Molecular Ecology (2003) **12**, 3069–3075 doi: 10.1046/j.1365-294X.2003.01981.x

Recent evolutionary history of the bluethroat (*Luscinia svecica*) across Eurasia

ROBERT M. ZINK,* SERGEI V. DROVETSKI,† SOPHIE QUESTIAU,‡ IGOR V. FADEEV,§ EVGENIY V. NESTEROV,§ MICHAEL C. WESTBERG* and SIEVERT ROHWER¶

*J. F. Bell Museum of Natural History, University of Minnesota, St Paul, MN 55108, USA, †Department of Ecology, Evolution, and Behavior, University of Minnesota, St Paul, MN 55108, USA, ‡Ecologie Animale, Université d'Angers, Belle-Beille, 2, boulevard Lavoisier, F-49045 Angers cedex, France, §State Darwin Museum, 57 Vavilova Street, Moscow 117292, Russia, ¶Burke Museum and Department of Zoology, University of Washington, Seattle, WA 98195–3010, USA

Abstract

We analysed mitochondrial DNA (mtDNA) sequences from 154 bluethroats (*Luscinia svecica*) sampled at 21 sites throughout much of their Eurasian range. A previously reported, single base-pair mtDNA difference between *L. s. svecica* and *L. s. namnetum* was inconsistent upon expanded geographical sampling. A significant F_{ST} value (0.29) and an isolation-by-distance effect show the existence of geographical differentiation. Phylogenetic analysis of haplotypes revealed northern and southern groups, although lineage sorting is incomplete. There was no geographical structure to the haplotype tree within groups, and currently recognized subspecies were not supported. A minimum evolution tree based on pairwise mtDNA genetic distances among average samples showed the same two broadly distributed northern and southern groups. These groups abut in the centre of the latitudinal range, and were possibly isolated by forest that developed and spread westward over the last 15 000 years. Pairwise F_{ST} values averaged 0.16 in the southern group, 0.04 in the northern group, and 0.42 between groups. Mismatch distributions suggested population growth in each group, with that in the south being more recent. In the northern group, the geographical pattern in tau suggested northward and eastward expansion. Analysis of nucleotide diversity suggested westward expansion in the southern group. The northern group had higher nucleotide diversity than the southern group, consistent with a larger current population size in the north. Given the significant F_{ST}, incompletely sorted haplotype tree, and broadly patterned minimum evolution tree, *L. svevica* appears to represent a species at an intermediate stage of differentiation between panmixia and reciprocal monophyly.

Keywords:

Received 29 April 2003; revision received 13 August 2003; accepted 13 August 2003

Introduction

The bluethroat (*Luscinia svecica*) is a widespread migratory Eurasian species, ranging from arctic to steppe habitats at middle latitudes. It is polytypic, with major clades recognized by the colouration of the throat (red or white spot, uniform); up to 10 subspecies have been described, depending upon the author consulted. Across Eurasia, many of the subspecies grade into one another (Cramp 1988), and sometimes co-occur (SVD, pers. obs.). The nature of this geographical variation is of interest for evolutionary and systematic reasons. In North America, phylogeographical differentiation appears greater in the mountainous regions of the west (Zink 1997). Although many species have been found to show genetic signatures of Quaternary habitat shifts in Europe, little is known about patterns of phylogeography across Eurasia (Bensch & Hasselquist 1999; Fedorov *et al*. 1999; Hewitt 2000; Kryukov & Suzuki 2000). Thus it is unknown whether patterns of mitochondrial DNA (mtDNA) variation are consistent with potential isolating barriers in Eurasia, such

Correspondence: Robert M. Zink, J. F. Bell Museum of Natural History, University of Minnesota, St Paul, MN 55108, USA.

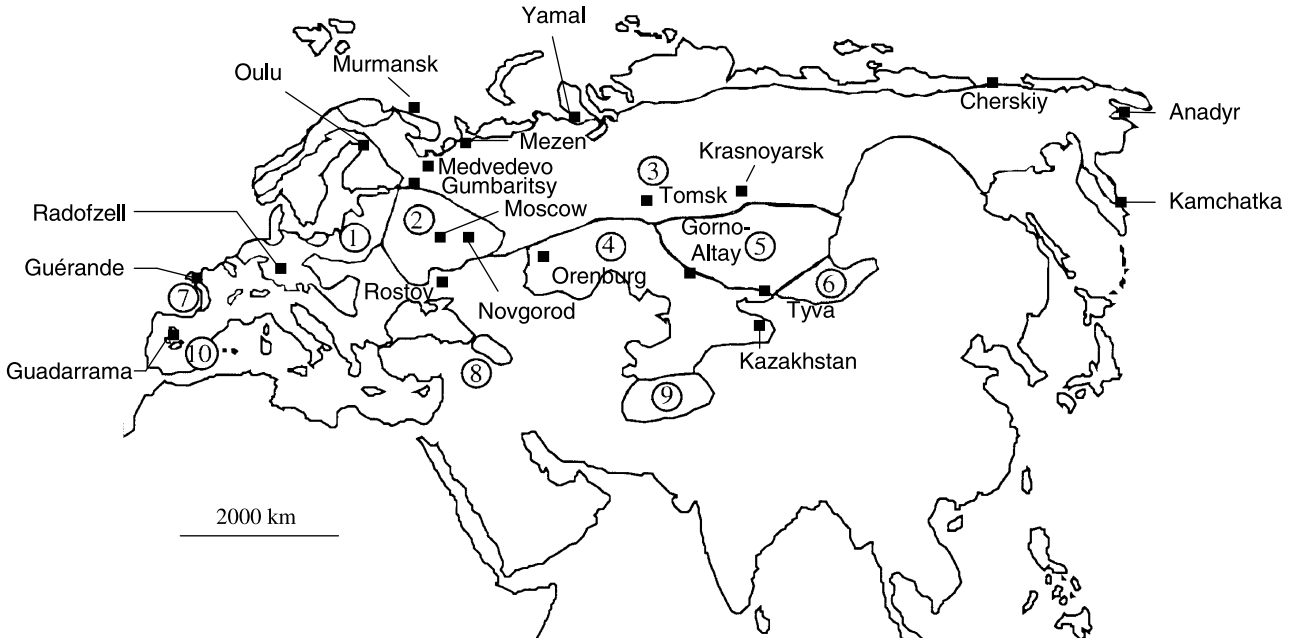

Fig. 1 Map showing general location of sample sites and distribution of subspecies (shown by outlining): 1: *cyanecula*, 2: *volgae* 3: *svecica*, 4: *pallidogularis*, 5: *saturatior*, 6: *kobdensis*, 7: *namnetum*, 8: *magna*, 9: *abotti* or *tianshanica*, and 10: *azuricollis*.

as the Ural Mountains, or exhibit signatures of Quaternary range alterations (Merilä *et al*. 1997; Hewitt 2000). The lack of information from the eastern Palearctic is unfortunate, because its glacial history differs from that of the western Palearctic. In particular, the most recent glacier extended much farther south in Europe than in Asia (Hewitt 2000), suggesting that species' eastern ranges were less affected.

Another topic of interest is the degree to which subspecies boundaries are consistent with mtDNA gene trees. In North America, subspecific taxonomy suggests far more divisions than those revealed in mtDNA gene trees (Ball & Avise 1992; Zink *et al*. 2000). Whether this is true for Eurasian birds is unknown. Questiau *et al*. (1998) compared mtDNA sequences from population samples representing two subspecies, *L. s. namnetum* and *L. s. svecica*. These subspecies, from western France and from Scandinavia across northwestern Russia, respectively, are characterized by differing size and the colour of the throat spot. A single base pair (bp) difference out of 1725 bp of mitochondrial control region and cytochrome *b* allowed diagnosis of samples of 10 individuals of each subspecies (from France and Russia, respectively) although the average distances within and between subspecies were equivalent. We extended the phylogeographical analysis of this species across Eurasia, adding sequences we gathered to those of Questiau *et al*. (1998), testing subspecies limits and providing a picture of mtDNA variation over a span of approximately 180° longitude, representing much of the species range.

Material and methods

We report sequence information from breeding individuals representing 21 Eurasian localities (Fig. 1), and one small sample of wintering birds from the Tagus Estuary, Portugal, which was combined with breeding birds from Guerande, France (Constant & Eybert 1995). Sequences came from the study of Questiau *et al*. (1998), Questiau (unpubl. data), and newly collected specimens, resulting in a total of 154 individuals. Samples from Finland and Questiau *et al*. (1998; S. Questiau unpubl. data) lack museum voucher specimens, whereas the remaining samples have tissue and associated museum vouchers housed in the Burke Museum, State Darwin Museum (Moscow), Bell Museum (University of Minnesota) or Moscow State University Zoological Museum. GenBank Accession nos include those in Questiau *et al*. (1998), AY352077–352210 and AY354540–354673.

We amplified and sequenced parts of the mitochondrial control region (CR) and the protein-coding cytochrome-*b* using protocols and primers noted in Zink *et al*. (1998). For the control region, we used LCR4 (5′-CTCACGAGAACCGAGCTACT-3′) and H1248 (5′-CATCTTCAGTGTCATGCT-3′) for polymerase chain reaction (PCR) and LCR4 and LCON2 (5′-CTTCCTCTTGACATGTCCAT-3′) for sequencing. For cytochrome *b* we used L14841 (5′-CCATCCAACATCTCAGCATGATGAAA-3′) and H15299 (5′-GGAGGAAGTGCAGGGCGAAGAATCG-3′) for PCR and sequencing. Sequences from the two genes were combined. We used ARLEQUIN (Schneider *et al*. 2000) to compute

AMOVA (F_{ST}), nucleotide diversity (π, θ_0 and θ_1, τ, Fu's 1997) *F*-value, and mismatch distributions for larger samples (greater than five individuals). F_{ST} reflects the amount of genetic divergence distributed among populations (and includes divergence among haplotypes). Nucleotide diversity measures genetic variation in terms of numbers of haplotypes and their degrees of difference; in essence, it is a reflection of the depth of haplotype trees. Values of θ represent the product of $2\mu N_0$ and $2\mu N_1$ (μ is the mutation rate and N the effective population size), respectively, and the difference between them provides a relative index of whether populations have expanded over time. Tau is a relative measure of the time (in generations) since population expansion. Fu's (1997) *F*-value tests whether sequences conform to neutral expectations. Mismatch distributions were tested against a model of sudden population expansion (Schneider & Excoffier 1999). Mantel's (1967) test was used to test for an isolation-by-distance effect, by comparing matrices of pairwise F_{ST}-values and straight-line geographical distances (in km).

Because analyses in ARLEQUIN depend on equilibrium conditions, we also used the software MDIV (Nielsen & Wakely 2001), which employs Bayesian statistics to estimate simultaneously posterior probabilities for four population parameters: $\theta = 2N_e\mu$ (where μ is the mutation rate), $M = N_e m$ (where m is the migration rate between populations), $T = N_e t$ (where t is the time since divergence of populations), and time since most recent common ancestor (TMRCA) for each pair of populations being compared. The modes of the distributions provide estimates of the parameters. MDIV does assume equal sizes of ancestral and current populations, and compares populations only pairwise. We compared the estimate of T-mode and M-mode with the F_{ST} value produced in ARLEQUIN, and to geographical distance, restricting comparisons to samples of five or greater.

PAUP* (Swofford 2000) was used to generate a haplotype tree using maximum parsimony (base positions weighted equally). Using a neighbour-joining (NJ) tree, we estimated base frequencies, percentage of invariant sites, gamma shape parameter and the transition:transversion ratio. These parameters were used to find a maximum likelihood (ML) tree. The same parameters were estimated on this tree, and a second heuristic search was conducted to find a ML tree. Lastly, we used the NJ method implemented in Mega2 (Kumar *et al.* 2001) with Kimura (1980) two-parameter distances and a correction for among-site rate heterogeneity (alpha = 0.8, estimated from PAUP*). The NJ tree was bootstrapped 500 times. We used a single sequence of *Erithacus rubecula* (GenBank Accession nos Y08057, Y08058) as an outgroup; however, it was very distant from haplotypes of *L. svevica* and rooting did not depend on the outgroup. Using the minimum-evolution method, we examined relationships among samples based on their pairwise distances (corrected for within-group divergence) using MEGA2 (Kumar *et al*. 2001).

Results

Of the 1071 bp sequenced for all individuals, 59 were variable and 28 were parsimony informative (outgroup excluded). Of the 154 individuals, 65 haplotypes were resolved. One widespread southern haplotype occurred in Kazakhstan ($n = 1$), Rostov (2), Gorno-Altay (2), Orenburg (2), Moscow (5) and 28 (of 48) individuals from Tyva, as well as in Medvedevo ($n = 1$). A widespread northern haplotype was found in Anadyr (2), Yamal (3), Oulu, Murmansk, Medvedevo and Mezen, as well as Kazakhstan and Tyva (3). Considering all 154 individuals, haplotype diversity was 0.86, nucleotide diversity was 0.0023, the average number of bp differences (k) was 2.44 and the uncorrected sequence divergence averaged 0.30%. The sequence of *E. rubecula* differed on average from haplotypes of *L. svecica* by 10%. Nucleotide diversity values ranged from 0.0009 (Rostov, Guerande) to 0.0042 (Mezen). F_{ST} was 0.29 ($P < 0.001$). Mantel's test was significant ($P = 0.006$, correlation coefficient = 0.27). No structure was apparent in the strict consensus (not shown) of more than 5000 maximum parsimony trees. A ML tree (not shown) and a NJ tree (Fig. 2), however, reveal a northern and a southern group. The widespread northern group includes samples from Mezen, Medvedevo, Oulu, Anadyr, Gumbaritsy, Yamal, Kamchatka, Murmansk and Cherskiy, whereas the southern group includes the other samples. However, the division is incomplete, with some haplotypes occurring in the 'wrong' group. Furthermore, these basic groups received only 30% bootstrap support; no other geographically coherent groups received higher bootstrap support. Thus, the haplotype tree provides a glimpse of a stage in the process of lineage sorting (Avise 2000). The tree (Fig. 3) grouping samples based on pairwise distances shows the same northern and southern groups, which abut in the latitudinal centre of the sampled distribution. An AMOVA based on partitioning samples into the two groups yielded an F_{ST} of 0.37 ($P < 0.001$).

Nearly all samples show large differences between θ-values (Table 1), suggesting population growth. Fu's *F*-values were mostly negative, and in samples larger than five were significantly so, except for the sample from Guerande. Unimodal mismatch distributions for the large sample from Tyva (Fig. 4), the northern group, the southern group and all samples combined (not shown) are consistent with a pattern of sudden population expansion according to the test proposed by Schneider & Excoffier (1999). The southern group has a smaller mismatch mean (2.2) than the northern group (3.4), suggesting an older expansion in the north. A multiple regression with latitude and longitude as independent variables did not explain

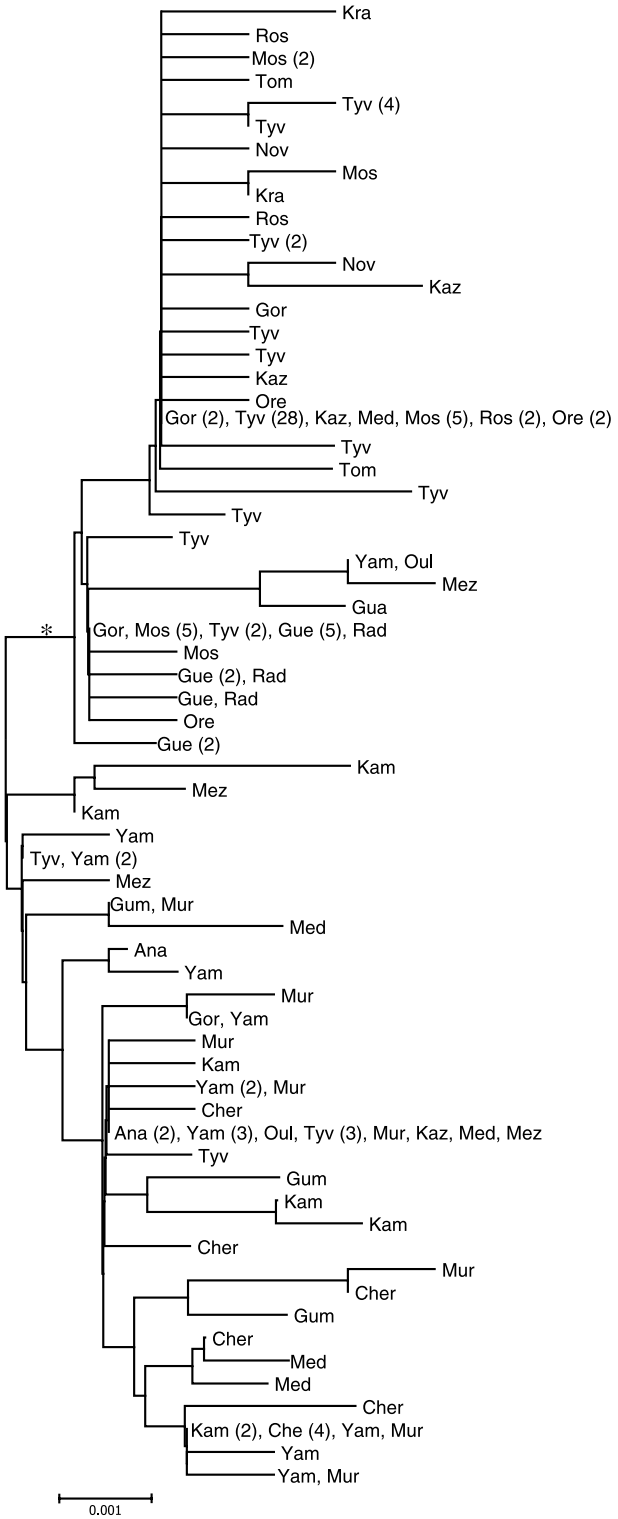

Fig. 2 NJ tree showing two general groups. The asterisk marks the division between two incompletely sorted groups, northern and southern, with lack of geographical structure within groups. Based on Kimura's (1980) two-parameter distance.

($P = 0.38$) a significant proportion of variation in π overall. There was a significant ($P = 0.048$) regression of π on longitude in the southern group, but no latitudinal or longitudinal pattern to π in the northern group. Overall, and in the southern group, there were no significant geographical patterns in tau. Tau was related significantly to latitude and longitude in the northern group ($P = 0.0025$), decreasing to the north and east.

The correlation coefficient between F_{ST} and M-mode was 0.33 ($P > 0.05$), whereas that for T-mode and F_{ST} was 0.64 ($P < 0.05$). A plot of M-mode and geographical distance (Fig. 5) suggested little evidence of gene flow (M-mode) between most localities, and for those pairwise comparisons that had values greater than one, they tended to occur between samples in the two groups.

Discussion

Phylogeographical history

The overall F_{ST} of 0.29 suggests relatively high differentiation. Hence, it was perhaps not surprising to discover two groups when analysing sequences (Fig. 2) and when grouping individuals at the population level (Fig. 3). Examining pairwise F_{ST} values supports the two broad groupings of samples. Within the northern clade F_{ST} averages 0.04, within the southern clade the value is 0.16, and between the two groups of samples a value of 0.42 was obtained. This general pattern of differentiation is consistent with current population densities. In the north, bluethroats are relatively more common than in the south, where there are more potentially competing species (such as the *Luscinia calliope*; Dement'ev & Gladkov 1954). Nucleotide diversity reflects a difference in relative abundance, averaging 0.0027 in the north (56 individuals, 34 haplotypes) and 0.0016 in the south (98 individuals, 34 haplotypes). Similarly, values of M-mode greater than one were found only within groups, with one exception (Gorno-Altay vs. Medvedevo). Hence, although the haplotype tree does not show a strong geographical pattern of reciprocal monophyly, it appears that an intermediate stage of differentiation has been achieved. That is, one would predict that in the future there would be two reciprocally monophyletic groups of haplotypes, and that at present lineage sorting is incomplete. This conflicts with findings in other Eurasian species in which no structure was evident (Taberlet *et al*. 1998; Zink *et al*. 2002a,b).

The samples in the northern group are interspersed in the dendrogram (Fig. 3), whereas those in the south are more geographically coherent. This is consistent with the pattern of F_{ST}, and is probably a result of the southern populations being *in situ* for a longer period. A potential isolating barrier was the emergence of a belt of forest across the centre of Eurasia (Kremenetski 1995). Although this is

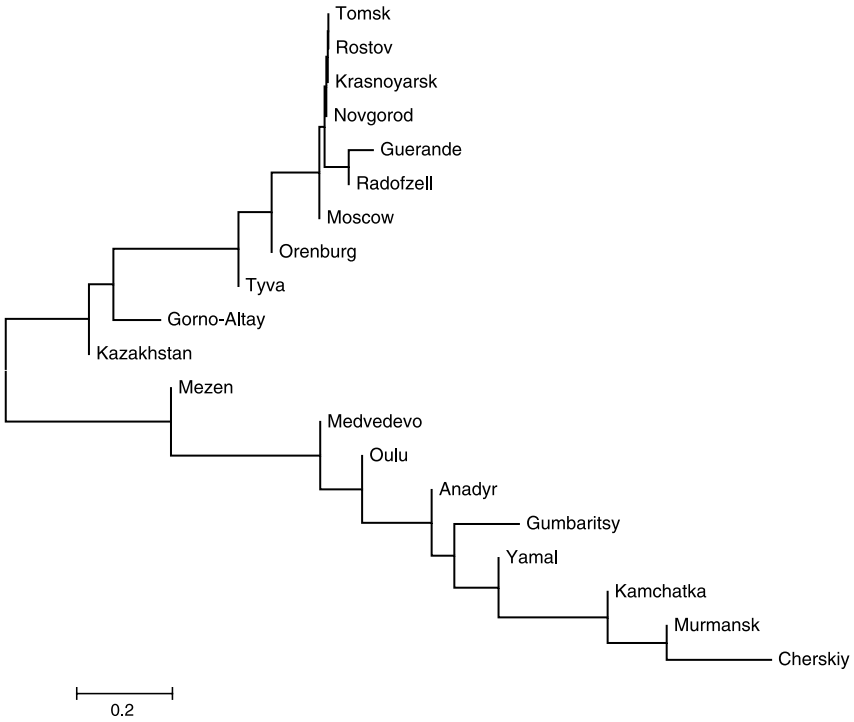

Fig. 3 Minimum-evolution tree based on pairwise genetic distance values among samples, showing existence of a southern group (top) and northern group (bottom; see Fig. 1 for localities). The Guadarrama site was excluded because it included a single individual.

Table 1 Genetic characteristics of samples of *Luscinia svecica*. Subspecies names from Peters (1948)

Locality	Subspecies	*n*	No. Hap.	π	τ	θ_0	θ_1	Fu's *F*
Anadyr	*svecica*	3	2	13	2.3	0	11	1.1
Murmansk[1]	*svecica*	8	8	28	3.3	0	6730	–5.7**
Kamchatka	*svecica*	7	6	32	4.1	0	217	–1.9
Moscow	*volgae*	14	5	11	—	—	—	–1.1
Gorno-Altay	*svecica*	5	4	21	6.1	0	4	–0.84
Krasnoyarsk	*svecica*	2	2	28	—	—	—	1.1
Cherskiy	*svecica*	9	6	19	1.7	1.5	14.0	–2.1
Yamal[2]	*svecica*	14	10	22	2.8	0	6841	–5.8**
Oulu	*svecica*	2	2	19	—	—	—	0.7
Tyva	*kobdensis*	48	15	18	2.5	0	5.1	–10.3**
Guerande[3]	*namnetum*	10	4	9	1.6	0	3439	–1.2
Gumbaritsy	*svecica*	3	3	31	4	0	4765	–0.1
Tomsk	*svecica*	2	2	28	—	—	—	1.1
Novgorod	*volgae*	2	2	28	—	—	—	1.1
Kazakhstan	*pallidogularis*	4	4	33	5.7	0	3075	–1.0
Guadarrama	*azuricollis*	1	1	—	—	—	—	—
Radolfzell[4]	*cyanecula*	3	3	13	1.6	0	3374	–1.2
Mezen	*svevica*	4	4	42	4.0	1.6	4795	–0.66
Medvedevo	*svevica*, *cyanecula*	5	5	38	5.0	0	3839	–1.7
Rostov	*volgae*, *cyanecula*	4	3	9	1.3	0	2645	–0.9
Orenburg	*pallidogularis*	4	3	19	2.3	0	1895	–0.3

n: Number of individuals per locality. No. Hap.: number of different haplotypes present. Nucleotide diversity (π) × 10 000. [1]Includes three individuals from Kola Peninsula (from Questiau *et al*. 1998). [2]Includes four individuals from Oural (from Questiau *et al*. 1998). [3]Includes two individuals from Briere (from Questiau *et al*. 1998) and three migrants from the Tagus Estuary. [4]Includes one individual from Hiddensee.

speculative, it could explain the current abutment of the two relatively weakly differentiated groups.

Mismatch distributions for the northern and southern groups, the Tyva sample (Fig. 4) and the entire sample are consistent with population expansions. However, the overall pattern of π does not conform to that expected for a signature of leading edge expansion (Hewitt 2000) — that is, it does not decrease progressively in a linear way from

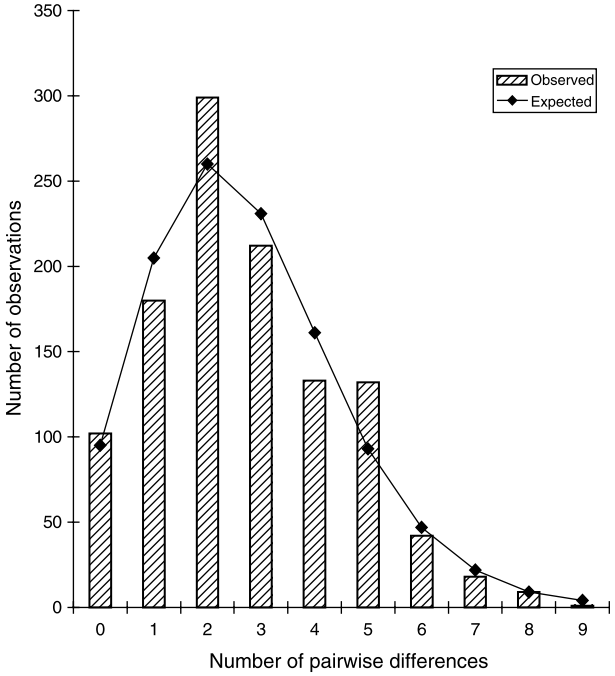

Fig. 4 Mismatch distribution for sequences from the Tyva sample showing consistency with population growth. Expected distribution based on model of sudden population expansion.

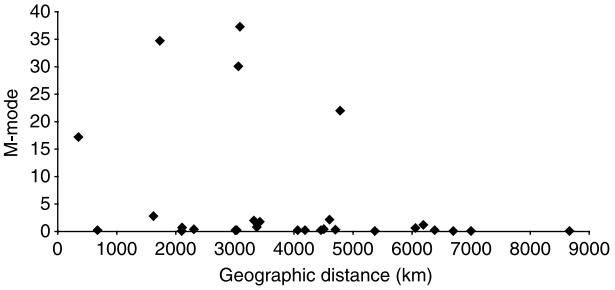

Fig. 5 Plot of the migration parameter obtained from the nonequilibrium approach of Nielsen & Wakeley (2001) vs. geographical distance (km) of eight samples (Cherskiy, Guerande, Medvedevo, Tyva, Yamal, Kamchatka, Gorno-Altay, Moscow).

south to north. However, π-values in the southern group (Fig. 3) decrease ($P = 0.028$) from Kazakhstan to the west, suggesting a westward expansion, which according to the mismatch distribution was more recent than the expansion in the northern group. Recent westward expansion would be expected as Europe was deglaciated. No clear geographical pattern in π exists in the northern group, although the highest values occur in the neighbouring samples of Mezen and Medvedevo. It is uncertain why a westward expansion would also not have occurred in the northern group. However, in the northern group, τ (time since expansion) does decrease significantly to the north and east, which is consistent with a wave of population expansions. Thus, it is possible that the northern group expanded north and east, but longer ago than the westward expansion in the southern group. In any case, bluethroat populations appear not to have expanded simply northward from southern refugia, as has been documented for some European species (Hewitt 2000). Instead, it is possible that there were southeastern and northern refugia, with subsequent recent population growth and longitudinal range expansion. The fact that the mismatch distribution for the entire sample is unimodal suggests that the differentiation between the two refuges was small.

Inferences about population history derived from analyses in ARLEQUIN, such as F_{ST}, are based on the assumption that populations are at equilibrium. If the recent history of *L. svevica* parallels that of other species (Hewitt 2000), we expect that populations have recently undergone expansions and range shifts, as climates ameliorated. Use of the MDIV program provides alternative estimates of gene flow to the equilibrium methods in ARLEQUIN. The general agreement in this study between results derived from MDIV and ARLEQUIN suggests that departures from equilibrium were not sufficiently large to bias results.

Taxonomy

The haplotype tree (Fig. 2) did not support any of the seven sampled subspecies (Table 1). We grouped samples into subspecies and recomputed F_{ST}, finding that the value decreased to 0.24. Thus, the subspecies do not predict evolutionary history (i.e. reciprocally monophyletic groups of haplotypes) represented by the mtDNA gene tree. Questiau *et al*. (1998) reported that individuals representing the subspecies *L. s. svevica* possessed an 'A' at the third position in their cytochrome *b* sequences, whereas those to the west representing *L. s. namnetum* possessed a 'G'. However, we found individuals with a G to the east in Moscow, Gorno-Altay, Krasnoyarsk, Yamal and Tyva (44 of 48 specimens). Although the Gs in samples from outside the range of *L. s. namnetum* could be derived independently, the single bp distinction between *L. s. svecica* and *L. s. namnetum* was not corroborated upon greater sampling.

The population-level tree (Fig. 3) also failed to support recognized subspecies. Lack of mtDNA support for subspecies is often observed in birds (Zink *et al*. 2000). If populations have been isolated for less than $2N_{ef}$ generations (on average), where N_{ef} is the inbreeding effective size of the female population, the mtDNA gene tree will not show a pattern of reciprocal monophyly. However, it is possible for plumage patterns to show geographical structure coincident with subspecies boundaries. This is because the evolution of polygenic phenotypic traits can be more rapid than that of reciprocal monophyly in the single-locus mtDNA genome. However, it is also possible that subspecies simply do not correspond to independently evolving

groups. In some other Eurasian species (Zink *et al*. 2002a,b; Pavlova *et al*. 2003) and most North American species (Ball & Avise 1992; Zink *et al*. 2000), subspecies names also do not predict evolutionary groupings. We predict that multivariate analysis of a suite of morphological characters will not reveal a pattern consistent with the existence of subspecies.

Acknowledgements

We thank the Burke Museum for curatorial assistance; S. Birks subsampled tissues. We thank S. Farrell, T. Line and R. C. Blackwell-Rago for laboratory assistance. A. Andreev, D. Banin, E. Koblik, I. Karagodin, B. Schmidt, V. Sotnikov, G. Voelker and C. Wood provided logistical help with expeditions or collecting. E. Koblik and A. Pavlova provided useful advice. D. Franz, A. Gaginskaya and M. Orell provided some tissues. G. Eddy supported expeditions and some laboratory work; additional laboratory support came from the NSF (DEB 9707496) and P. Taberlet. SVD was supported by Eddy, Leaders Five fellowships from the Burke Museum and a postdoctoral fellowship from the Department of Ecology, Evolution and Behaviour.

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This research is part of an ongoing series of studies of avian phylogeography in Eurasia by R. Zink, S. Rohwer and S. Drovetski. I. Fadeev and E. Nesterov are research scientists at the State Darwin Museum. S. Questiau is interested in questions dealing with avian breeding ecology and sexual selection. M. Westberg is a research associate working in avian genetics.
