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Selective neutrality of mitochondrial ND2 sequences, phylogeography and species limits in *Sitta europaea*

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Abstract

Variation and geographic differentiation in mitochondrial DNA (mtDNA) was studied in the widespread and phenotypically variable Eurasian nuthatch (*Sitta europaea*). To assess whether sequences were evolving in a selectively neutral fashion, we used McDonald–Kreitman [Nature 351 (1991) 652] tests and a tree-based method, which suggested that although ND2 sequences are affected by natural selection against slightly deleterious alleles, the effects do not compromise phylogeographic inferences. Three phylogenetic species-level clades of nuthatches were discovered, corresponding to the Caucasus, southern Europe, and northern Europe plus Asia. Unimodal mismatch distributions within each clade suggest that populations have undergone recent growth. A westward range expansion was inferred from the geographic pattern in nucleotide diversity. Although samples were insufficient, it is possible that nuthatches in England and Japan are recently differentiated. Two specimens of the subspecies *S. e. arctica* formed a sister group to all other *S. europaea*, differing by ca. 10% uncorrected sequence divergence, pointing the need for additional study of this phenotypically distinct taxon. As with other species, mtDNA data support major phenotypic distinctions, but not subspecies.

Keywords: Natural selection; Phylogeography; McDonald–Kreitman test; Eurasian nuthatch; Mismatch distribution; Mitochondrial DNA; Species limits; Population expansion

1. Introduction

Analyses of mitochondrial DNA (mtDNA) sequences are frequently used to assess species boundaries and elucidate phylogeographic patterns (Avise, 2000). The inference of phylogeographic history from mtDNA sequences carries the assumption that the sequences evolve in a selectively neutral fashion, which might be violated in some cases (Ballard and Whitlock, 2004). It is often observed that mtDNA haplotype trees for bird species are shallow and geographically unstructured. Such trees could result from recent demographic expansion (Harpending et al., 1998) or natural selection. Although researchers have tested for the influence of natural selection on mtDNA sequences, few have appraised its effect on phylogeographic inference. In this article, we sequenced the ND2 gene and part of the mitochondrial control region (CR) from 137 individuals identified as *Sitta europaea* (Eurasian nuthatch), representing 22 Eurasian localities. We performed statistical tests on the ND2 sequences to determine whether they have been influenced by natural selection, and if so, whether phylogeographic and taxonomic inferences were affected.

The Eurasian nuthatch is a wide-ranging polytypic species. Taxonomy below the species level depends on the author consulted, as with many species. Some authors recognize varying numbers of subspecies groups, whereas others elevate some forms to species status. For example, Harrap and Quinn (1995) note that the species is often divided into three groups: *caesia* (western Europe, North

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Africa, Asia Minor, the Caucasus, and Iran), *sinensis* (central and eastern China), and *europaea* (Scandinavia to Japan, south to the eastern Tien Shan mountains, northern China). Within the *caesia* and *europaea* groups are numerous subspecies, ranging from forms that differ subtly in plumage coloration to subspecies such as *S. e. arctica*, which is "very distinct, and there are records of *arctica* and *asiatica* being collected together at the same site. It may be an incipient species…" (Harrap and Quinn, 1995, p. 113). Furthermore, Harrap and Quinn (1995) suggest that *S. europaea* is in a "superspecies complex" with *S. nagaensis*, *S. castanea*, and *S. cashmirensis*, which have been variously treated as one or four species. Cramp and Perrins (1993) states that there are five main groups: *europaea*, *caesia*, *sinensis*, *cashmirensis*, and *castanea*; numerous contact zones are recognized. A molecular appraisal of these taxonomic schemes is warranted.

A growing number of phylogeographic studies exist for Eurasian birds (Drovetski et al., 2004a; Irwin et al., 2001; Kryukov and Suzuki, 2000; Kvist et al., 2003; Merila et al., 1997; Pavlova et al., 2003, 2005a,b; Zink et al., 2002a,b, 2003), but general evolutionary patterns in this region are unclear. The Eurasian nuthatch is a useful species for study because of its obligate forest-dwelling habits, which presents the opportunity to compare its phylogeographic pattern to knowledge of habitat change, and genetic patterns in other co-distributed forest species.

2. Materials and methods

2.1. Sampling

We obtained sequence information from 137 breeding individuals, representing 22 Eurasian localities (Fig. 1). GenBank numbers DQ219499–219780. Most specimens have associated study-skin vouchers which are housed in the Burke Museum (University of Washington), State Darwin Museum (Moscow, Russia), Bell Museum (University of Minnesota), or Moscow State University Zoological Museum. Outgroups included *S. canadensis*, *S. crueperi*, *S. carolinensis*, *S. himalayensis*, and *S. pygmaea*.

2.2. Laboratory methods

We amplified and sequenced parts of the mitochondrial CR and the protein-coding ND2 gene using primers and protocols in Zink et al. (2002a) and Drovetski et al. (2004b).

2.3. Data analysis

Sequences from the two gene regions were combined because of the linkage of the mitochondrial genome. We used Arlequin (Schneider et al., 2000) to compute AMOVA (Φ_{ST}), nucleotide diversity (π), θ_0 and θ_1,τ, Tajima's (1989) *D* value, Fu's (1997) *F* value, and mismatch distributions for larger samples (greater than three individuals). Φ_{ST} reflects genetic divergence among populations, accounting for both haplotype frequency differences and haplotype divergence. Nucleotide diversity is a measure of genetic variation, incorporating both the frequency of haplotypes and their sequence divergence. Values of θ_0 and θ_1 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; the relative difference between them provides an index of whether populations have expanded over time. Tau is a relative measure of the time (in generations) since population expansion. Fu's *F* and Tajima's *D*-values reveal whether sequences conform to neutral expectations. Mismatch distributions were tested against a model of sudden population expansion with a bootstrap resampling procedure (Schneider and Excoffier, 1999).

PAUP* (Swofford, 2000) was used to generate a haplotype tree using maximum parsimony (base positions

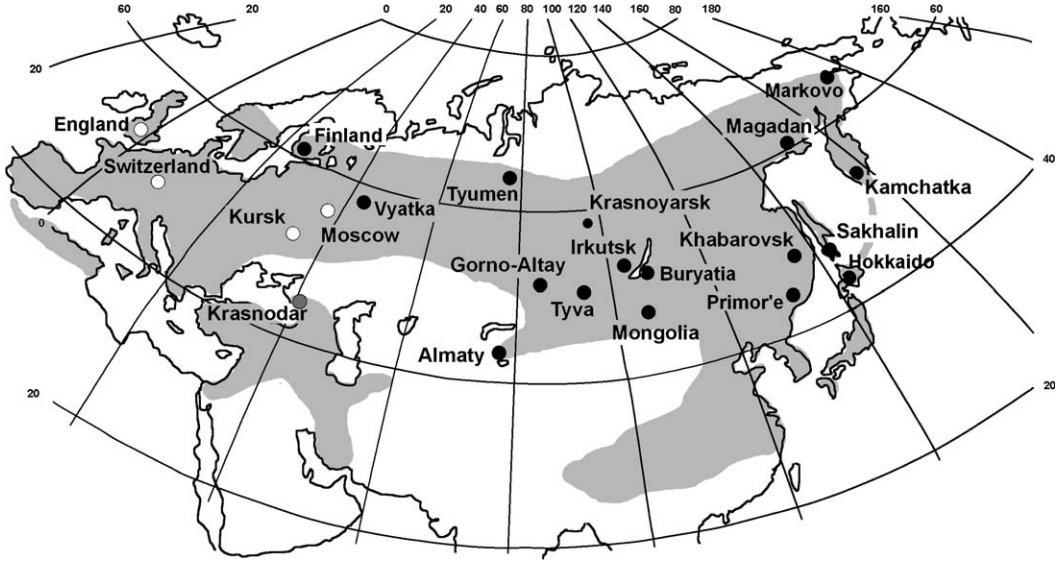

Fig. 1. Breeding distribution of *Sitta europaea*. Solid circles indicate sample sites in large eastern and northern clade, open circles signify the western clade, and Krasnodar (Caucasus) is gray.

weighted equally). Using Modeltest (Posada and Crandall, 1998), we estimated parameters used as input into PAUP* for estimating a maximum likelihood (ML) tree. We used Mega2 (Kumar et al., 2001) to derive a neighbor-joining (NJ) tree based on *p* distances.

Using DnaSP (Rozas et al., 2003), we performed McDonald and Kreitman (1991) (M–K) tests for selection on the ND2 sequences alone for the major groups revealed by phylogenetic analysis. The M–K test compares synonymous and nonsynonymous variation within and between pairs of taxa (or groups of individuals). Under neutrality, the ratio of nonsynonymous (RI)- to synonymous (SI)-fixed substitutions between species should be the same as the ratio of nonsynonymous (RV) to synonymous (SV) polymorphisms within species. A *G*-test or Fisher's exact test (Sokal and Rohlf, 1981) is used to test whether ratios differ statistically. As M–K tests are pairwise, it is difficult to ascertain whether significant M–K tests result from deviations from selective neutrality in one or both species. Furthermore, it is possible to have a nonsignificant M–K test but a nonrandom distribution of substitutions over a tree. Thus, many have noted that a tree-based method is valuable (Creevey and McInerney, 2002; Jansa et al., 2003) for exploring deviations from neutrality. We used the methods of Creevey and McInerney (2002) to test for positive (adaptive or directional) and negative (purifying) selections. In this method, a rooted NJ tree (distance measure computed using only synonymous changes) is computed and assumed to be correct. Hypothetical ancestral sequences are constructed for each node and substitutions are classified as either nonsynonymous or synonymous and their locations identified on the tree. For each internal branch of the tree, substitutions are counted from the branch to the descendant tips, resulting in four values: RI, RV, SI, and SV. A *G*-test or Fisher's exact test (Sokal and Rohlf, 1981) is used to compare the ratio of SV:RV to SI:RI (the expected value under selective neutrality).

Creevey and McInerney (2002) also describe a method to detect negative or purifying selection, which we used on the ND2 sequences. Here, the ratio of replacement to silent sites is computed for each lineage in the haplotype tree, and the number of replacement and silent changes counted on each branch. The expectation is that the ratio of replacement to silent changes on each branch will be predicted by the overall ratio of the two types of sites on the entire tree. Computations are implemented in software "Crann" written by C. Creevey (available at http://bioinf.may.ie/crann/). We recognize that other tests for selection are available, mostly designed to detect positive selection (e.g., Yang, 1998); because we did not detect this type of selection, we did not pursue other methods.

Wise et al. (1998) determined that transmembrane and surface portions of ND2 were subjected to differing levels of selection. We divided the ND2 gene into transmembrane (666 bp) and surface (372 bp) using a structural model suggested by Persson and Argos (1994). Surface codons include: 1–3, 67–81, 142–183, 244–264, 325–345, 406–426, 487–561, 637–711, 796–816, 901–963, and 1024–1038; the remaining codons are transmembrane.

We used DnaSP (Rozas et al., 2003) to compute F_{ST} values among populations separately for surface, transmembrane, synonymous, and nonsynonymous sites. The reason was to determine whether substitutions in a particular data partition might be nonneutral and thereby affect phylogeographic interpretations.

3. Results

3.1. Genetic variation

A total of 65 haplotypes was resolved in the 137 individuals surveyed. A single widespread haplotype was found in 32 individuals from Magadan ($n=3$), Sakhalin ($n=1$), Khabarovsk ($n=2$), Primor'ye ($n=10$), Mongolia ($n=1$), Buryatia ($n=3$), Irkutsk ($n=6$), Tyva ($n=1$), Krasnoyarsk ($n=1$), Tyumen ($n=3$), and Vyatka ($n=1$). One haplotype occurred in eight individuals from Mongolia, Irkutsk, and Tyumen. Nucleotide diversity (Table 1) ranged from 0.0003 (Buryatia and Kursk) to 0.002 (Magadan). Values of τ (Table 1) mirrored nucleotide diversity ($R^2=0.66$; $P=0.002$). A plot (not shown) of nucleotide diversity versus latitude showed no significant pattern, whereas that for longitude (Fig. 2) showed a decreasing trend ($R^2=0.37$, $P=0.046$); multiple regression of nucleotide diversity on latitude and longitude failed to establish a significant relationship ($P=0.15$). Similarly, there was no trend between τ and latitude, whereas smaller values of τ were found in western sites ($r=0.20$, $P=0.027$). The relationship between longitude, and τ and π suggests a westward expansion of nuthatch populations. Comparisons of θ suggested population growth for most samples excluding Tyumen, Khabarovsk, and Krasnodar (Caucasus).

Overall, transmembrane sites were more variable than surface sites, although the combined eastern samples had higher nucleotide diversity for the surface sites (Table 2). The only significant Tajima's *D* and Fu's *F* values were for the combined eastern samples.

3.2. Genetic differentiation

The same overall tree topology (Fig. 3) was revealed by all methods of tree building and showed three major clades, each with bootstrap support exceeding 95%, corresponding to the Caucusus (Krasnodar), western Eurasia (United Kingdom, Switzerland, Kursk, and Moscow), and eastern and northwestern Eurasia (all remaining sample sites). The relationships among the three groups were unresolved. Within each clade, there were no significant clusters of haplotypes, indicating an overall absence of geographic structure; therefore, the individual haplotypes were collapsed into three clades (Fig. 3). However, the single sample from Japan differed by 1% uncorrected sequence divergence from the remainder of the eastern group and was sister to it; further sampling is needed to assess the status of the nuthatches in Japan. Two samples from Kamchatka plus one from

Table 1
Genetic characteristics of samples (greater than four) individuals of *Sitta europaea*

Locality	Subspecies	*N*	Number of haplotypes	π	τ	θ_0	θ_1	Fu's *F*
Buryatia 51°35′N 106°50′E	*asiatica*	4	2	3	0.8	0	1045	0.2
Irkutsk 56°41′N 105°46′E	*asiatica*	12	3	5	0.9	0	2138	0.0
Khabarovsk 51°01′N 137°25′E	*amurensis*	8	5	13	2.4	0	9.9	−1.1
Kursk 51°41′N 34°55′E	*europaea*	4	2	3	0.8	0	1045	0.2
Gorno-Altay 51°02′N 85°38′E	*asiatica*	5	5	18	3.1	0	4646	−2.4*
Krasnodar 44°42′N 38°49′E	*caucasica*	11	4	8	1.8	0	5.7	−0.2
Magadan 59°08′N 150°47′E	*asiatica*	11	9	20	3.0	0	4500	−4.5**
Moscow 54°55′N 39°22′E	*europaea*	13	5	6	1.1	0	2414	−2.0*
Primore' 44°22′N 133°02′E	*amurensis*	42	29	17	2.7	0	6656	−26.6**
Tyumen 63°29′N 74°52′E	*asiatica*	5	3	10	3.8	0	2.5	0.3
Tyva 50°47′N 94°26′E	*asiatica*	4	3	12	2.5	0	312	0.1

Sample locations, subspecies designations and sample sizes for other localities: Switzerland, 47°48′N 08°53′E (cisalpine, 1), United Kingdom, 51°53′N 0°25′W and 51°08′N 0°08′W (*caesia*, 2), Finland, 60°11′N 22°56′E (*europaea*?, 2), Japan, 43°00′N 143°84′E (unknown, 1), Mongolia, 46°43′N 102°51′E and 48°07′N 100°22′E (asiatica, 3), Sakahlin, 46°22′N 141°53′E (*sakhalinensis*, 2), Kamchatka, 53°05′N 157°46′E (*albifrons*, 2), Vyatka, 56°23′N 51°08′E (*europaea*, 1), Markovo, 64°41′N 170°25′E (*arctica*, *albifrons*; 2, 1), Krasnoyarsk, 57°28′N 97°18′E (*asiatica*, 1), Almaty (*asiatica*, 1).
Subspecies names from (Harrap and Quinn 1995). *N* is number of individuals per locality. No. Hap. Refers to the number of different haplotypes present. Nucleotide diversity (π) times 10,000. Tau refers to the relative timing of population expansion, θ_0 refers to before expansion and θ_1 after expansion, and Fu's, 1997 *F* is a measure of selection or population expansion.
$^*P < 0.05$, $^{**}P < 0.01$.

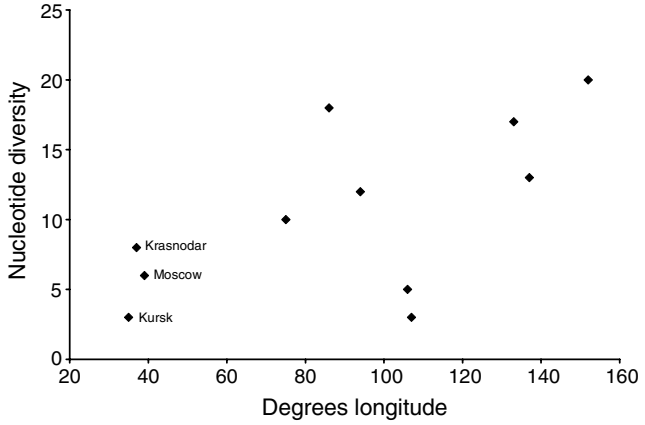

Fig. 2. Plot of nucleotide diversity versus degrees east longitude.

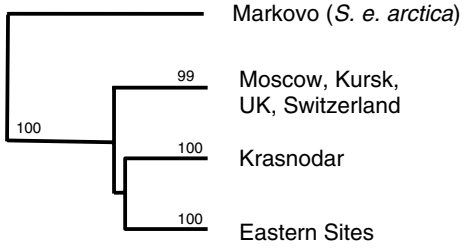

Fig. 3. Phylogenetic tree of major clades showing bootstrap values, rooted with a composite outgroup (see text); the same topology resulted from neighbor-joining, maximum parsimony and maximum likelihood analyses. Because there is no phylogeographic structure in each major clade, individual haplotypes are collapsed.

Table 2
Measures of genetic variability for the surface ("s") and transmembrane ("t") portions of ND2 for the Caucasus, and combined eastern (excluding Japan) and combined western samples

Site	π	θs	Tajima's *D*	Fu's *F*
Caucasus-t	0.0019	0.0015	0.8	−0.23
Caucasus-s	0.0	0.0	0.0	0.0
Eastern-t	0.0014	0.0070	−2.4**	−27.50**
Eastern-s	0.0017	0.0094	−2.33**	−24.64**
Western-t	0.0026	0.0038	−1.08	−2.7
Western-s	0.0003	0.0008	−1.16	−0.88
Overall-t	0.0147	0.017	−0.43	−3.87
Overall-s	0.0133	0.0145	−0.29	−2.78

** $P < 0.001$.

Markovo grouped together in 96% of bootstrap replicates. This suggests that the subspecies *albifrons* might be evolving independently, and more samples are required. Finally, of three samples collected at Markovo, two were identical but highly divergent (ca. 10% uncorrected sequence divergence) from the third, and constituted the sister group to all other samples of *S. europaea*. We performed multiple extractions and polymerase chain reaction amplifications with different primers, always obtaining the same sequence. These two specimens are phenotypically identified as *S. e. arctica* (the third specimen from Markovo was *S. e. albifrons*).

Considering all populations independently, AMOVA indicated that 90% of the genetic variance was distributed among populations. When the samples were organized into the three clades (Caucasus, Eastern, and Western Palearctic), 93.5% of the variance was distributed among clades, 1.9% among populations within clades, and 4.6% within populations. However, the four samples in the western clade were relatively differentiated, with Φ_{ST} values (not shown) above 0.6. Small sample sizes preclude additional analyses.

3.3. Tests for selection

McDonald–Kreitman tests among combined eastern, combined western, and Krasnodar samples were not significant (Table 3), providing no evidence for major deviations from selective neutrality. However, it was clear in some instances that the number of variable replacement substitutions was relatively high. For instance, the SI:RI ratio for

Table 3
McDonald–Kreitman tests among combined eastern, western, and Caucasus' samples

		Eastern		Western	
		Fixed	Variable	Fixed	Variable
Western	Synonymous	19	41		
	Nonsynonymous	4	10		
Caucasus	Synonymous	20	36	21	8
	Nonsynonymous	2	10	4	5

None of the ratios are significant ($P > 0.1$) although it is apparent that variable, nonsynonymous substitutions are often more common than expected.

western versus the Caucasus samples was 21:4, whereas the SV:RV ratio was 8:5. M–K tests (not shown) for the surface and transmembrane sites were also not significant, but again there was a relatively high number of polymorphic replacement substitutions. The NJ tree (Fig. 4) shows two instances (two asterisks) of positive or adaptive changes and several instances (single asterisk) in which a significant deficit of replacement changes was observed, most of which were relatively basal in the tree.

Plots of F_{ST} for surface versus transmembrane sites showed a high correspondence with exception of comparisons involving Tyva and Moscow-Kursk (Fig. 5). In these comparisons, a high transmembrane F_{ST} was associated with a low surface value. F_{ST} values involving Tyva, Moscow, and Kursk were higher for synonymous than nonsynonymous sites (Fig. 6).

3.4. Population growth

Mismatch distributions (Fig. 7) for the Caucasus, combined eastern samples, and combined western samples did not differ from the expectation of sudden population expansion ($P > 0.10$).

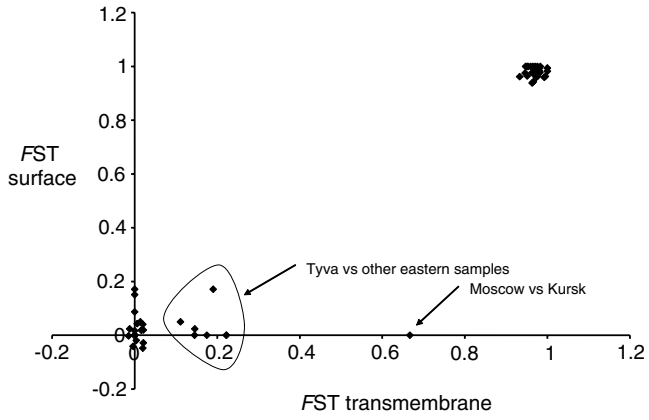

Fig. 5. F_{ST} values for surface versus transmembrane sites. Place in which the two sets of sites give different values are indicated.

4. Discussion

4.1. Selection

Ornithologists have used mtDNA as a marker for discovering population history for more than a decade. An assumption in such studies is that mtDNA sequences evolve in a selectively neutral manner. Thus, geographic patterns in mtDNA can be assumed to represent the demographic and distributional history of populations and not adaptive responses to geographically varying selective pressures. Many studies test for selection on mtDNA coding sequences by computing Tajima's (1989) D value or Fu's (1997) F value (or both). Significantly

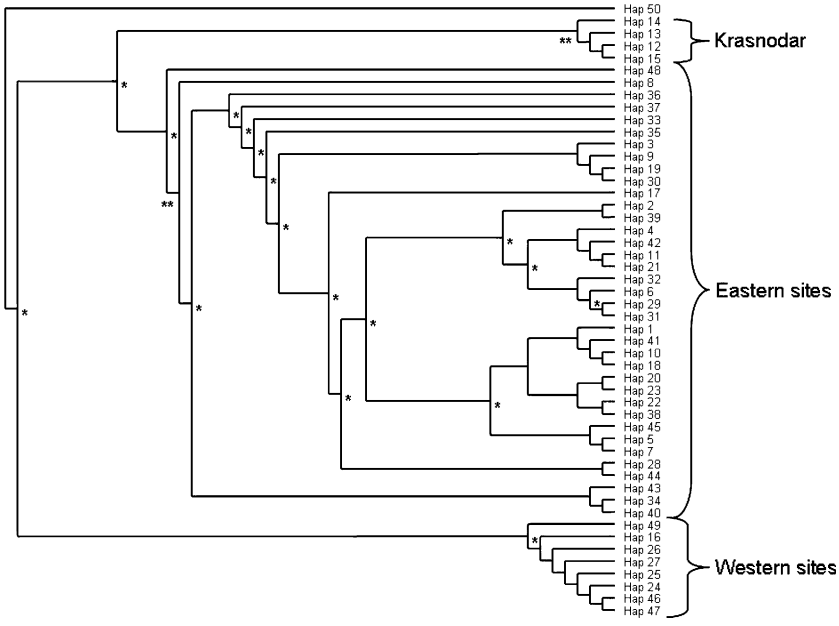

Fig. 4. Neighbor-joining tree for unique haplotypes of ND2 (only) showing phylogenetic distribution of nodes with significant deficits of replacement substitutions (single asterisk). Double asterisks show two places in which positive selection was inferred.

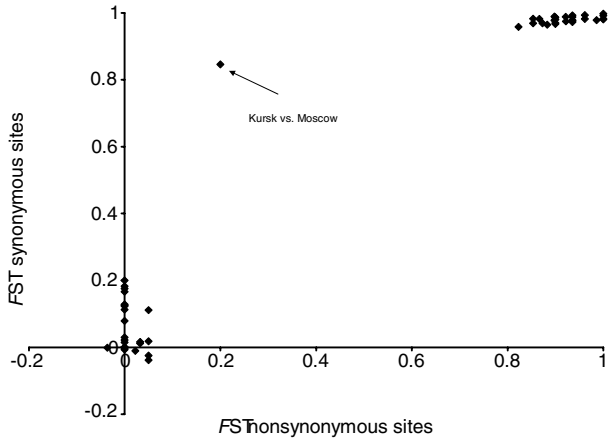

Fig. 6. F_{ST} values for synonymous versus nonsynonymous sites. Places with higher synonymous values involve the Tyva, Moscow, and Kursk locations as in Fig. 5.

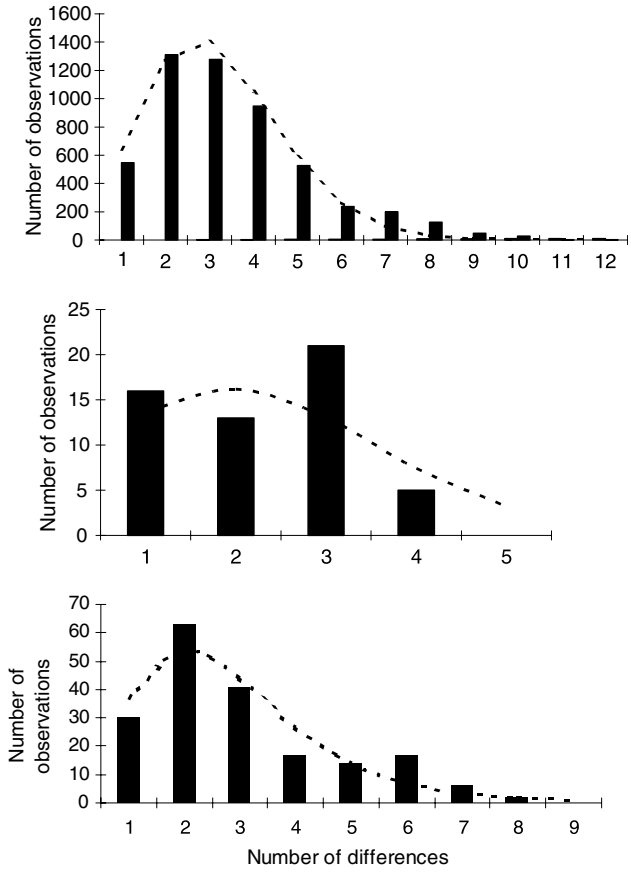

Fig. 7. Mismatch distributions for the three main clades (top—eastern, middle—Krasnodar, bottom—western). In each case, the distribution does not differ significantly from that expected under a model of sudden population expansion (dashed line).

negative F or D values, however, can be explained in two ways: either populations have expanded and sequences are evolving in a neutral fashion, or natural selection affects sequence evolution. It is difficult to distinguish these two potential causes of departures from expectations for selective neutrality.

Documented instances of nonneutral evolution on mtDNA sequences often suggest selection against slightly deleterious alleles (Wise et al., 1998). In this mode, replacement substitutions are underrepresented toward the base of the tree, whereas synonymous substitutions occur more-or-less randomly throughout the tree. The inference is that the replacement substitutions are removed more rapidly by natural selection and segregating nonsynonymous substitutions are on average younger than synonymous changes, which occur more evenly throughout the tree. Although the effect of selection has been investigated on the coalescent (Barton and Etheridge, 2004; Neuhauser and Krone, 1997), investigators typically do not consider the potential impact of selection on phylogeographic analyses, because there has been no clear course of analysis. Here, we attempted to evaluate the effects of the observed selection for phylogeographic inference.

In our study, M–K tests showed no significant difference between ratios of synonymous and nonsynonymous substitutions within and among the three major groups. As these are closely related groups ("phylogroups" sensu Avise and Walker, 1998), it suggests that the early stages of mtDNA lineage divergence in nuthatches is a stochastic process involving mostly genetic drift. That is, natural selection does not appear to be driving genetic divergence among these newly evolved taxa.

The distribution of replacement substitutions on the nuthatch tree (Fig. 4) fits a model of slightly deleterious substitutions, because they tend to be underrepresented toward the base of the tree [true for alternative rootings and other NJ trees (not shown)]. This pattern is consistent with that found in other studies (Nielsen and Weinreich, 1999; Zink, 2004a) and suggests that nonsynonymous substitutions are removed more quickly by selection than synonymous changes that are lost by drift. Although the M–K tests suggest there is not a statistically significant excess of polymorphic replacement substitutions, those that do exist are not randomly distributed over the tree. Thus, the M–K and tree-based methods do not actually conflict; that is, nonsignificant M–K tests do not necessarily rule out nonneutral sequence evolution.

To assess the effect of observed selection on the ND2 sequences, we compared F_{ST} for synonymous and nonsynonymous sites. Several values for synonymous sites (up to 0.20) are larger than those for nonsynonymous sites (Fig. 6). However, this does not mean that the overall F_{ST} values are compromised by selection, which would require nonsynonymous changes to outnumber synonymous ones. In fact, in the early stages of divergence from a common ancestor, one expects synonymous changes to precede nonsynonymous ones, and in the nuthatches, this is so.

Tajima's (1989) D and Fu's (1997) F (Table 2) values were significant for the surface and transmembrane sites for the combined eastern samples. In ND2 data on *Pan* and *Homo*, Wise et al. (1998) found an excess of replacement polymorphisms only within the transmembrane portion of the gene using the M–K test. This would not be expected if

demographic expansions were the cause of the significant Fu's F values because each region should be similarly affected. The fact that both surface and transmembrane sites for nuthatches had significant values (Table 2) for the combined eastern samples suggests that population expansion was responsible, a conclusion apparent in mismatch distribution, for all base pairs (Fig. 7) and for each data partition separately (plots not shown).

A potential difference in the surface and transmembrane data was noted. The plot of F_{ST} values for each partition shows that the substitutions in the transmembrane sites are not mirrored in the surface sites in comparisons of Kursk and Moscow, and Tyva versus eastern sites (Fig. 6). This could indicate that selection is biasing the interpretation of geographic differentiation because the pattern does not exist in each partition. However, the substitutions in the transmembrane sites are in fact synonymous changes. Given that there are more base pairs in the transmembrane parts of the gene (666–372), it is not surprising that there might be more substitutions in this region. In particular, because these substitutions are synonymous, it again fails to establish a bias caused by selection. It appears that incipient divergence has occurred at synonymous positions in the transmembrane partition by chance.

In summary, the tendency for replacement substitutions to be relatively less old (Fig. 4) does not appear to bias inference of population differentiation, nor population growth because mismatch distributions (not shown) for each set of sites were consistent with population growth. In fact, balancing or positive selection would be more likely to influence the shape of the coalescent tree and bias phylogeographic reconstruction than the type of selection documented in nuthatches. Thus, we conclude that although there is evidence of selection against slightly deleterious substitutions, it is insufficient to alter phylogeographic interpretations.

4.2. Systematics

Our haplotype trees (Figs. 3 and 4) reveal at least three independent evolutionary units currently classified as *S. europaea*, corresponding to the Caucasus, western (United Kingdom, Switzerland, Moscow, and Kursk), and eastern/northwestern (remaining sample sites). These groups differ on average by 2% uncorrected sequence divergence. Thus, current taxonomy is an inadequate guide to evolutionary patterns (Zink, 2004b). These three groups might be classified as phylogenetic species (Cracraft, 1989), upon better understanding of zones of contact and Scandinavian populations. Their status as biological species awaits analysis of mate choice behavior.

The samples available to us, although covering nearly 180° longitude, are insufficient to determine finer patterns of geographic differentiation within and between the two main east-west groups. For example, the two samples from the United Kingdom group together in some trees, but without bootstrap support. In addition, it will be important to clarify the status of the nuthatches on Hokkaido and Honshu, where several named subspecies exist, and our sample is ca. 1% divergent. Unfortunately, we cannot ascertain the subspecific identify of our (tissue only) specimen. Although the subspecies *arctica* is phenotypically distinct, it has not been postulated by previous authors to be the sister group to all other *S. europaea*. However, in our phylogenetic analyses (Fig. 3), the two identical specimens of *S. e. arctica* are sister to all other *S. europaea* (including the other individual from Markovo). Unfortunately, we lack enough specimens to determine whether, in fact, this taxon is extremely divergent, and what its geographic limits might be.

The classification based on phenotype variation is, in general, inconsistent with the mtDNA gene tree, relative to subspecies limits. Although the subspecies name (*caucasica*) for the Caucasus sample reflects a group with an independent evolutionary history, other subspecies (Table 1, Fig. 3) do not reflect reciprocally monophyletic groups, as is typical for other avian subspecies (Zink, 2004b). For example, the Vyatka and Finland sites supposedly represent *S. e. europaea* but cluster with eastern samples.

4.3. Phylogeography

To investigate whether the three main groups (Caucasus, western Europe, and eastern Eurasia, excluding Japan, Kamchatka, and all individuals from Markovo) have had different recent population histories, we evaluated mismatch distributions (Fig. 7). Each distribution shows consistency with the pattern expected from population growth, although the sample from Krasnodar (Caucasus) is somewhat ragged (but not statistically so, according to the bootstrap test in Arlequin). The European nuthatch is an obligate forest species. Adams (2002) notes that western Siberia was covered by tundra at the height of the last glaciation. The plot of nucleotide diversity versus longitude (Fig. 2) suggests that a recent westward range expansion has occurred. Hence, as forests returned to western Siberia, recent westward colonization in the obligate forest-dwelling nuthatches is logical. However, it is also apparent that the lowest values occur in the western clade of haplotypes, which weights the overall trend. Thus, a better interpretation might be that Krasnodar and the western group were either recently founded or bottlenecked, or both. In other words, the pattern is likely phylogenetic rather than a result of range expansion.

The phylogeographic pattern observed in European nuthatch was not observed in two other obligate forest species, the great spotted woodpecker (*Dendrocopos major*) and three-toed woodpecker (*Picoides tridactylus*) (Zink et al., 2002a,b). Thus, there is not a consistent phylogeographic pattern yet observed for obligate forest species across Eurasia. However, endemic (or nearly so) clusters of haplotypes in the Caucasus have been documented for other species: Winter wren (*Troglodytes troglodytes*; Drovetski et al., 2004a), White wagtail (*Motacilla alba*; Pavlova et al., 2005b), and Common Rosefinch (*Carpodacus erythrinus*;

Pavlova et al., 2005a). The Caucasus appear to be an area of avian divergence.

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