

10–0.5 Hz with the smooth model code<sup>26</sup> results in a much larger resistivity gradient at 30–40 km depth for the same r.m.s. misfit, implying that the data sensitive to that depth range are better fitted with a large gradient in conductivity.

Seismic reflection<sup>26</sup>, refraction<sup>27</sup> and teleseismic<sup>28</sup> studies along the same profile are consistent in interpreting Moho depth at ~36 km. Thus, we can associate the conductivity change in the layered-Earth model with the seismically defined crust–mantle boundary. Re-performing the smooth model inversion but allowing a stepwise change in conductivity at 35.5 km yields the third model shown in Fig. 4. This model has a lower minimum misfit than the model without the step change and closely resembles the layered-Earth model below 10 km, featuring an order-of-magnitude increase in conductivity across the boundary. Varying the depth of the permitted step from 28 to 42 km (Fig. 4 inset) yields a minimum achievable r.m.s. misfit at a depth of  $35.5 \pm 1.4$  km for both the full dataset (1,000–0.1 Hz), and the reduced range dataset (10–0.2 Hz).

This is, to our knowledge, the first definitive identification of a change in electrical conductivity at depths corresponding to the crust–mantle boundary, and the required resolution is a consequence of the absence of conducting material in the crust. It is also, to our knowledge the first time that the conductivity of the uppermost continental mantle directly beneath the Moho has been obtained, which also can be attributed to the lack of a conducting lower crust<sup>11</sup>. The upper mantle beneath the Slave craton is two orders of magnitude more conducting than laboratory studies on olivine would suggest<sup>29</sup>, inferring that there must be a connected conducting phase.

The conductivity for the lower crust of the Slave Province, at around  $0.00025 \text{ S m}^{-1}$ , is consistent with laboratory studies on candidate dry rock assemblages at appropriate facies conditions<sup>30</sup>. In contrast, other mid- to late-Archaean cratons (Superior, Siberian, Baltic, Kaapvaal) have conducting material in their lower crust<sup>3</sup>, with a minimum conductance (conductivity-thickness product) of 20 S compared to less than 1 S for the Slave. Although the cause of the enhanced conductivity seen everywhere else remains contentious, what is clear is that whatever processes caused its existence elsewhere were not operating as crust was formed in the Slave craton. Recalling that the Slave craton hosts the oldest dated rocks, we speculate that this difference addresses questions of early Earth development and tectonic processes, and the applicability of plate-tectonic theory to the early- to mid-Archaean. If the enhanced conductivity in the continental lower crust of late-Archaean, Proterozoic and Palaeozoic terranes is emplaced by plate-tectonic processes of subduction and imbrication of sedimentary material (see, for example, ref. 31), then either such material was not available during the early Archaean or tectonic processes were operating differently. □

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**Speciation in a ring**

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The evolutionary divergence of a single species into two has never been directly observed in nature, primarily because speciation can take a long time to occur. A ring species, in which a chain of intergrading populations encircles a barrier and the terminal forms coexist without interbreeding, provides a situation in which variation in space can be used to infer variation in time<sup>1–3</sup>. Here we reconstruct the pathway to speciation between two reproductively isolated forms of greenish warbler (*Phylloscopus trochiloides*). These two taxa do not interbreed in central Siberia but are connected by a long chain of intergrading populations encircling the Tibetan Plateau to the south<sup>4</sup>. Molecular data and climatic history imply that the reproductively

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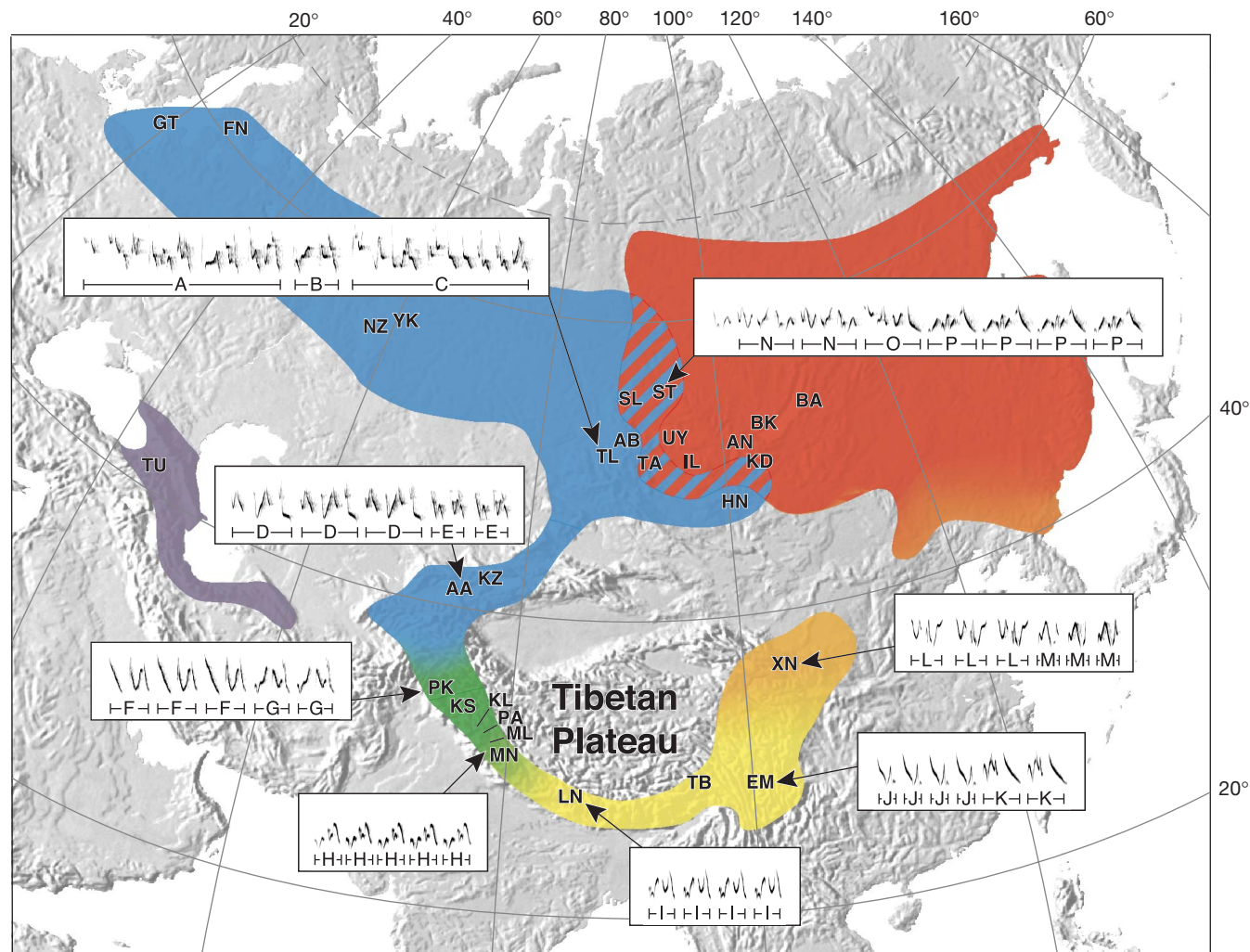
isolated taxa came into contact following expansions northward around the western and eastern sides of the plateau. Parallel selection pressures for increased song complexity during the northward expansions have been accompanied by divergence in song structure. Playback experiments show that the two Siberian forms do not recognize each other's songs. Our results show how gradual divergence in a trait involved in mate choice leads to the formation of new species.

The greenish warbler, *Phylloscopus trochiloides*, is a small (~7 g) insectivorous leaf-gleaning bird that breeds in forests over a range spanning much of the Palaearctic (Fig. 1). In 1938 Ticehurst<sup>4</sup> identified five subspecies encircling the treeless Tibetan Plateau that intergrade with each other, except in central Siberia to the north of the plateau (Fig. 1). On the basis of morphology and plumage patterns, both Ticehurst<sup>4</sup> and Mayr<sup>1</sup> felt that the greenish warbler was a ring species, with evolution of reproductive isolation between the terminal forms resulting from spread around the Tibetan plateau.

Warblers in the genus *Phylloscopus* use song in species recognition<sup>5,6</sup>. In a previous study<sup>7</sup> we documented song variation in the greenish warbler by measuring spectrograms of songs

recorded at 15 sites throughout its geographic range. There is gradual variation in song characteristics around the ring (Fig. 2), but on both the west and east sides of the ring songs are longer and more complex in the north than in the south (Figs 1, 2). The songs of the putatively reproductively isolated forms (*viridanus* in the west and *plumbeitarsus* in the east) differ discontinuously in central Siberia. While *viridanus* constructs songs out of long song units with a high frequency range, *plumbeitarsus* constructs songs out of short units with low frequency range<sup>7</sup> (Figs 1, 2).

We used song playback experiments to investigate the potential for reproductive isolation around the ring. Response by males to the playback of song is a widely used measure of whether different groups view each other as potential mates or competitors, and has been used to assign species status to several *Phylloscopus* taxa<sup>6</sup>. We conducted experiments in ten populations (PK, KL, PA, ML, MN and LN in the Himalayas, AA, YK, ST and AN further north) using recordings from other sites. Males responded strongly to recordings made up to 1,000–1,500 km away, but not to recordings made at further distances (Fig. 3). However, playbacks between *viridanus* and *plumbeitarsus* (shown by open circles in Fig. 3), even when separated by a relatively short distance, resulted in very little

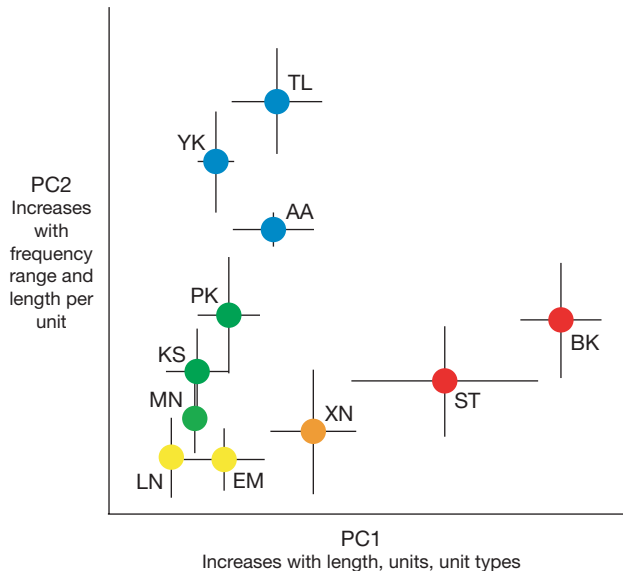


**Figure 1** Geographic range of the greenish warbler species complex, along with research sites and representative song spectrograms. Different colours illustrate the ranges of six taxa commonly considered to be subspecies of *Phylloscopus trochiloides*<sup>4</sup>: purple, *nitidus*; blue, *viridanus*; green, *ludlowi*; yellow, *trochiloides*; orange, *obscuratus*; red, *plumbeitarsus*. Colours grade together in regions where Ticehurst<sup>4</sup> described gradual change between subspecies. Research sites are indicated by their two-letter designation.

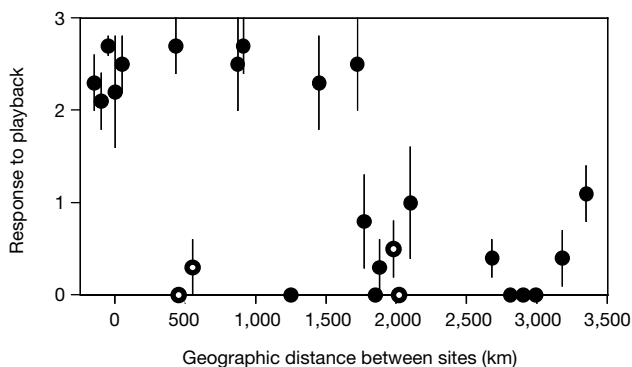
Also shown are representative song spectrograms (horizontal axis is time, vertical is frequency, darkness is amplitude) from eight locations<sup>7</sup>. Letters and brackets below the spectrograms indicate distinct song units. Song structure (for example, length of each unit, repetition of units, frequency range) differs between *viridanus* and *plumbeitarsus*, but there is a gradient in song around the southern side of the ring<sup>7</sup>.

response, indicating that the two taxa do not consider each other's song to be from their species. These results suggest that divergence of song and accompanying song recognition have been important in the evolution of reproductive isolation between the terminal forms of the ring.

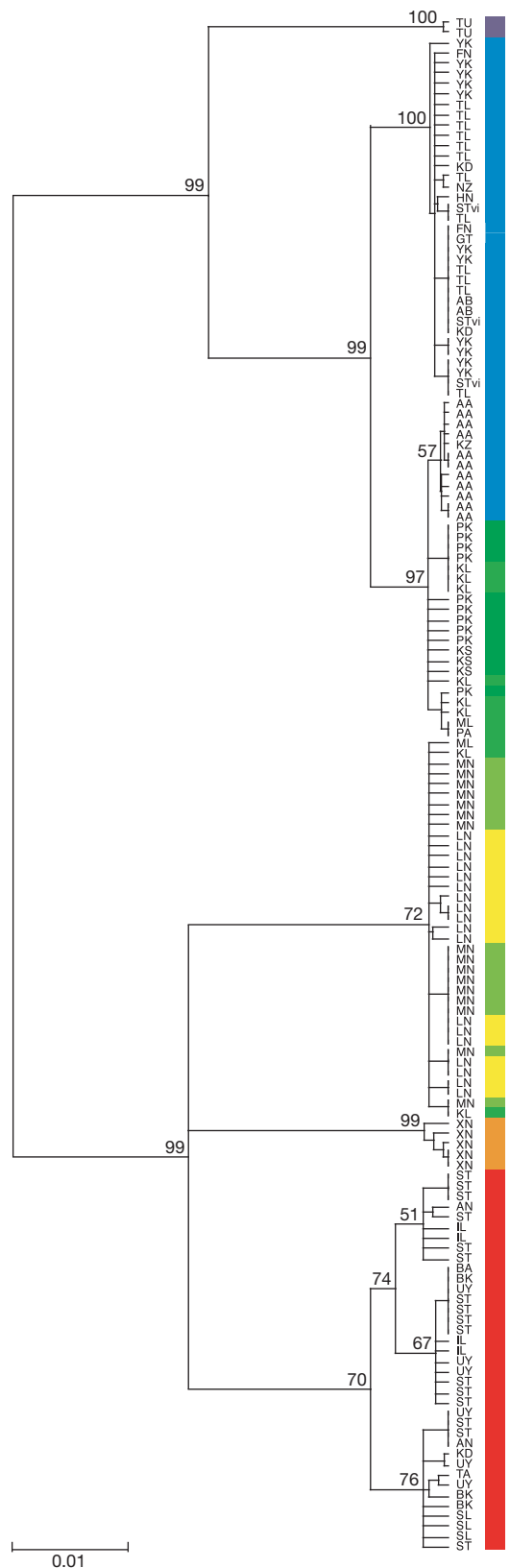
We used molecular markers to reconstruct the biogeographical history of the ring. We constructed a mitochondrial DNA (mtDNA) gene tree for 149 individuals collected throughout the species' range, based on sequences of approximately 1,200 base pairs in the neighbourhood of the control region (Fig. 4). The gene tree



**Figure 2** Geographic variation in the song of the greenish warbler as quantified by principal components analysis. Shown are population means and standard deviations, modified from ref. 7 with additional data. Songs distinctly differ between *viridanus* (blue) and *plumbeitarsus* (red), but change gradually through populations to the south (in order around the ring: YK-TL-AA-PK-KS-MN-LN-EM-XN-BK-ST). Both PC1 and PC2 are axes of complexity, and Himalayan populations (yellow and green) have the simplest songs (low PC1 and PC2).



**Figure 3** Relationship between geographic distance and song recognition. The horizontal axis shows the distance between the population in which song recordings were made and the population in which those recordings were used in a playback experiment. The vertical axis shows the response (mean  $\pm$  s.e.) to those recordings. Open circles are playbacks of *viridanus* recordings to *plumbeitarsus* populations or vice versa, and filled circles are all other experiments. The cluster of points at a geographic distance of zero represent trials in which songs recorded in a population were played to birds in that population (although never to the same bird as the recording or his neighbour). Generally, birds respond strongly to recordings from populations up to 1,500 km away, but *viridanus* and *plumbeitarsus* across Siberia do not respond to each other even when only 500 km apart. Number of males tested for each point varied from 2 to 15 (mean 5.25).



**Figure 4** Mitochondrial DNA gene tree based on variation in  $\sim$ 1,200 bp in the neighbourhood of the control region and ND6 gene. The two-letter designation and colour bar at each tip of the tree indicate the research site the individual is from and the taxon to which it belongs (the three STvi individuals were *viridanus* males at site ST; the other ST individuals were all *plumbeitarsus*). We were unable to obtain sequence data for sites TB and EM. Numbers at the nodes are support values in per cent for major clades. The tree was produced using maximum-likelihood quartet puzzling<sup>21</sup> assuming clock-like branch lengths (see Methods). Scale bar represents the substitution rate along a lineage.

shows strong geographic structure. Two major clades correspond to western and eastern individuals. We sampled intensively across about 500 km in each of the regions where the two clades meet. In central Siberia the concordance between haplotype (western or eastern clade) and song (*viridanus* or *plumbeitarsus*) was perfect (17 western birds and 35 eastern birds), providing no evidence for mitochondrial introgression between the two Siberian taxa. In the south, the region where the two mitochondrial clades meet does not correspond with a subspecies boundary, but instead occurs within the range of *ludlowi* (Figs 1 and 4). Songs of individuals carrying the different haplotypes are not distinguishable (for example, compare the similarity of KS and MN in Fig. 2), and playback experiments indicate that birds do not distinguish among them in this region. For example, birds at PK responded to MN song, even though the two are from different mitochondrial clades. And in the two populations where birds of both mitochondrial clades have been found to co-occur (KL, ML), individuals respond strongly to songs recorded at both PK (western haplotype) and MN (eastern haplotype) ( $n = 10$  playback experiments).

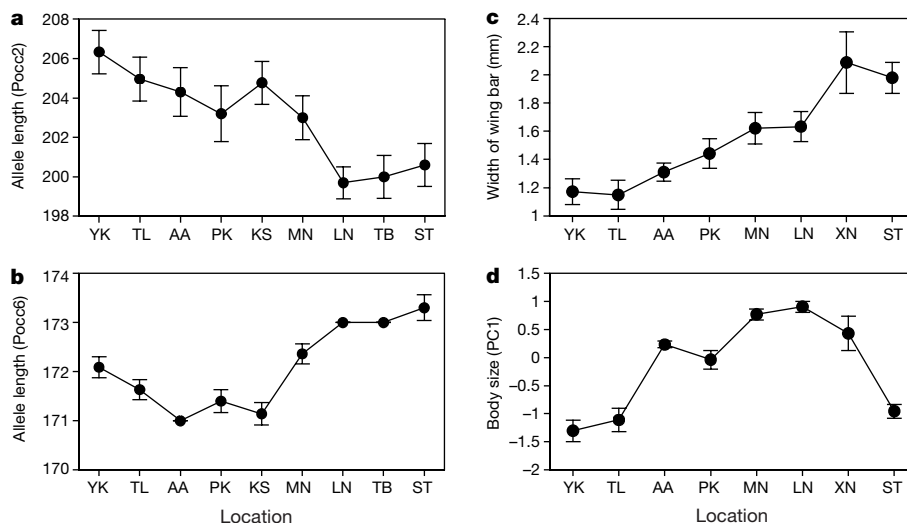
Genetic variation in two microsatellite markers (Pocc2 and Pocc6) matches that of the mtDNA tree (Fig. 5a, b). For both microsatellites, mean length varies significantly over the range (analysis of variance (ANOVA) across localities: Pocc2,  $F_{8,181} = 4.4$ ,  $P < 0.0001$ ; Pocc6,  $F_{8,191} = 21.6$ ,  $P < 0.0001$ ). The central Siberian forms at TL and ST differ in mean allele length (*a posteriori* tests: Pocc2,  $P < 0.01$ ; Pocc6,  $P < 0.0001$ ). There is also significant variation in the south between Kashmir and Nepal (comparison of KS to LN: Pocc2,  $P < 0.01$ ; Pocc6,  $P < 0.0001$ ) but the central population in northwest India (MN) is clearly intermediate to the Kashmir and Nepal populations, suggesting continuing gene flow in that region. There is little variation from south to north along either side of the ring.

The microsatellite data and the mtDNA tree are consistent in showing high genetic change both in the north, between the central Siberian taxa, and in the south, across the western Himalayas. Song variation, the mtDNA tree and the microsatellite data together indicate that there is current gene flow across the southern part of the range, but not the northern. These results suggest two possible biogeographical histories. In the first, an ancestral species was split into two populations: one to the west and the other to the east.

These populations then expanded and met each other in the south, where they interbred, and in the north, where they did not interbreed. In the second model, an ancestral species was confined to the Himalayas and developed some genetic structure as a result of isolation by distance. The species then expanded northwards along two pathways: one from the western side of its range into central Asia and west Siberia, and one from the eastern side into China and east Siberia. Simulations (D.I., unpublished data) indicate that both biogeographical histories are consistent with the data. In particular, strong geographic structuring of mitochondrial gene trees readily arises in isolation-by-distance models, and cannot necessarily be taken as indication of past geographic separation of populations. With either model, it is clear that the Siberian populations have resulted from recent northward expansions, because ice ages have rendered most of Siberia treeless for long periods through the Pleistocene<sup>8</sup>.

The greenish warbler, like other ring species<sup>3,9,10</sup>, has probably had a complex biogeographical history involving allopatric populations and secondary contact. Indeed, currently there is a gap in distribution in northeast China that is probably due to almost complete destruction of forests in that region by humans over the past several millennia<sup>11,12</sup>. The recentness of this break is indicated by the relatively little differentiation in genetics, song and plumage across the gap (Figs 2, 4 and 5), and birds in east Siberia ( $n = 3$ ) responded strongly to songs recorded in central China. The presence of past geographical breaks does not detract from the basic value of ring species as indicators of the way in which continuous variation within species is transformed to discontinuities between them<sup>9</sup>. Around the greenish warbler's ring the two reproductively isolated forms in Siberia are connected by continuous variation in all measured phenotypic traits, including songs (Fig. 2), plumage patterns (Fig. 5c) and body size (Fig. 5d).

Two of the measured phenotypic traits (songs and plumage patterns) are used in courtship and territory defence<sup>7,13,14</sup>. Both of these traits are distinctly different between the two reproductively isolated forms in Siberia. In the case of song, a thorough analysis of the geographic variation implicates reduced natural selection costs and/or increased sexual selection pressures as the cause of parallel evolution of greater complexity towards the north on both sides of the Tibetan Plateau<sup>7</sup>. The increased complexity has



**Figure 5** Variation in four traits (mean  $\pm$  s.e.) around the ring of greenish warbler populations. Populations (see Fig. 1) are arranged in geographical order from west Siberia through the Himalayas to east Siberia. **a, b**, The lengths of two microsatellite loci (9–14 individuals per site); **c**, width of the greater covert wing bar (4 males at XN, 6 at PK, 10 at

all others); **d**, body size (4–28 males per site). Populations TL (*viridanus*) and ST (*plumbeitarsus*), both in central Siberia, are among the most divergent of all populations in the two microsatellites and wing bar. They are, however, similar in body size.

been accomplished in different ways on the west and east sides of the ring, resulting in song divergence<sup>7</sup>. A plumage characteristic, the size of the pale wing bar, also differs between populations, being smaller in west Siberia than in east Siberia (Fig. 5c; ANOVA across localities:  $F_{7,62} = 9.6$ ,  $P < 0.0001$ ; *a posteriori* comparison of TL and ST:  $P < 0.0001$ ). Differences in wing-bar size among the *Phylloscopus* species have been related to habitat<sup>13</sup>. However, the habitat occupied by the reproductively isolated taxa in central Siberia is very similar, and an explanation for the divergence in wing bars remains to be uncovered.

In contrast to the patterns shown by song and plumage, body size is similar between the two reproductively isolated taxa. Differences in body size among species in the genus *Phylloscopus* have been related to ecological traits such as food type and feeding methods<sup>14,15</sup>. Body size varies significantly around the ring ( $F_{7,98} = 43.5$ ,  $P < 0.0001$ ; Fig. 5d) but differs little between the northerly populations (*a posteriori* tests, comparison of TL and ST:  $P = 0.44$ ). Apparently, parallel natural selection pressures across the west and east sides of the species range have resulted in parallel evolution of body size, from the larger southern type to the smaller northern types. Ecological similarity of the two reproductively isolated forms in the north is also suggested by the relatively small penetration of each into the other's range.

Much recent interest has been directed towards a possible role of natural selection in speciation<sup>16,17</sup>, but sexually selected traits are most obviously divergent between the two reproductively isolated taxa in Siberia. It appears that by magnifying small differences in southern populations into species-level differences between the Siberian taxa, sexual selection has had a prime role in speciation. Sexual selection has been invoked as a powerful force in speciation partly because sexual selection often favours novelty and/or complexity, resulting in many possible outcomes<sup>18</sup>. We suggest that similar selection pressures for greater song complexity in both northward expansions has resulted in song divergence largely because there are many different ways to evolve greater complexity. As illustrated here for body size, natural selection may often lead to parallel or convergent evolution, resulting in little difference between incipient species when they meet. □

## Methods

### Fieldwork

We visited most sites for several weeks during at least one breeding season (May through early July). Sites TU, FN, NZ, KZ, TB, EM, HN, KD and BA were visited by others. Some sites were visited more than once (KS, six seasons; MN, four seasons; LN, two seasons; and ST, four seasons).

### Collection of samples for molecular analysis

To obtain most of the DNA samples, we captured each bird in a mist net, collected a few drops of blood, and released the bird. We also obtained 12 skin samples from the British Museum (collected at site TB in the years 1922 to 1947) and 7 muscle samples from the Burke Museum (one each from sites NZ, KZ, BA and HN, and three from KD). DNA was extracted from samples using the QIAamp Tissue Kit (Qiagen).

### Mitochondrial DNA gene tree

Because of a gene rearrangement<sup>19</sup>, *Phylloscopus* warblers have an unusual mitochondrial gene order surrounding the control region (cyt b, tRNAThr, control region, tRNAPro, ND6, tRNAGlu, non-coding region, tRNAPhe, 12S rRNA). We amplified a fragment from this region using the primers DLL3 (control region) and 12SH2 (12S rRNA). This fragment was sequenced with an ABI 377 DNA Sequencer at the UCSF Cancer Center in two reactions, using primers DLL3 and DLLF2 (5'-GGATCAGCTGCTAACGACAC-3'), which is located near the centre of the fragment. Sequences ranged in size from 1,006 to 1,298 bp, both because of insertions/deletions and because some sequences were incomplete at the ends. We aligned the sequences using the program Clustal W<sup>20</sup>. To construct the gene tree, we used the program PUZZLE 4.0.2 (ref. 21) using an HKY model of substitution, parameters estimated from the data set (by the approximate method) and gamma-distributed rates (gamma-distribution parameter  $\alpha = 0.32$ ). Gaps (from insertions/deletions) were treated as missing data. Out of the 149 individuals sequenced, there were 101 unique haplotypes and only these were used to construct the tree. The tree assuming a molecular clock (Fig. 4) is not significantly less likely than the no-clock tree (likelihood ratio test,  $\chi^2_{99} = 99.35$ ,  $P = 0.47$ ). Aligned sequences from all samples have been

deposited in GenBank (accession numbers AF316171–AF316319) and can also be obtained from the authors.

### Microsatellites

We genotyped individuals for two microsatellite loci, Pocc2 and Pocc6 (ref. 22). We labelled primers with <sup>32</sup>P or <sup>33</sup>P, and used PCR conditions as in ref. 22. Products were resolved on Sequagel XR (National Diagnostics) polyacrylamide gels and exposed to film for 1–72 h.

### Wing bar size

We collected the fourth greater covert feather from the right wing of most of the birds that we caught. Using a microscope, we measured the length of pale (non-melanized) colour along the shaft of the feather at the feather's tip (males only).

### Body size

For every bird we caught, we measured six morphological traits<sup>15</sup> (tarsus length, wing length, tail length, and beak length, depth and width). These variables were used in a principal components analysis (males only). The first principal component (PC1) weights all variables about equally, and is used as a measurement of body size.

### Playback experiments

We prepared each playback tape by recording a singing male for 10 min. Whenever possible, three recordings (each from a different bird) from a source population were used in the playback experiments. At each target population, we conducted playbacks by locating a singing male, placing a speaker directly below the tree he was in, randomly determining which source tape to play (choosing from three tapes of each possible source population), and playing the tape for 7–10 min. We judged the response of the target bird on a scale of 0 (no response) to 3 (strong response, aggressively approaching the speaker).

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