution?

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Abstract

Divergence in signalling systems might play a central role in speciation. To assess the importance of possible causes of signal divergence, we examine two types of vocalizations within a geographically variable species complex, the greenish warblers (*Phylloscopus trochiloides* Sundevall). Calls, which are used by both sexes throughout the year, and songs, which are sung primarily by breeding males, differ distinctly between two distinct Siberian forms. Through a ring of southern populations that connect the northern forms, signal divergence is correlated with both geographic distance and genetic divergence. Calls and songs differ in their particular patterns of geographic variation, probably because of the larger influence of sexual selection on songs than on calls. These patterns are supportive of neither acoustic adaptation nor morphology being major drivers of divergence in vocalizations. Rather, these results support the importance of stochastic evolution of communication systems in the evolution of new species.

Introduction

Closely related species often differ dramatically in the signals that they use to communicate, and these differences might play a central role in causing reproductive isolation (West-Eberhard, 1983; Henry, 1985; Jones, 1997; Slabbekoorn & Smith, 2002a; Mendelson & Shaw, 2005; Price, 2008). Understanding the causes of signal evolution is therefore crucial to the understanding of how biodiversity arises. Although a variety of possible causes have been hypothesized, their relative importance remains unknown. Herein, we consider geographic variation in vocalizations of greenish warblers (*Phylloscopus trochiloides* Sundevall), a species complex that is particularly well suited to the study of evolutionary divergence (Irwin *et al.*, 2001a,b; Irwin *et al.*, 2005), with the goal of determining which processes appear to be most important in driving signal divergence.

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One group of hypotheses emphasizes deterministic processes in causing signal divergence. Habitat differences might cause selection for signal divergence, because different frequencies of sound travel best in different environments or because different habitats have different types of ambient noise (the 'acoustic adaptation hypothesis'; Morton, 1975; Wiley & Richards, 1982; Ryan & Brenowitz, 1985; Sorjonen, 1986; Badyaev & Leaf, 1997; Buskirk, 1997; Slabbekoorn & Smith, 2002a,b; Slabbekoorn, 2004; Seddon, 2005; Baker, 2006; Nicholls & Goldizen, 2006). Signal divergence might also be caused by morphological divergence, for example, because organisms of different sizes and shapes have different constraints on the sounds that they can produce (Ryan & Brenowitz, 1985; Podos, 1996; Podos & Nowicki, 2004; Seddon, 2005). Selection for species recognition might lead to signal divergence through reproductive character displacement or reinforcement (Dobzhansky, 1940; Miller, 1982; Howard, 1993; Seddon, 2005), acting deterministically to cause whatever signal differences already exist between two species to become more extreme. Each of these hypotheses generates clear predictions about the direction of evolution in particular situations.

Another group of hypotheses emphasizes stochastic processes in causing signal divergence. For example, sexual (or social) selection might cause rapid evolution of signals because of the attractiveness of novelty, the potential for runaway change and the absence of well-defined optima (Fisher, 1930; West-Eberhard, 1983; Andersson, 1994; Iwasa & Pomiankowski, 1995; Jones, 1997; Mendelson & Shaw, 2005). Signal divergence might also occur in the absence of any kind of selection because of genetic and cultural mutation and drift (Lemon, 1975; Mundinger, 1982; Lynch, 1996; Martens, 1996; Payne, 1996). According to these hypotheses, signal divergence is a highly stochastic and unpredictable process.

Finally, some hypotheses equally emphasize both stochastic and deterministic factors. For example, signals could evolve in response to a balance between sexual selection for elaboration of a signal and natural selection opposing elaboration, and ecologically caused changes in that balance could lead to divergence (e.g. Kirkpatrick, 1987; Price, 1998; Irwin, 2000). A related hypothesis is that intra- and intersexual selection can oppose each other (Howard, 1974), and that ecological factors can alter the relative strengths of those selective forces (Catchpole, 1980, 1982; Irwin, 2000). In these hypotheses, sexual selection plays a highly stochastic role, but the environment can play a predictable role in influencing the context in which sexual selection acts.

Greenish warblers are particularly useful for the study of signal evolution because they display many levels of geographic variation, from small differences between neighbouring populations to differences between separate species. Morphological, behavioural and molecular variation indicates that the greenish warbler complex is a ring species: two forms coexist without interbreeding in central Siberia, but these forms are connected by a chain of intergrading populations encircling the uninhabited Tibetan Plateau to the south (Fig. 1; Ticehurst, 1938; Mayr, 1942; Irwin *et al.*, 2001a,b, 2005). Molecular genetic data and considerations of Pleistocene climatological history indicate that the west Siberian (*P. t. viridanus*) and east Siberian (*P. t. plumbeitarsus*) forms of greenish warblers each resulted from northward expansions of southern forms along separate routes into west and east Siberia (Irwin *et al.*, 2001a, 2005). Thus, geographical variation in signals can be interpreted in a historical context in terms of evolution from the traits of the southern forms to those of the northern forms.

Variation in signals can also be compared with known patterns of habitat variation throughout the breeding range of greenish warblers (Irwin, 2000). The two Siberian forms breed in remarkably similar habitats consisting of dense, lowland forests. By contrast, southern populations in the Himalayas breed in sparsely wooded areas near treeline. At intermediate latitudes, in central Asia and central China, greenish warblers inhabit forests of intermediate tree density at intermedi-
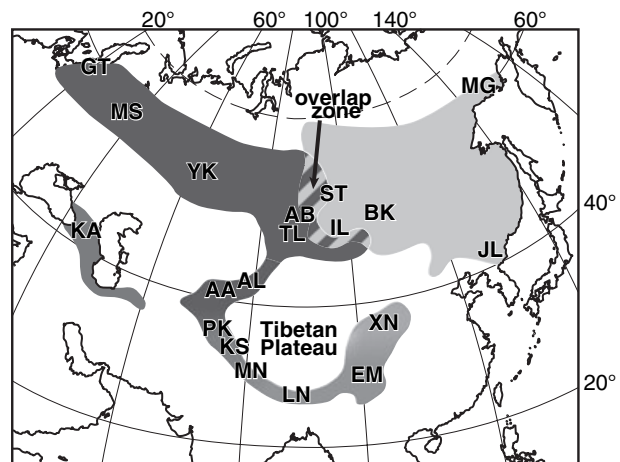

Fig. 1 Map of Asia showing the breeding range of the greenish warbler species complex. Shades of grey correspond roughly to genetic similarity of different populations of greenish warblers. West Siberian *Phylloscopus trochiloides viridanus* and east Siberian *P. t. plumbeitarsus* meet in a narrow overlap zone in central Siberia, where the two forms are reproductively isolated (Ticehurst, 1938; Irwin *et al.*, 2001a, 2005). These forms are connected by a chain of progressively intermediate forms that inhabit the mountains encircling the Tibetan Plateau (*P. t. ludlowi* in the western Himalayas, *P. t. trochiloides* in the central and eastern Himalayas and *P. t. obscuratus* in central China). The gap between *P. t. obscuratus* and *P. t plumbeitarsus* in northern China is probably because of recent habitat destruction (Irwin *et al.*, 2001a), and those two taxa are relatively closely related relative to variation throughout the species complex. The geographically disjunct form *P. (t.) nitidus* inhabits the Caucasus Mountains in the west. Locations where vocalizations were recorded are indicated by two-letter codes (Table 1).

ate elevation. Thus, greenish warblers underwent parallel habitat shifts during the two northward expansions into Siberia. If habitat-induced acoustic adaptation were the major force in the evolution of greenish warbler vocalizations, we would expect parallel changes in vocalizations during the two expansions.

Likewise, a similar prediction results from the hypothesis that morphometric change drives changes in vocalizations. Morphometrically, *viridanus* and *plumbeitarsus* are more similar to each other than they are to their southern relatives, which are larger (Irwin *et al.*, 2001a). Thus, *viridanus* and *plumbeitarsus* have experienced parallel decreases in body size, presumably because of the parallel ecological shifts during the two expansions into Siberia. Thus, if either habitat shifts or morphometric change is the primary driver of changes in vocalizations, we would expect *viridanus* and *plumbeitarsus* to have similar vocalizations, while together differing from those in the south.

An advantage of using songbirds to study evolution of signals is that most songbird species have two general types of vocalizations, calls and songs, which differ in their social role (Catchpole & Slater, 1995; Marler, 2004).

Calls tend to be short, simple vocalizations that are produced by both sexes throughout the year. Songs are generally longer, more complex vocalizations produced primarily by males in the breeding season. Songs play an important role in mating, serving both to attract females and to repel rival males, whereas calls are used in a variety of contexts that are not usually directly related to mating. Most studies of geographic variation in passerine bird vocalizations have focused on song, and relatively little attention has been given to calls (Mundinger, 1982; Martens, 1996; exceptions include Marler & Mundinger, 1975; Brown & Farabaugh, 1991; and Laiolo & Tella, 2006).

Avian vocalizations, thus, consist of two kinds of signals that can be expected to differ in the amount they are subject to sexual selection, potentially causing calls and songs to show different patterns of evolution. On the other hand, because both songs and calls are acoustic signals, they may be subject to similar habitat-based selection due to the acoustic properties of the environment (Morton, 1975; Wiley & Richards, 1982; Slabbekoorn & Smith, 2002a,b; Slabbekoorn, 2004). Furthermore, calls and songs may be subject to similar physiological constraints because they are produced by the same vocal organs (Podos, 1996; Podos & Nowicki, 2004) and by overlapping neurological pathways. These factors may cause songs and calls to evolve in similar ways.

In this paper, we quantify variation in the most commonly vocalized call type of adult greenish warblers and compare call variation with song variation, which was originally analysed in a previous paper (Irwin, 2000). The calls we investigate are used by both males and females in both the breeding and nonbreeding seasons (Price, 1981; Katti, 2001); they are used as territorial calls in winter and during the breeding season to express ownership of a territory and also as alarm calls to announce the presence of a predator (D. Wheatcroft and T. Price, pers. comm.). Thus, we refer to them as 'territorial alarm calls'. Greenish warblers have a variety of other calls that are used less often, in the context of close communication between family members or enhanced aggression between territorial rivals (D. Wheatcroft and T. Price, pers. comm.). Territorial alarm calls are much shorter than songs and are easily distinguished from them.

We use call variation to answer three primary questions. First, is call variation consistent with acoustic adaptation in the breeding season or morphometric change being the primary driver of call evolution? Greenish warblers have experienced parallel habitat and morphometric shifts during the two northward expansions into Siberia (Irwin, 2000); the hypothesis that acoustic adaptation and/or morphometric change are playing important roles leads to a clear prediction: we would expect some amount of parallel evolution of calls during the two northward expansions. Second, how coupled are call evolution and song evolution? Close

similarity in patterns would suggest that the same forces have influenced call and song evolution. By contrast, differences in patterns of song and call variation might be a result of different social roles of the two forms of vocalization and thereby point to an important role of sexual and social selection in the divergence of vocalizations. Third, how closely does vocalization divergence correspond to genetic divergence? A close coupling of call and genetic divergence would suggest that calls might be evolving unpredictably because of the stochastic forces of mutation, drift and social selection.

Materials and methods

We obtained recordings of territorial alarm calls from 39 greenish warblers at 14 locations distributed throughout their breeding range (Fig. 1; Table 1). Most recordings were made by DI, who used an Audio-Technica (Stowe, OH, USA) 815a shotgun microphone and a Sony TCD-D7 digital audio tape recorder (Sony, Park Ridge, NJ, USA). We also analysed a number of recordings contributed by other researchers (Table 1).

Spectrograms of calls were produced using the program CANARY 1.2 (Mitchell et al., 1995) on default settings. For each individual, we chose the five most cleanly recorded calls to analyse further [for the following four individuals, we had fewer than five recorded calls: one bird from site TL (three calls), one from AB (two calls), one from LN (three calls), one from EM (four calls)], for a total sample of 187 calls in the whole study. We measured the following six variables from each call: maximum frequency, minimum frequency, peak frequency (the frequency at which the amplitude of sound is the greatest), peak time (the time at which the amplitude is the greatest, measured from the start of the call), length, and the number of distinct upward or downward strokes in the call. The first five variables were measured from on-screen spectrograms using the measurement panel of CANARY 1.2, after adjusting the amplitude of each call to a standard level so that measurements were independent of recording volume. The measurements of peak frequency and peak time were fully automated in CANARY; after the researcher determined the length of each call and the maximum and minimum frequencies visually, CANARY determined the frequency and time during the call at which amplitude was the highest. The measurements of maximum frequency, minimum frequency and call length were based on the part of the spectrogram that appeared to be clearly made by the bird (that is, ignoring background noise, calls of other individuals or species and sound reverberations); we have found that different observers are quite consistent in measuring these variables for a given set of calls. For each individual, mean values were calculated for each of the variables. We tested whether each variable showed statistically significant differences between sites using Kruskal–Wallis tests. We used principal components analysis (PCA) on the

Table 1 Locations where calls and songs of greenish warblers were recorded, and the number of birds recorded at each and included in the call and song analyses.

Location	Subspecies	Map symbol	Latitude (°N)	Longitude (°E)	Birds recorded (calls)	Birds recorded (songs)	Recorded by
Kazbegi	*nitidus*	KA	42	45	1	1	P. Alström & U. Olsson
Gotland	*viridanus*	GT	57	18	–	1	DI
Moscow	*viridanus*	MS	56	38	–	1	B. Veprintsev
Yekaterinburg	*viridanus*	YK	57	60	1	5	DI & J. Irwin
Maly Abakan	*viridanus*	AB	52	90	2	-	DI
Teletsk Lake	*viridanus*	TL	52	88	2	5	DI
Almaty	*viridanus*	AL	43	77	2	-	P. Alström & U. Olsson
Ala Archa	*viridanus*	AA	43	74	4	5	DI
Pakistan	*ludlowi*	PK	35	74	–	5	S. Gross & T. Price
Kashmir	*ludlowi*	KS	34	75	–	2	K. Marchetti & T. Price
Manali	*ludlowi*	MN	32	77	2	5	Z. Benowitz-Fredericks & K. Marchetti
Langtang	*trochiloides*	LN	28	85	5	5	DI
Emeishan	*trochiloides*	EM	30	103	3	3	P. Alström & U. Olsson
Xining	*obscuratus*	XN	37	102	5	5	DI & J. Irwin
Jilin	*plumbeitarsus*	JL	44	127	1	1	P. Alström & U. Olsson
Magadan	*plumbeitarsus*	MG	60	151	–	1	T. Price & K. Marchetti
Baikal	*plumbeitarsus*	BK	52	105	5	4	DI & P. Alström
Ilinka	*plumbeitarsus*	IL	51	96	2	–	DI & J. Irwin
Stolbi	*plumbeitarsus*	ST	56	93	4	5	DI

correlation matrix of individual mean values to summarize broad patterns of geographic variation in calls. Each variable that showed significant differences between sites was used in the PCA (all except peak time).

To quantitatively test for evidence that habitat or morphology has an effect on call evolution, we tested whether the first principal component of variation in calls was correlated with: (1) habitat; and (2) morphometrics. For a quantitative measure of habitat, we measured 'habitat openness' at five research sites (YK, AA, LN, IL and ST) at which detailed maps of greenish warbler habitat occupancy had already been made (data for four of these was presented by Irwin, 2000). This was performed by surveying transects through habitat occupied by greenish warbler territories. Transects were chosen by randomly choosing a point within greenish warbler habitat, and then randomly determining direction (by spinning a compass). Observations of canopy cover were then made every 10 m along the transect. If the transect reached the edge of greenish warbler habitat, a new transect was again randomly chosen starting within the habitat. 'Habitat openness' was defined as the per cent of observation points that had no vegetation above 2 m in height. The number of sampling points ranged from 49 to 139 (mean of 95.8) among the five sites. Confidence intervals on the estimate of per cent habitat openness were made using the Agresti–Coull method (Whitlock & Schluter, 2009). It should be noted that although habitat openness was only quantified at five research sites, much informal observation of greenish warbler habitat confirms that these sites well repre-

sent the habitat characteristics of the larger regions each is in. Most noticeably, northern populations of greenish warblers inhabit tall, closed-canopy Siberian taiga in which habitat openness is low, whereas southern populations inhabit bushy treeline habitat in which habitat openness is high (Irwin, 2000).

Morphometric variation was presented previously in a greatly summarized form by Irwin *et al.* (2001a), who presented only the first principal component resulting from a principal components analysis of six morphometric traits. Here, for the first time, we present variation in each of the six traits as well as the first two principal components. The six traits include (all according to Pyle, 1997): tarsus length, wing length (flattened), tail length, bill length (from the anterior end of the nostril to the tip) and bill depth and width (both measured at the anterior end of the nostrils). We also include data from more sites than the earlier study (Irwin *et al.*, 2001a), as that study only presented morphometric PC1 from eight major research sites. We now include data from 25 additional birds from eight additional research sites distributed primarily in central Siberia.

Geographic variation in songs of greenish warblers was summarized by Irwin (2000). To provide a direct comparison with patterns of call variation, we reanalysed song variation using the data of Irwin (2000) supplemented with measurements from an additional six individuals (one from site KA and five from XN; Table 1). Songs are constructed out of song units, which are distinct series of sounds that generally are sung without interruption; songs can consist of one or more unique

units, each one possibly repeated a number of times (Irwin, 2000). Ten songs were measured from each individual, and the following five variables were measured (following Irwin, 2000): length, maximum frequency, minimum frequency, number of song units, and number of unit types. A PCA of song variation was carried out in a similar manner as for call variation.

For purposes of illustrating the relationship between call variation and geographic location around the ring, we measured 'corrected' geographic distances according to the ring species hypothesis, which was supported by genetic analyses (Irwin *et al.*, 2001a, 2005): it was assumed that there is a barrier to gene flow between *viridanus* and *plumbeitarsus* in central Siberia and that the interior of the ring (i.e. Tibet and deserts to the north) cannot be crossed. Thus, corrected distances between west (*viridanus*) and east Siberian (*plumbeitarsus*) populations were measured through the ring of populations connecting them to the south. To determine a single axis representing geographic location around the ring, we performed a principal coordinates analysis on the matrix of these corrected geographic distances. The first principal coordinate explained 89.4% of the variation in the corrected geographic distances, and this was then used as a measure of geographic location around the ring.

We used Mantel tests to determine whether call and song divergence were correlated and whether divergence in each type of vocalization was correlated with genetic distance as measured by corrected AFLP (Amplified Fragment Length Polymorphism) distances from Irwin *et al.* (2005). Note that although an analysis of song variation was presented previously (Irwin, 2000), an analysis of the correlation between song and AFLP variation has never before been published. Corrected AFLP distances are the number of AFLP markers that differ in presence/absence between individuals of different populations minus the average AFLP difference between individuals within each population. In two pairwise comparisons in which this estimate was slightly negative, we changed those negative values to the minimum possible genetic distance of zero. To determine whether call and song divergence were correlated after accounting for the correlation of each with genetic distance, we conducted a partial Mantel test (Smouse *et al.*, 1986). All statistical analyses were conducted using *R* (R Development Core Team, 2006).

Results

Each territorial alarm call consists of a short series of vocalizations, which we call 'strokes', each of which is usually frequency modulated sharply upward or downward (Fig. 2). Each of the measured variables except one (peak time) shows significant geographic variation (Fig. 3), and these differences between call notes from different regions can be recognized rather easily in the field by a human observer.

The two Siberian forms have the most divergent calls in the entire complex. Calls in west Siberia (*viridanus*; sites YK and TL) are generally the shortest and simplest (i.e. have the fewest strokes) and use the highest frequencies, whereas calls in east Siberia (*plumbeitarsus*; sites BK, IL and ST) are the longest and most complex and use the lowest frequencies. Calls in the southern areas (*ludlowi*, *trochiloides* and *obscuratus*; e.g. sites MN, LN, EM and XN) tend to be progressively intermediate in length, complexity and frequency. Figure 3, in which populations are arranged according to their geographic proximity through the ring of populations, shows that call variables tend to change gradually around the ring, from west Siberia south through central Asia to the Himalayas, then eastward to southern China, and finally northward to eastern Siberia. An exception to this pattern is that calls from the geographically disjunct form *nitidus* (site KA) are similar in some traits (e.g. length and strokes) to east Siberian (*plumbeitarsus*) calls rather than calls from geographically closer areas (*viridanus* and *ludlowi*). Calls of *nitidus* and *plumbeitarsus* also differ in some traits (e.g. peak frequency and overall appearance of the calls).

These broad patterns in call variation can be further explored in the results of a principal components analysis (Fig. 4a; Table 2). This analysis was used to summarize variation in the five call variables that showed significant geographic variation (all except peak time; see caption to Fig. 3). Most of the variance (64%) in the underlying variables is summarized by the first principal component (call PC1), which increases as call length and strokes increase and as the three frequency variables decrease. Call PC1 shows significant geographic variation (Kruskal–Wallis test: $H_{13} = 34.81$, $P = 0.0009$), being low in west Siberia and gradually increasing through the ring to the south and then into east Siberia (Figs 5 and 6). Call PC2 summarizes a much smaller amount of the variation (17%) and does not show significant geographic variation (Kruskal–Wallis test: $H_{13} = 15.52$, $P = 0.28$). Thus, calls show a clear pattern of isolation by distance (Fig. 6).

Calls do not show the pattern predicted by the hypotheses of morphological constraint or acoustic adaptation, that calls should be similar in the two Siberian forms because of the similarity of their morphometric traits and habitat. The morphometric data and principal components analysis confirm that the two Siberian forms are quite similar in body size and together differ from populations to the south (Fig. 7, Table 3). Morphometric PC1, which is essentially a measure of body size, does not differ significantly between *viridanus* and *plumbeitarsus*, whereas each does differ from southern forms, with northern forms having roughly 10% smaller body size (Fig. 7). There is a statistically significant difference in morphometric PC2, consisting primarily of beak depth, between *viridanus* (TL*) and *plumbeitarsus* (ST*) in central Siberia, but the
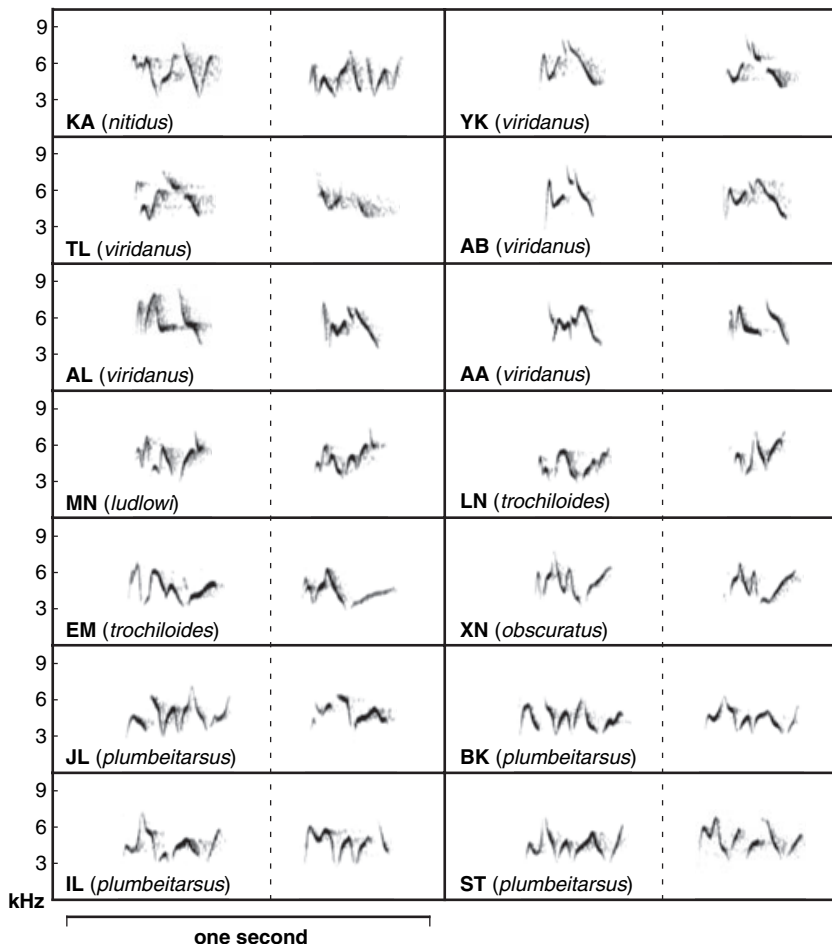
Fig. 2 Representative greenish warbler call spectrograms from 14 sites. Two calls, usually from different birds, are shown from each site. Calls were chosen for illustration based on the clarity of the recording.

effect size is small, with a great deal of overlap between the two taxa (see standard deviations in Fig. 7). It would be difficult to argue that the differences in beak depth could have driven the call difference between the taxa, as the overlap in call variables is much less than in beak depth: the mean morphometric PC2 differs between *viridanus* and *plumbeitarsus* in central Siberia by only 1.77 pooled standard deviations, whereas the mean call PC1 differs by 4.37 pooled standard deviations (based on *viridanus* calls from YK, TL and AB, and *plumbeitarsus* from BK, IL and ST). Habitat openness is also very similar between *viridanus* and *plumbeitarsus*, with both inhabiting denser forest than southern forms (Fig. 8a). Across study sites, call PC1 is not correlated with either habitat openness or morphometric PC1 (Fig. 8). The lack of a correlation is not because of a lack of geographic variation within a variable, as each variable is strongly structured across space. Rather, call divergence appears to be driven by factors other than habitat and morphometrics.

Whereas most geographic variation in calls is explained by a single axis of variation, two major principal component axes explain variation in songs (Fig. 4b; Table 4).

Song PC1, which increases with length, units and unit types per song, explains 54% of the variance in the underlying variables, whereas song PC2, which increases with frequency range and length per unit, explains 36% of the variance. There is significant geographic variation in both principal components and each of the five song variables (Kruskal–Wallis tests: PC1, $H_{15} = 46.26$, $P = 0.00005$; PC2, $H_{15} = 46.48$, $P = 0.00004$; maximum frequency, $H_{15} = 45.65$, $P = 0.00006$; minimum frequency, $H_{15} = 40.10$, $P = 0.0004$; length, $H_{15} = 49.28$, $P = 0.00002$; units, $H_{15} = 48.65$, $P = 0.00002$; unit types, $H_{15} = 44.60$, $P = 0.00008$). Song PC1 is high in east Siberia and lower elsewhere, whereas song PC2 is high in west Siberia and lower elsewhere, and there is gradual change around the southern side of the ring.

Genetic distance as estimated with AFLP markers (Irwin *et al.*, 2005) is correlated with divergence in both kinds of vocalizations. Call distances (i.e. Euclidean distances based on PC1 and PC2) between sites are highly correlated with genetic distances (Fig. 9a, Mantel test: $r = 0.8257$, $P = 0.0006$). Song distances, calculated in a similar way, are also correlated with genetic distances (Fig. 9b, Mantel test: $r = 0.7249$, $P = 0.0007$).
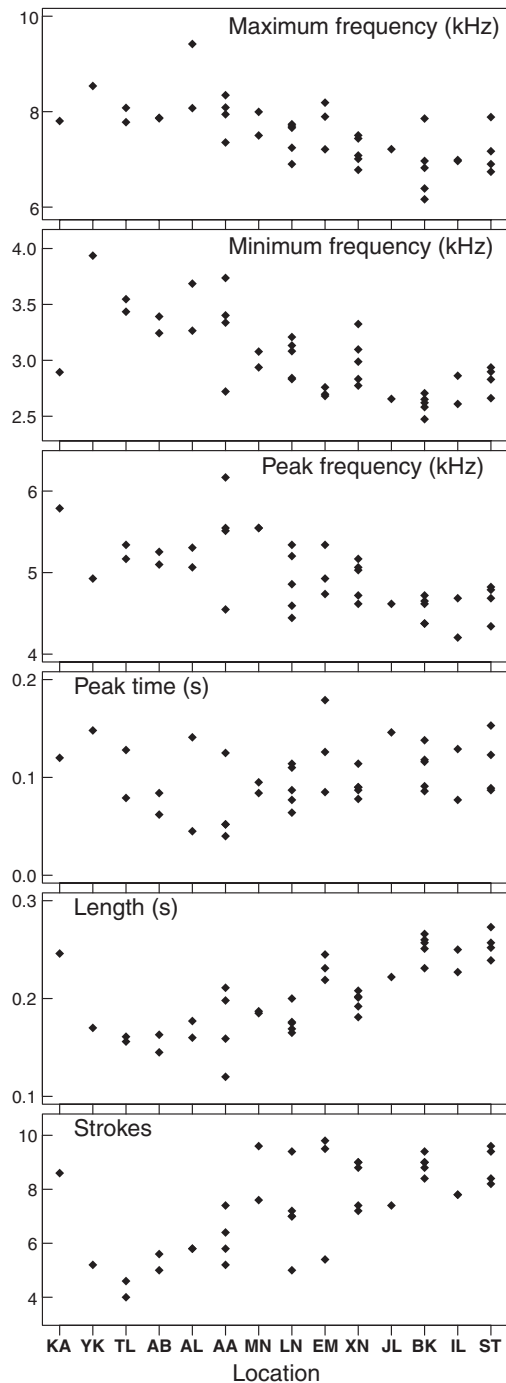
Fig. 3 Geographic variation in six call variables. Sites are arranged along the horizontal axis in rough geographic order around the ring (see Fig. 1 for locations). Each point represents the mean for a single individual [based on two to five (mean 4.8) calls per individual]. All variables except peak time show significant geographic variation (Kruskal–Wallis tests: maximum frequency, $H_{13} = 24.04$, $P = 0.031$; minimum frequency, $H_{13} = 29.33$, $P = 0.006$; peak frequency, $H_{13} = 22.67$, $P = 0.046$; peak time, $H_{13} = 14.39$, $P = 0.35$; length, $H_{13} = 32.87$, $P = 0.002$; strokes, $H_{13} = 24.35$, $P = 0.028$).
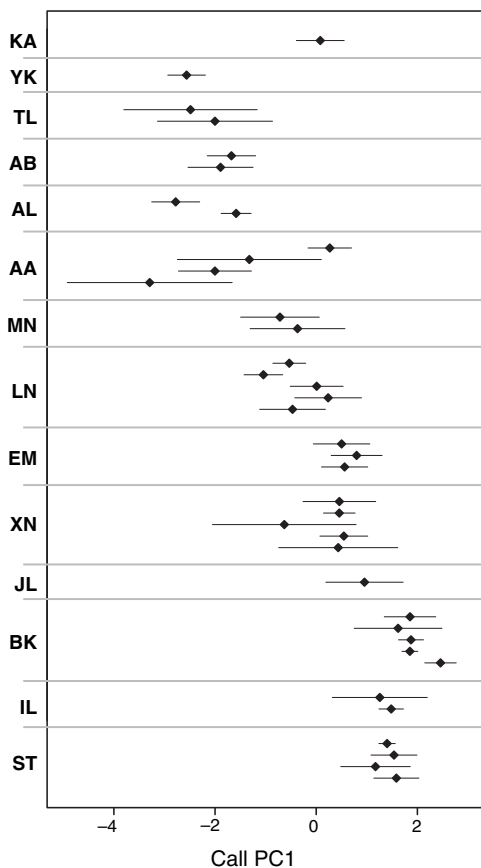

Fig. 4 Principal components analysis reveals that both (a) calls and (b) songs change gradually around the ring of greenish warbler populations, with the two Siberian forms differing distinctly. Calls change gradually around the ring along a single axis of variation, whereas songs show geographic variation along two axes. Each graph shows site mean values of both principal components, and error bars show standard deviations. At some sites, there are no error bars because only a single bird was recorded. Per cent figures on each axis correspond to the amount of variance in the data set that is explained by that axis. Shades of grey and the two-letter site codes correspond to the map shown in Fig. 1. See Tables 2 and 4 for variable loadings on each principal component.
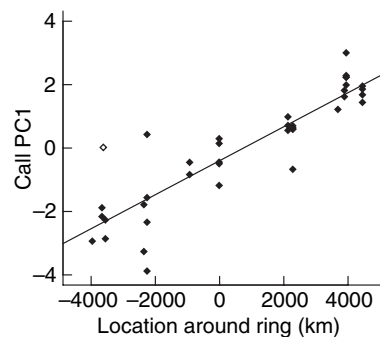
Call distances and song distances between populations are correlated (Mantel test: $r = 0.6850$, $P = 0.0001$). However, as shown above, divergence in each type of vocalization is correlated with genetic distance, making it possible that the correlation of call and song divergence is simply a result of the influence of genetic distance on each. When the effect of genetic distance is controlled for, the correlation between call and song divergence is not statistically significant (partial Mantel test: $r = 0.3048$, $P = 0.09$).

Table 2 Per cent variance explained and call variable loadings on the first two call principal components.

	Call PC1	Call PC2
Variance explained (%)	64.0	16.8
Variables		
Maximum frequency	−0.412	−0.435
Minimum frequency	−0.509	0.094
Peak frequency	−0.400	−0.572
Length	0.498	−0.185
Strokes	0.403	−0.664

Fig. 6 The major component of variation in calls (i.e. call PC1, the first principal component from Fig. 4a) varies gradually around the ring of greenish warbler populations, but differs distinctly between the terminal forms that come into contact in central Siberia. Each symbol represents average calls of a single bird. Geographic location around the ring explains a high amount of the variation in calls (Pearson's $r = 0.887$, $t_{37} = 11.67$, $P = 6 \times 10^{-14}$). See Materials and methods for details on how locations around the ring were generated. The open diamond represents the *P. p. nitidus* individual from site KA; it appears to be an outlier, but because *P. t. nitidus* is geographically disjunct from the main ring of populations, it is not surprising that it does not fit the general pattern (it was, however, fully included in the statistical analyses).

Fig. 5 Within-individual, within-population and geographic variation in the major component of variation in calls (PC1). Each point represents a single individual, with error bars showing standard deviations. Individuals are then grouped into locations, as indicated on the vertical axis, which are arranged in order around the ring, starting with *nitidus* (KA) and *viridanus* (YK, TL, AB, AL and AA), then going south through *ludlowi* (MN), *trochiloides* (LN and EM) and *obscuratus* (XN), and finishing with *plumbeitarsus* (JL, BK, IL and ST). It is apparent that west Siberian *viridanus* and east Siberian *plumbeitarsus* differ in calls, and that there is gradual variation around the ring.

We acknowledge that sample sizes of call recordings for several research sites are lower than we would have preferred, but we emphasize that our goal is not to precisely describe the calling behaviour of any single population or any single bird, but rather to reveal broad patterns of geographic variation and to test whether those patterns are related to geographic variation in potentially causal factors. We believe our sample size is sufficient for this purpose, as our statistical tests show highly significant relationships between call characteristics and location ($P = 6 \times 10^{-14}$; Fig. 6), as well as between call characteristics and genetic distance ($P = 0.0001$; Fig. 9). If sample sizes were insufficiently low for the questions being addressed in this paper, it would be highly unlikely to observe such strong relationships.

Discussion

The approach we take in this paper is to quantify variation in two types of vocalizations throughout a large, geographically variable species complex, and then to attempt to infer the processes that have led to the observed variation. We compare the observed patterns with the expected patterns that would be produced by deterministic causes of divergence, such as acoustic adaptation and morphometric change, and by stochastic causes of divergence, such as social selection, mutation and drift. We attempt to infer which forces have played the largest role in signal divergence.

Little of the overall variation in vocalizations seems to be explained by acoustic adaptation. If breeding habitat structure played a dominant role in the evolution of

greenish warbler vocalizations, we would expect vocalizations to show geographic variation corresponding to geographic variation in habitat: vocalizations of the two Siberian forms should be similar but they should both differ from the southern forms. Such a pattern is seen in morphometric traits, which have apparently evolved in response to habitat shifts. Contrary to this expectation, the two Siberian forms have the most divergent calls in the whole species complex, and their divergence in songs is also large. Of the call and song variables that we measured, we might expect the frequency variables to be most directly influenced by the acoustic environment. High frequencies tend to be scattered by vegetation more than low frequencies (Morton, 1975; Wiley & Richards, 1982; Slabbekoorn, 2004). We would then predict that greenish warblers in the north, where vegetation is denser, would use lower frequencies than those in the south. The overall patterns are not supportive of this prediction. In calls, all three frequency variables (maximum, minimum and peak) are high in west Siberia, intermediate in the southern areas, and low in east Siberia. In songs, maximum frequency is high in west Siberia and low elsewhere, whereas minimum frequency is the highest in the Himalayas and lower elsewhere.

The similar patterns of song and call variation in maximum frequency point to the possibility of a single factor influencing the evolution of both. One possibility is that there has been a correlated response to selection;
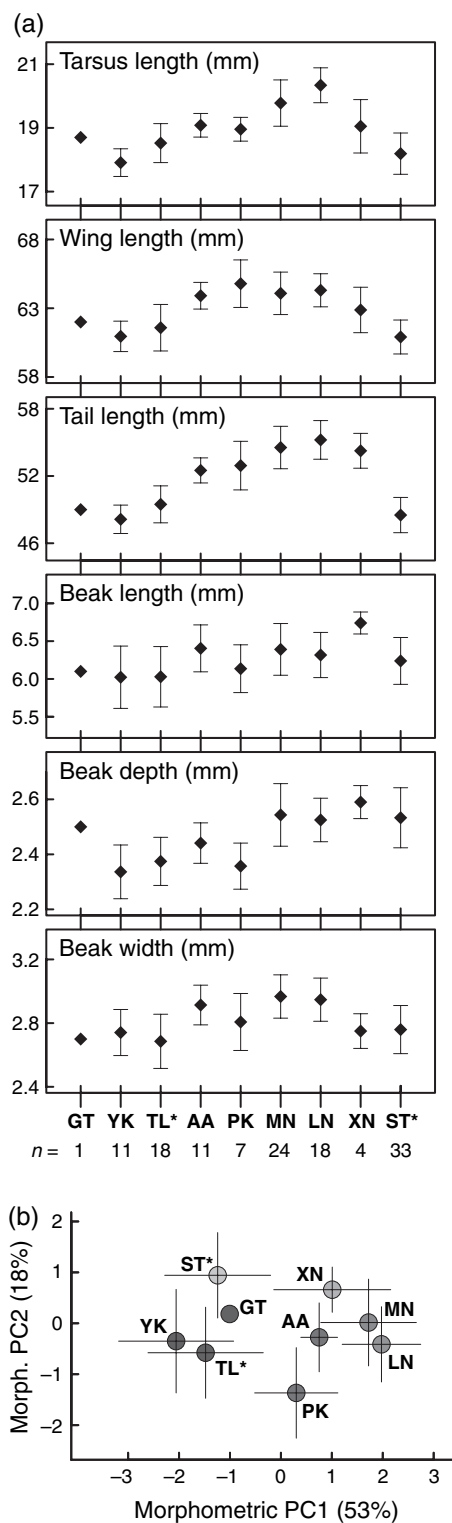

Fig. 7 Morphometric variation throughout the greenish warbler species complex. Research sites are referred to by their two-letter codes, according to Fig. 1 and Table 1. The asterisks indicate that nearby research sites were grouped together: TL* includes 12 *viridanus* from site TL, three from AB, and three from ST, whereas ST* includes 17 *plumbeitarsus* from site ST, six from site UY, four from IL, three from SL, two from BK and one from TA (see Irwin *et al.*, 2001a for locations). Variation in both (a) individual variables and (b) a principal components analysis including all of these variables reveals that the two Siberian forms, *viridanus* (sites YK and TL) and *plumbeitarsus* (site ST), are similar morphometrically, while together differing from more southern forms. Mean values and standard deviations for each research site are shown. Sample sizes (number of individuals) for each site are shown at the bottom of (a). There is significant variation among sites in each of the raw variables as well as the first two principal components (ANOVA: tarsus length, $F_{8,118} = 29.20$, $P < 2.2 \times 10^{-16}$; wing length, $F_{8,118} = 19.76$, $P < 2.2 \times 10^{-16}$; tail length, $F_{8,118} = 46.90$, $P < 2.2 \times 10^{-16}$; beak length, $F_{8,118} = 3.71$, $P = 6.8 \times 10^{-4}$; beak depth, $F_{8,118} = 10.87$, $P = 2.2 \times 10^{-11}$; beak width, $F_{8,118} = 8.37$, $P = 6.0 \times 10^{-9}$; PC1, $F_{8,118} = 39.33$, $P < 2.2 \times 10^{-16}$; PC2, $F_{8,118} = 9.94$, $P = 1.7 \times 10^{-10}$). Variable loadings on the principal components are shown in Table 3. The two Siberian forms differ significantly only in tail length, beak length, beak depth and PC2, although tail length and beak length would not be significant after Bonferroni correction (*t*-tests comparing TL* and ST*: tarsus length, $t_{49} = 1.78$, $P = 0.082$; wing length, $t_{49} = 1.63$, $P = 0.11$; tail length, $t_{49} = 2.04$, $P = 0.047$; beak length, $t_{49} = -2.08$, $P = 0.043$; beak depth, $t_{49} = -5.30$, $P = 2.8 \times 10^{-6}$; beak width, $t_{49} = -1.60$, $P = 0.12$; PC1, $t_{49} = -0.753$, $P = 0.46$; PC2, $t_{49} = -6.05$, $P = 2.0 \times 10^{-7}$). PC1 was previously shown by Irwin *et al.* (2001a), but raw variables and PC2 are shown here for the first time.

Table 3 Per cent variance explained and morphometric variable loadings on the first two morphometric principal components.

	Morph. PC1	Morph. PC2
Variance explained (%)	52.8	18.2
Variables		
Tarsus length	0.458	−0.198
Wing length	0.472	−0.353
Tail length	0.480	−0.332
Beak length	0.341	0.358
Beak depth	0.252	0.718
Beak width	0.397	0.287

Table 4 Per cent variance explained and song variable loadings on the first two song principal components.

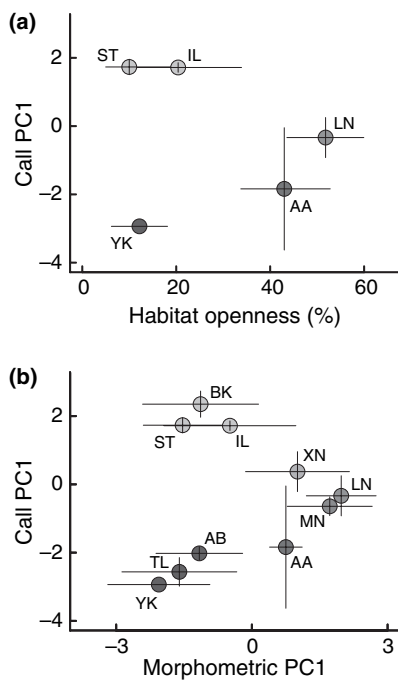|	Song PC1	Song PC2
Variance explained (%)	53.8	35.5
Variables		
Maximum frequency	−0.137	0.671
Minimum frequency	−0.300	−0.577
Length	0.563	0.183
Units	0.469	−0.426
Unit types	0.595	0.027

Fig. 8 Plots of the dominant axis of variation in calls (call PC1) vs. (a) habitat openness, and (b) the dominant axis of variation in morphometric traits (Morphometric PC1, Fig. 7b, Table 3). Each point represents a research site, as indicated by its two-letter code according to Fig. 1. Error bars show standard deviations, except in the case of habitat openness, in which case they are 95% confidence intervals (see Materials and methods). Call characteristics are not correlated with either habitat (Pearson's $r = 0.19$, $t_3 = −0.34$, $P = 0.758$) or morphometrics (Pearson's $r = 0.099$, $t_8 = 0.28$, $P = 0.79$).

Fig. 9 Genetic distance between research sites is highly correlated with both (a) call and (b) song divergence. Linear regressions are shown, but statistical significance was evaluated using Mantel tests (see Materials and methods and Results). Genetic distance was estimated using AFLP markers (Irwin *et al.*, 2005).

selection for a certain change in one type of vocalization might cause the other type of vocalization to change in the same way, because both are produced by similar neurological and physiological processes. Another possibility is that there is acoustic adaptation to wintering habitat. West Siberian breeding populations winter largely in western India, Himalayan breeding populations winter further east in Bangladesh and nearby
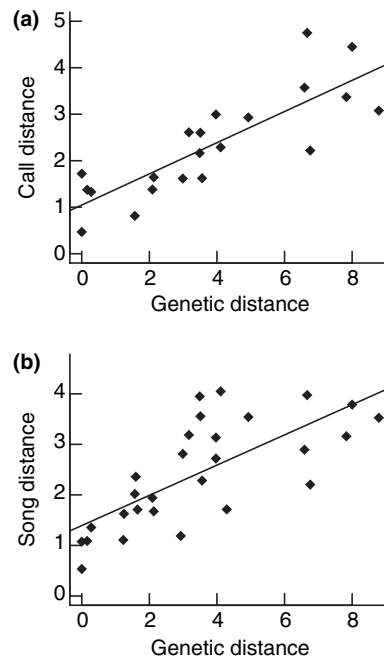
areas, and east Siberian breeding populations winter in south-east Asia (Ticehurst, 1938; Irwin & Irwin, 2005). Hence, the 'ring' of breeding populations around Tibet translates into a 'string' of wintering populations across southern Asia, through which there could be a gradient in habitat characteristics that might influence call variation. Calls play an important role in establishing and maintaining winter territories (Price, 1981; Katti, 2001; D. Wheatcroft and T. Price, pers. comm.), suggesting that selection on calls is potentially strong during winter. Songs are also used occasionally in winter (Price, 1981), although to a much lesser extent than in the breeding season. Nevertheless, our results at least suggest that the acoustic characteristics of the

breeding environment have relatively little direct influence on the evolution of greenish warbler vocalizations. This conclusion is in accord with Daniel & Blumstein's (1998) finding, in a review of papers that have examined the acoustic adaptation hypothesis, that the acoustic environment has a relatively small role in shaping signal structure. Likewise, Badyaev & Leaf (1997) found that acoustic adaptation explained a relatively small (although statistically significant) amount of variation among songs of *Phylloscopus* warblers. One caveat with our study and others is that they are based on present habitat distributions, whereas present traits of organisms may have been influenced by past habitats that may have differed from those in the present, especially if recent human activity has significantly altered habitats. We suspect that this is not a major problem in our study, as greenish warblers tend to be found in the breeding season only in areas that have remained relatively undisturbed by humans, such as treeline habitat in the Himalayas, steep slopes of forested mountains in China and central Asia, and mature taiga forest in Siberia.

Another possible cause of divergence in vocalizations is morphological divergence. As with habitat divergence, patterns of morphological divergence in greenish warblers are not closely associated with patterns of divergence in calls or songs. The two Siberian forms are very similar morphologically, but are both smaller in body size than southern forms, having apparently evolved in parallel in response to parallel habitat shifts during northward range expansions. This pattern contrasts with the patterns of differentiation in vocalizations: both calls and songs differ most between the morphometrically similar Siberian forms. Generally, organisms with larger body sizes broadcast sounds of lower frequencies (Ryan & Brenowitz, 1985). When applied to the greenish warblers, this principle leads to the prediction that southern, larger birds should make lower frequencies than northern, smaller birds. In calls, however, southern birds are intermediate between the two Siberian forms in all three frequency measurements. In songs, southern birds have higher minimum frequencies than northern birds, which results in a smaller range of frequencies in the south (Irwin, 2000). We suggest that the larger frequency ranges in the songs of Siberian birds, which run counter to predictions based on both habitat structure and body size, could simply be a result of sexual selection; increasing the frequency range is one way to increase song complexity.

Overall, the patterns of geographic variation in calls and songs of greenish warblers are roughly similar. In both types of vocalizations, the two Siberian forms (*viridanus* and *plumbeitarsus*) are highly distinct, and there is a gradient through the ring of populations to the south. For both calls and songs, geographic distance around the ring correlates strongly with divergence in vocalizations. These patterns support Ticehurst's (1938) hypothesis,

supported by genetic studies and playback experiments (Irwin *et al.*, 2001a), that there is a species boundary in Siberia, but not through the southern chain of populations. In general, the further apart the two populations are through the ring of breeding populations, the more different their vocalizations are.

Although call and song divergence are correlated, there are striking differences in the particular patterns of evolution they exhibit. Most of the variation in calls can be explained by a single principal component, which is highly correlated with geographic location around the ring. By contrast, song variation is explained by two major axes of variation, each of which is highly correlated with geography across part of the range. Southern populations have low values of both song PC1 and PC2, both of which are measures of song length and complexity; song PC1 increases moving north to the east of Tibet, whereas song PC2 increases moving north to the west of Tibet. Hence, during the two northward expansions into Siberia, songs have experienced both parallel evolution (in length and complexity) and divergent evolution (in the form of complexity), whereas calls have undergone only divergent evolution. Furthermore, south-to-north differences are generally much larger in songs than in calls. For example, whereas songs are, on average, 104% longer (i.e. 2.04 times longer) in west Siberia and 200% longer in east Siberia than they are in the southernmost research site (site LN; Irwin, 2000), calls are only 9% shorter in west Siberia and 40% longer in east Siberia than they are the south.

The difference between the patterns of call and song variation may be explained by the greater importance of sexual selection in the evolution of songs. Songs of both the west and east Siberian forms are longer and more complex than the short and simple Himalayan forms. These differences in length and complexity parallel many ecological differences: populations of both Siberian forms experience higher vegetation density, higher food abundance, lower population density and shorter breeding seasons than southern populations (Irwin, 2000). Female passerine birds often prefer males who sing long, complex songs (Kroodsma, 1976; Catchpole, 1987; Hasselquist *et al.*, 1996; Searcy & Yasukawa, 1996) and there are many ways in which the parallel ecological shifts might have altered the context in which sexual selection operates, leading to the evolution of long, complex songs in both west and east Siberia (Irwin, 2000). Calls show no such parallel increase in length and complexity in the north, perhaps because they do not play a primary role in mate attraction and are hence not as subject to sexual selection. In fact, calls are the shortest and have the simplest structure in west Siberia (*viridanus*).

If changes in habitat, body size and song do not explain patterns of call variation, what might? One possibility is that selection for species recognition

between the forms in central Siberia has caused divergence of calls (and songs as well) of the two Siberian forms. Evaluating this hypothesis in the greenish warblers is difficult, because a pattern of character displacement (i.e. more difference in sympatry than in allopatry; Howard, 1993) is confounded with the pattern expected in a ring species (i.e. gradual variation between two extreme forms). Nevertheless, in a west-to-east transect across Siberia (hence avoiding the ring around Tibet), there is little evidence for a pattern of character displacement; calls do not appear to differ more between the forms in sympatry than in allopatry. It also seems unlikely that the strong gradual variation throughout the entire southern ring of populations could be caused primarily by interactions in the narrow overlap zone between the Siberian forms.

A remaining possibility for the primary cause of call evolution is that calls are evolving in response to stochastic forces of divergence rather than deterministic ones. The strong pattern of isolation by distance in calls around the ring, as well as the strong correlation between genetic divergence and call divergence, support this suggestion; if calls were evolving primarily in response to deterministic causes, such as acoustic adaptation or morphological change, we would not expect such a strong correlation between genome-wide genetic divergence and call divergence; each of these deterministic factors predicts different patterns than the observed ones. Stochastic causes of change include mutation and drift, but could also involve social selection; signals evolving under social selection can show rapid and unpredictable evolutionary change within populations (West-Eberhard, 1983). If such social selection works at a relatively constant rate, it would lead to the observed pattern of a strong correlation between signal divergence and genetic divergence. Many researchers who study avian vocalizations have emphasized the importance of mutation and drift in causing signal evolution, especially when vocalizations have a cultural, or learned, component (Lemon, 1975; Mundinger, 1982; Lynch, 1996; Martens, 1996; Payne, 1996). We suggest that the exact form of a species' call is relatively arbitrary and unconstrained; many forms of the call would work, as long as they are recognized by both signaller and receiver. Songs are likewise subject to stochastic forces, but are also subject to the deterministic forces of sexual selection for elaboration and natural selection for simplification. Because of the almost infinite variety of possible sounds that can be made and the relatively equal effectiveness of many different sounds in conveying information, both calls and songs have high potential for evolutionary divergence.

Acknowledgments

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