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Evolution and genetic structure of the great tit (*Parus major*) complex

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The great tit complex is divided into four groups, each containing several subspecies. Even though the groups are known to differ markedly on morphological, vocal and behavioural characters, some hybridization occurs in the regions where they meet. The great tit has often been referred to as an example of a ring species, although this has later been questioned. Here, we have studied the genetic structure and phylogenetic relationships of the subspecies groups to clarify the evolutionary history of the complex using control region sequences of the mitochondrial DNA. The subspecies groups were found to be monophyletic and clearly distinct in mitochondrial haplotypes, and therefore must have had long-independent evolutionary histories. This conflicts with the ring species assignment and supports the formation of secondary contact zones of previously temporarily isolated groups. According to the phylogenetic species concept, all the subspecies groups could be considered as separate species, but if the definition of the biological species concept is followed, none of the subspecies groups is a true species because hybridization still occurs.

Keywords: *Parus major*; subspecies groups; mitochondrial control region; phylogeography

1. INTRODUCTION

The great tit, *Parus major*, is one of the most intensively studied bird species. It has been the subject for over one thousand evolutionary and ecological studies, mostly in Europe but also in Asia (Biosis Previews database produced 1349 hits from 1969 to 2002). It has been a popular study object for many reasons; it is a very familiar bird inhabiting a variety of habitats, it is sedentary and accepts nest-boxes and food provided by man. During the breeding season it is territorial, but otherwise lives in social flocks of single or mixed species. Its distribution range is the widest among the *Parus* species, covering Eurasia from the Atlantic to the Pacific and from northern Fennoscandia to southern Indonesia.

The great tit complex has traditionally been classified into three or four subspecies groups: *major* (Europe, Siberia and northwest Africa), *bokharensis* (central Asia), *cinereus* (from Iran east to India and southeast Asia) and *minor* (China, Japan and eastern Russia; figure 1). These groups comprise approximately 30 subspecies (Cramp & Perrins 1993; Harrap & Quinn 1996). According to the classification of Harrap & Quinn (1996), there are 11 subspecies in the *major* group, 13 in the *cinereus* group and nine in the *minor* group. The *bokharensis* group, with three subspecies (Harrap & Quinn 1996), has sometimes been treated as a separate species, the Turkestan tit (*Parus bokharensis*), and sometimes as a subspecies group in the great tit complex. Russian authors treat all four groups as separate species (Stepanyan 1990). Some hybridization between these groups is known to occur in the regions where they meet. The *major* and *minor* groups mix in the middle Amur valley in far-eastern Siberia, the *cinereus* and *minor* groups in southern China, *major* and *bokharensis* in southwestern Mongolia, and *major*, *cinereus* and *bokharensis* in northeast Iran (Eck & Piechocki 1977; Gosler 1993; Harrap & Quinn 1996; Martens 1996; figure 1). The great tit complex has often been referred to as an example of a ring species with circular distribution and gene flow from one subspecies to another. According to Martens (1996), this assessment is not correct because there is no continuous distribution of forms, the hybrid zones present rather a secondary contact where the hybridizing forms are very distinct, both morphologically and vocally. In addition, there is a bridge formed by the *bokharensis* group in the middle of the otherwise circular distribution range embracing the Tibetan Plateau.

Even though the great tit has been so widely studied, genetic studies on populations and subspecies groups are scarce, and wider studies of the relations between and within subspecies groups are non-existent. However, it is important to know the relationships of different populations and subspecies, for example when ecological and evolutionary studies are compared. Therefore we have undertaken a broad phylogeographic study of the great tit with several specific questions in mind. We wanted to determine: (i) how the subspecies groups are related phylogenetically to each other; (ii) whether there are differences in the genetic structure of the subspecies groups; (iii) if there is evidence of continuous distribution or of a secondary contact at the hybrid zones of the subspecies groups; and (iv) what the genetic structure reveals about the evolutionary history of the great tit complex. These

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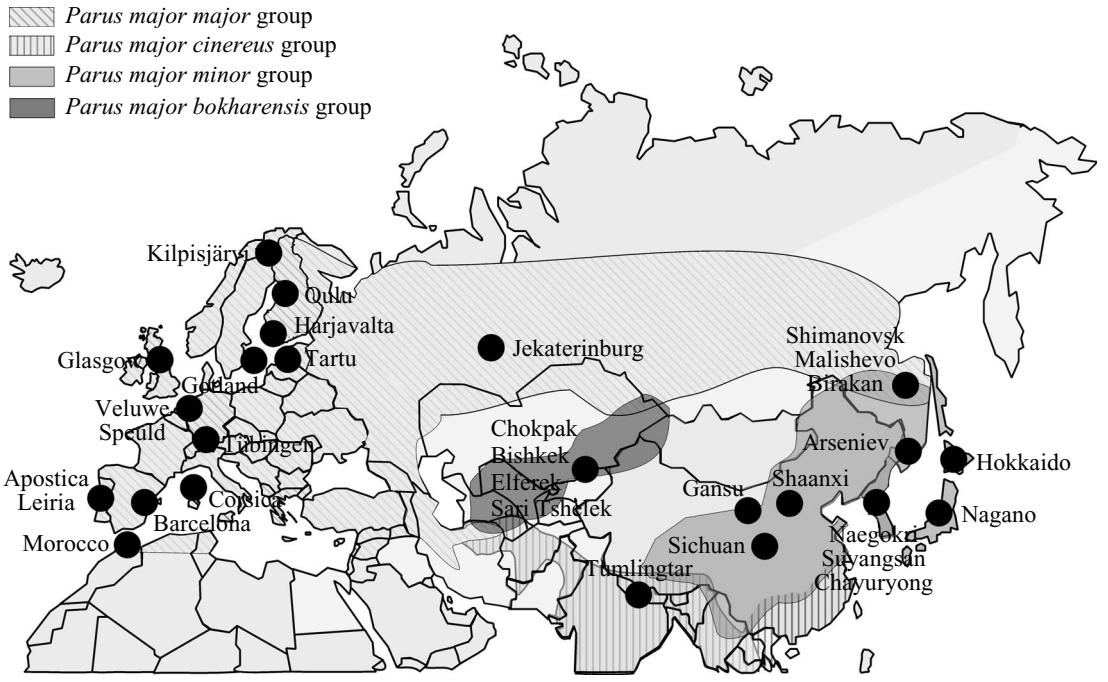

Figure 1. The distribution ranges of the great tit subspecies groups and sampling sites. The sampling sites were grouped and named as follows. *major* group: Glasgow, UK; Veluwe and Speuld, The Netherlands; Leiria and Apostica, Portugal; Barcelona, Spain; Tübingen, Germany; Tartu, Estonia; Harjavalta, Oulu and Kilpisjärvi, Finland; Gotland, Sweden; Corsica, Corsica; Morocco, Morocco; Jekaterinburg, the Urals; Shimanovsk and Birakan, Amur; Chokpak (a *bokharensis* bird possessing *major* genotype) and Bishkek, Kirghizia; *minor* group: Nagano and Hokkaido, Japan; Naegokri, Suyangsan and Chayuryong, North Korea; Birakan, Malishevo and Arseniev, Amur; Sichuan, Shaanxi and Gansu, China; *bokharensis* group: Elterek, Sari Tshelek and Chokpak and *cinereus* group, Tumlingtar (see also electronic Appendix A).

questions were studied by using control region sequences of the mitochondrial DNA.

2. MATERIAL AND METHODS

(a) *Sampling and laboratory methods*

The samples were collected in the wild during 1992–2001 from the sampling sites presented in figure 1 (see also electronic Appendix A; available on The Royal Society's Publications Web site). The samples were blood, feathers or embryonic plates, except samples from North Korea, which were toe pads from museum birds caught during 1978–1986. Parts of the European samples and the samples originating from the Amur valley have been included in previous studies (Kvist *et al.* 1999*a*, 2003; Kvist 2003). DNA from blood samples was extracted using the standard phenol–chloroform procedure. The DNA from feathers and toe pads was isolated by cutting the toe pads or the tips (calamus) of the feathers into tiny pieces and placing them in 100 µl of buffer containing 0.1 M Tris–HCl (pH 8.5), 0.5 mM of EDTA, 0.2% SDS, 0.2 M NaCl and 0.03 mg of proteinase K. The tips or toe pads were incubated for 3 h at 56 °C and centrifuged for 10 min at 10 000 r.p.m. after which the DNA was precipitated from the supernatant with 200 µl of ice-cold ethanol and 10 µl of 3 M Na-acetate (pH 5.2), washed and diluted in 100 µl of sterile water. Enriched mitochondrial DNA was isolated from the embryonic plates as described in Kvist *et al.* (1998).

Amplification of the mitochondrial control region was performed with primers L16700 (5′ATCATAAATTCTCGCCGGGACTCT3′) and H636 (5′GAGATGAGGAGTATTCAACCGAC3′). The amplified region covered the first, and part of the second, domain of the control region. Some DNA samples isolated from the feathers, and all the samples isolated from the toe pads needed to be amplified in two parts using primer pairs designed to amplify the *minor* (L16700 + H328minor 5′-GGGACATTATTCGTATACTGG-3′ and L288minor 5′-CGTACATACAAACTCCACCAG-3′ + H636) or *major* (L16700 + H351major 5′-CTTTAGGAGGTGGGCTTCATGC-3′ and L288major 5′-ACAAACTCCACTCTAGTATACGGA-3′ + H636) haplotypes. PCR reactions were performed in a 50 µl volume containing *ca.* 250 ng of template DNA, 1.0 µM of each primer, 0.2 mM of each dNTP, 5 µl of 10× PCR buffer (2.5 mM $MgCl_2$) and 1.0 unit of Dynazyme (Finnzymes). The amplification profile was 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min and a final extension in 72 °C for 5 min. Sequencing reactions were performed with the primers H636, H328minor or H351major with Big Dye Terminator Cycle Sequencing Kit v. 2.0 and run with ABI 377 automatic sequencer.

(b) *Analysis of mitochondrial data*

The sequences were aligned by eye with program BioEdit v. 5.0.9 (Hall 1999). Pairwise Tamura–Nei distances (Tamura & Nei 1993) within and between the subspecies groups were estimated with program Mega v. 2.1 (Kumar *et al.* 2001) and the same program was used for constructing a neighbour-joining tree with 1000 bootstraps. For the population level analyses, some of the sampling sites were grouped (see figure 1 and electronic Appendix A).

DNAsp v. 3.51 (Rozas & Rozas 1999) was used for estimating nucleotide diversities (π; Nei 1987; eqn 10.5), θ (= $2N_e\mu$, where N_e is effective population size and μ is mutation rate), estimated from the numbers of polymorphic sites per nucleotide (Tajima 1996; eqn 10), haplotype diversities ($\hat{h}$; Nei 1987; eqns 8.4 and

Table 1. Tamura–Nei mean distances (%) for the subspecies groups of the great tit.
(On the diagonal within group average, above the diagonal between group average and below net between group average. Standard deviations (estimated by a bootstrap method, when the distance is estimated and the standard deviation of the original values is computed, 500 bootstraps) are given in parentheses.)

	major	*minor*	*bokharensis*	*cinereus*
major	0.330 (0.059)	5.942 (1.051)	2.810 (0.602)	5.520 (0.978)
minor	5.592 (0.995)	0.370 (0.113)	5.313 (0.894)	4.370 (0.841)
bokharensis	2.057 (0.535)	4.540 (0.829)	1.177 (0.359)	5.131 (0.849)
cinereus	4.945 (0.916)	3.776 (0.815)	4.133 (0.799)	0.819 (0.307)

8.12) and Tajima's *D*s (Tajima 1989*a*; eqn 38). The same program was also used for calculating the mismatch distributions under population expansion and raggedness statistics quantifying the smoothness of the observed mismatch distributions (Harpending 1994), thus allowing a distinction between expanded and stationary populations. Haplotype distributions, pairwise F_{ST}s and analyses of molecular variance (estimated using the haplotype frequencies and Tamura–Nei distances) were calculated with ARLEQUIN v. 2.00 (Excoffier *et al.* 1992), which was also used to construct minimum spanning trees from the *minor* and *major* subspecies groups. The three times rule (Palumbi *et al.* 2001) states that on average most nuclear loci will be monophyletic when the branch length leading to the mtDNA sequences of a species is three times longer than the average mtDNA sequence diversity within that species. The coalescence ratio, C_R (mitochondrial branch length for the subspecies group divided by the average intragroup nucleotide diversity) and the probability of coalescence of a randomly chosen nuclear locus by time t_n in the past were estimated according to Tavaré (1984; eqn 6.4) as modified by Palumbi *et al.* (2001).

(c) *Taxonomy*

In general, we relied on Cramp & Perrins (1993) and Harrap & Quinn (1996). However, in certain cases we followed the more detailed studies by Eck (1980, 1992).

3. RESULTS

(a) *Analyses between the subspecies groups*

Almost all the samples produced a typical sequence for its subspecies group, except one phenotypically *bokharensis* bird, which produced *major* sequence (therefore grouped with other *major* haplotypes in the analyses) and one phenotypically *minor* bird, which produced a heteroplasmic sequence of both *minor* and *major* type (Kvist *et al.* 2003). Both of these birds originate from hybrid zones (see figure 1). The largest difference between the subspecies groups was between the *major* and *minor* groups (table 1). In the neighbour-joining tree these groups were placed most distantly from each other accordingly, with the *cinereus* and *bokharensis* groups in between, *cinereus* being closer to the *minor* group. All four subspecies groups were monophyletic with high bootstrap support (figure 2). The coalescence ratios C_R were 4.0 for *major*, 4.5 for *minor*, 2.1 for *cinereus* and 0.25 for *bokharensis*. These ratios, when substituted in Tavaré's equation (1984; eqn 6.4) modified by Palumbi *et al.* (2001), give probabilities of 0.76 in the *major*, 0.81 in the *minor*, 0.18 in the *cinereus* and 0.01 in the *bokharensis* group for monophyletic nuclear loci.

(b) *Analyses within the subspecies groups*

In the alignment of 578 bp from 125 *major* birds there were 52 polymorphic sites, of which 25 were phylogenetically informative (38 transitions, 15 transversions and one deletion, in some sites there were both transitions and transversions), comprising 57 haplotypes. The most common haplotype was shared between 46 birds originating from almost all populations studied and additional seven haplotypes were shared between two or more populations (see electronic Appendix B). In the 44 *minor* birds there were 24 polymorphic sites (alignment length 575 bp), of which eight were phylogenetically informative (19 transitions, six transversions and one deletion), comprising 29 haplotypes. Only two haplotypes were shared between populations (see electronic Appendix B). In the three *cinereus* birds there were seven singleton polymorphic sites, and in the three *bokharensis* birds 11 singleton polymorphic sites and one indel. Nucleotide diversities, numbers of segregating sites per nucleotide, haplotype diversities and Tajima's *D*s are shown in table 2.

The analysis of molecular variance showed that in the *major* group 12.00% of the total variance comes from the variance between populations, whereas in the *minor* group the between population variance was 27.62%. In the *major* group the significant (permutation test, $p < 0.05$) pairwise F_{ST} values show that the Kirghizian–Kazakhstan (range of 0.2879–0.5392) and British (range of 0.1440–0.4040) populations are different from all the others and to some extent also the Corsican population (range of 0.2015–0.5392, but only two birds were sequenced from there). In the *minor* group all the pairwise F_{ST} values were significant (range of 0.0971–0.4162). The minimum spanning trees in figure 2 show that the relationships between the haplotypes form a different structure in the *major* and *minor* groups, the *major* group showing a typical star-like structure of an expanding population and the *minor* group forming a core of four haplotypes to which the other haplotypes are connected. The mismatch distributions for both subspecies groups were unimodal ($\theta_0 = 0$, $\theta_\infty = 1000$ and $\tau = 1.881$ for *major* and $\theta_0 = 0$, $\theta_\infty = 1000$ and $\tau = 2.110$ for *minor*, where θ is the expected pairwise difference that increases from θ_0 to θ_∞ in τ units of mutational time before present in units of half u generations and u is the sum of per-nucleotide mutation rate in the DNA). The raggedness index for the *major* group was 0.0454 and for the *minor* 0.0569. Distributions of the raggedness statistics based on 1000 simulations resulted in the mean raggedness index of 0.0223 for *major* and 0.0626 for *minor* (95% confidence intervals 0.0063–0.664 and 0.0130–0.2000, respectively). These distributions suggest

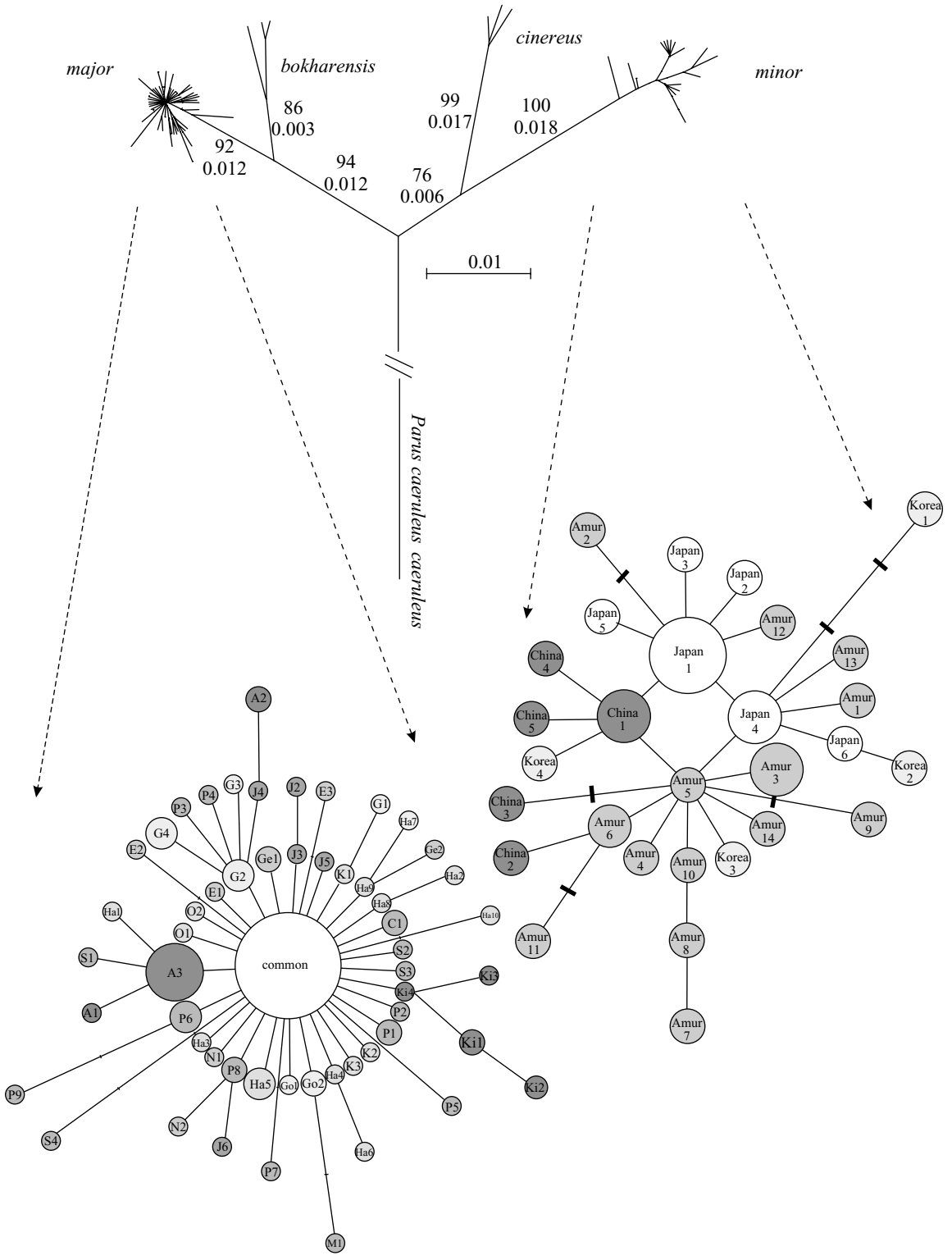

Figure 2. A neighbour-joining tree of (*a*) the great tit subspecies groups and (*b*) minimum spanning trees from the *major* and *minor* groups. The numbers on the branches of the tree represent the bootstrap values supporting the branch (above) and branch lengths (below). The codes in the minimum spanning tree are explained in electronic Appendix B, the sizes of the circles (haplotypes) are proportional to the number of individuals possessing the haplotype and one bar represents one substitution in the DNA sequence.

that 93% and 61% of the simulations produce values equal to or larger than the observed values, given a stationary population. Thus, we can conclude that the *major* group shows population expansion ($p = 0.07$), whereas we cannot reject the hypothesis of no population expansion for the *minor* group ($p = 0.39$).

4. DISCUSSION

(a) *Hybrid zones*

The great tit has been proposed as a ring species, in which case the genetic pattern should follow the isolation by distance model, with geographically adjacent individuals

Table 2. Number of individuals n, nucleotide diversity π (%), θ estimated from the number of segregating sites per nucleotide (%), haplotype diversity $\hat{h}$ and Tajima's D for the subspecies groups and populations of the great tit.

	n	π	θ	$\hat{h}$	Tajima's D
major ssp. group	125	0.325	1.698	0.855	-2.520, $p < 0.001$
UK	10	0.300	0.306	0.844	
Portugal	14	0.373	0.598	0.945	
Spain	9	0.384	0.637	0.917	
Germany	8	0.247	0.334	0.857	
The Netherlands	6	0.173	0.227	0.600	
Estonia	9	0.269	0.446	0.833	
Sweden, Gotland	8	0.247	0.334	0.893	
Finland	28	0.243	0.711	0.794	
Urals/Russia	9	0.336	0.509	0.917	
Kirghizia	6	0.277	0.303	0.933	
Amur/Russia	15	0.277	0.266	0.695	
minor ssp. group	44	0.368	0.921	0.908	-2.051, $p < 0.05$
Amur/Russia	18	0.383	0.608	0.948	
Japan	14	0.176	0.273	0.736	
Korea	4	0.725	0.759	1.000	
China	8	0.354	0.470	0.893	
bokharensis ssp. group Kazakhstan, Kirghizia	3	1.269	1.269	1.000	
cinereus ssp. group Nepal	3	0.810	0.810	1.000	

being more alike than distant individuals (see Irwin *et al.* 2001). However, we found large and discontinuous shifts from genotypes of one subspecies group to another even within sampling locations. This suggests secondary contacts of the subspecies groups in the hybrid zones, which exist between all the subspecies groups. According to Gosler (1993) the hybrid zones were formed as a consequence of post-glacial range expansions. The data presented here support the secondary contact explanation conflicting with the ring species assignment for the great tit.

The samples from the hybrid zone of *bokharensis* and *major* in Kazakhstan and Kirghizia come from a region where hundreds of *major* birds originating from Western Siberia (the exact location is unknown) were released on several occasions in the 1960s (Martens 1996), and they are known to hybridize quite freely in some places (Formozov *et al.* 1993), but in other places they live side by side without mixing (Martens 1996). A former hybridization event was also seen in our sample set of the *bokharensis* birds, as one individual possessed a *major* haplotype. All the birds with *major* plumage phenotype originating from this region possessed *major* haplotypes. In these birds, which are descendants of the introduced birds, one carried the common haplotype and the others carried haplotypes forming a monophyletic group within the major group. This differentiation has probably evolved already in the population from which the introduced birds originate because such differences in the mitochondrial DNA cannot have arisen in the short time-period after the introductions in the 1960s.

Hybridization is also known to occur relatively frequently in the contact zone between *major* and *minor* in the middle Amur valley, even though the differences between the *minor* and *major* birds in coloration, behaviour and vocalization are relatively large. At least the central European *major* birds do not accept *minor* songs in playback experiments (Martens 1996). Also, the habitat preferences are different: the *major* birds preferably occupy open agricultural and other human associated habitats, but *minor* lives in semi-open hilly woodlands (Martens 1996). J. Martens (unpublished data) noted that coloration, especially lipochromes of breast and belly, forms a continuous transition from pure *major* (bright yellow breast and belly) to pure *minor* (light greyish) even within a single village (see also Nazarenko *et al.* 1999). The hybrid birds may sing pure *major* verses, pure *minor* verses or songs where the note groups of these two are mixed, and they often choose agrarian habitats close to, or even within, villages (Martens 1993, 1996). The sampled birds, however, were genotypically from the same subspecies group as predicted from the phenotype, except one individual, which was shown to be heteroplasmic, possessing both *minor* and *major* haplotypes (Kvist *et al.* 2003).

(b) *Evolutionary history of the subspecies groups*

The levels of nucleotide diversity within the *major* and *minor* groups were almost identical (0.325% and 0.368%, respectively), but θ, as estimated from the number of segregating sites, shows a marked difference (1.698% and 0.921%, respectively). As θ is influenced more by the current population size than nucleotide diversity (Tajima 1989*b*), this difference suggests that population expansion in *major* has been far more extensive than in *minor* populations. The expansion in the *major* group may also have been more recent than in the *minor* group (time to expansion τ estimated from the mismatch distribution is 1.881 for the *major* group and 2.110 for the *minor* group). The few samples from the *cinereus* and especially from the *bokharensis* group suggest the populations have been stable for a long time because these subspecies groups possess more diversity than the *minor* and *major* groups and the estimates for nucleotide diversity and θ are identical. As the climate cooling during the Ice Ages was much more pronounced at northern latitudes, and especially in Europe rather than in Central and Eastern Asia, this pattern in

subspecies structure seems to be an inevitable result. The great tits of the *major* subspecies group in Europe were forced to retreat south to escape the advancing ice and coldness, and it has been proposed that all European great tits survived in a single refugium at the Balkans (Kvist *et al.* 1999*a*). The Asian *minor* subspecies group also had to reduce its distribution range. Even though the ice did not advance as far south in eastern Asia as in Europe, parts of the present distribution were covered by steppe–tundra and grassland vegetation (Adams 1997). The *cinereus* group, by contrast, had more or less suitable habitats for it all through the Ice Ages at the present distribution area. The *bokharensis* group differs from the rest by preferring plains, deserts and semi-deserts for habitats (Harrap & Quinn 1996) including a preference for riparian woodlands (J. Martens, personal observation), much the same kind of environment that existed during the Ice Ages within its present range (Adams 1997). Therefore there was less (or no) habitat reduction for the *cinereus* and *bokharensis* groups, than for the *major* and *minor* groups, and they could maintain relatively large population sizes, though the distribution range of *bokharensis* area may have never been as large as that of the three other subspecies groups.

Morphologically and vocally, the four subspecies groups are quite distinct. The coloration of the *major* birds is greenish above and yellow below, the under-parts of the three other groups are white or whitish but the upper-parts differ. The *minor* birds have greenish, the *cinereus* birds blue-grey and the *bokharensis* birds pale grey upper-parts (Harrap & Quinn 1996). The acoustic characters differ markedly, the songs of *major* and *bokharensis* are composed of only slightly changing whistles, whereas *cinereus* and *minor* songs contain rapidly falling and rising note forms (Martens 1996).

(c) *Taxonomic status of the subspecies groups*

The mitochondrial marker used in this study revealed that all the subspecies groups were monophyletic, with high bootstrap values. The average between-group distances between *minor* and *major* (5.94), *cinereus* and *major* (5.52), *bokharensis* and *minor* (5.31) and *cinereus* and *bokharensis* (5.13) are of the same level as distances obtained by using the same part of the control region between some well-recognized species of the subgenus *Poecile*, of the genus *Parus* (e.g. the distances between the willow tit, *Parus montanus*, and the marsh tit, *P. palustris*, is 5.39 and between the Siberian tit, *P. cinctus*, and the black-capped chickadee, *P. atricapillus*, is 6.03; Kvist *et al.* (2001)). The *bokharensis* group, the subspecies group that has most often been proposed to merit a species status, actually differs the least from the *major* group (distance 2.810). However, the distance between *major* and *bokharensis* is twice the distance between two subspecies of the blue tit (*P. caeruleus caeruleus* and *P. c. ogliastrae*, 1.310; Kvist *et al.* 1999*b*).

The need for defining what are actually 'good' species has led to the proposal of many different species concepts, of which the most widely known are the biological species concept (BSC) by Mayr (1963) and the phylogenetic species concept (PSC) by Cracraft (1983). According to the BSC, species are 'groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups' (Mayr 1963), whereas the PSC defines the species as a monophyletic group consisting of 'the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestors and descent' (Cracraft 1983). According to the definition of PSC all four subspecies groups should be considered as separate species, but according to the definition of BSC the situation is not so clear, because the subspecies groups are not completely reproductively isolated. However, the genetic distances show that the subspecies groups have spent quite a long time in allopatry before the formation of the present secondary contact zones. Secondary contacts between the subspecies groups were not possible before the warming of the climate after the last glaciation towards the modern temperatures around 8000 years ago, and northward expansions of the *major* and *minor* subspecies groups.

The times to common ancestor of the subspecies groups were estimated to be *ca.* 3 Myr for *major* and *minor*, 2.8 Myr for *major* and *cinereus*, 1.4 Myr for *major* and *bokharensis*, 2.7 Myr for *minor* and *bokharensis*, 2.2 Myr for *minor* and *cinereus* and 2.6 Myr for *cinereus* and *bokharensis* based on a substitution rate of 2% per Myr (Kvist *et al.* 1999*a*) and average Tamura–Nei distances between the subspecies groups. In other words, this means that no detectable gene flow has occurred between the populations from the late Pliocene or early Pleistocene until the present. According the three times rule (Palumbi *et al.* 2001), most nuclear loci will be monophyletic when the branch lengths leading to interspecific mitochondrial clusters are three times longer than the average sequence diversity observed within species. In the case of the great tit this coalescence ratio varied from 4.5 for *minor* to 0.25 for *bokharensis*. These ratios, when substituted in Tavaré's equation (1984; eqn 6.4) modified by Palumbi *et al.* (2001), suggest that 76% of the nuclear loci in the *major* group and 81% in the *minor* group should be monophyletic, but only 18% in the *cinereus* group and just 1% in the *bokharensis* group, the one that has often been assigned a species status. The reason for these differences in the coalescence ratios lies in the evolutionary histories of the subspecies groups. The genetic variation within *major* and *minor* populations reduced drastically as the populations went through bottlenecks due to the Ice Ages, leading to the growth of the coalescence ratios. On the contrary, the long-term stable population sizes of *bokharensis* and *cinereus* have led to relatively high levels of genetic variation within the subspecies groups and therefore also to low coalescence ratios. In fact, the interspecific branch lengths estimated for the subspecies groups were about the same in *major*, *minor* and *cinereus*, but the branch leading to *bokharensis* was only one-quarter to one-sixth of those (figure 2).

The evolutionary history of the *cinereus* and *bokharensis* groups revealed by our results is somewhat controversial compared with the previous views. According to Gosler (1993), the *cinereus* group is thought to have gone through a bottleneck and range expansion due to the Ice Ages like the *major* and *minor* groups. Even though our samples from the *cinereus* group are few, this seems unlikely because of the large variation in haplotypes. Gosler (1993) also suggests that, even though the *bokharensis* group shares recent ancestors with *major*, it presents an earlier

isolation than *minor* and *cinereus* groups. Our results, combined with the morphology and song characters, show that it is quite unlikely that the *bokharensis* group could have been isolated before the *cinereus* and *minor* groups.

5. CONCLUSIONS

The traditional view, that the great tit complex forms an example of a ring species, is not supported by the data presented here, because the hybrid zones between the subspecies groups are formed by secondary contacts after the expansion of the *major* and *minor* subspecies groups following the Ice Ages. The subspecies groups are clearly distinct in morphological, vocal, behavioural and genetic features and have long, independent evolutionary histories. The *major* and *minor* groups are probably monophyletic in most of their nuclear genes, but the *cinereus* and especially the *bokharensis* groups are not. According to the phylogenetic species concept, all the subspecies groups could be considered as separate species, but if the definition of the biological species concept is strictly followed, none of the subspecies groups is a true species because hybridization still occurs. However, it appears that the subspecies groups are on the way to becoming even more differentiated. In any case, determination of borders between such terms as populations, subspecies or species is arbitrary because there is an evolutionary continuum leading from one to another, which is exactly what is also seen in the case of the great tit complex.

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REFERENCES

Adams, J. M. 1997 *Global land environments since the last interglacial*. Oak Ridge, TN: Oak Ridge National Laboratory. See http://www.esd.ornl.gov/ern/qen/nerc.html.

Cracraft, J. 1983 Species concepts and speciation analysis. In *Current ornithology* (ed. R. F. Johnston), pp. 159–187. New York: Plenum Press.

Cramp, S. & Perrins, C. M. 1993 *The birds of the Western Palaearctic*, vol. VII, pp. 255–281. London: Oxford University Press.

Eck, S. 1980 *Parus major*—ein Paradebeispiel der Systematik? *Falke* **27**, 385–392.

Eck, S. 1992 Zur Systematik und Morphometrie der Kohlmeisen Taschkents und Mittelasiens (Aves, Passeriformes: Paridae). *Zool. Abh. Mus. Tierk. Dresden* **47**, 79–86.

Eck, S. & Piechocki, R. 1977 Eine Kontaktzone zwischen den *bokharensis*-Subspezies und den *major*-Subspezies der Kohlmeise, *Parus major*, in der Südwest-Mongolei. *Ann. Ornithol.* **1**, 127–136.

Excoffier, L., Smouse, P. E. & Quattro, J. M. 1992 Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479–491.

Formozov, N. A., Kerimov, A. B. & Lopantin, V. V. 1993 New hybridisation zone of the great titmouse and *Parus bokharensis* in Kazakhstan and relationships in forms of *Parus major* superspecies. In *Hybridization and the problem of species in vertebrates* (ed. O. L. Rossomolino), pp. 118–146. Moscow: Archives of the Zoological Museum Moscow State University. [In Russian with English summary.]

Gosler, A. 1993 *The great tit*. London: Hamlyn.

Hall, T. A. 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98.

Harpending, R. C. 1994 Signature of ancient population growth in a low resolution mitochondrial DNA mismatch distribution. *Hum. Biol.* **66**, 591–600.

Harrap, S. & Quinn, D. 1996 *Tits, nuthatches and treecreepers*, pp. 353–367. London: Christopher Helm Ltd.

Irwin, D. E., Irwin, J. H. & Price, T. D. 2001 Ring species as bridges between microevolution and speciation. *Genetica* **112–113**, 223–243.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. 2001 *Mega2: molecular evolutionary genetics analysis software*. Tempe, AZ: Arizona State University.

Kvist, L. 2003 On the phylogenetic status of the British great and blue tit (*Parus major newtoni* and *P. caeruleus obscurus*). *Avian Sci.* **3** (In the press.)

Kvist, L., Ruokonen, M., Thessing, A., Lumme, J. & Orell, M. 1998 Mitochondrial control region polymorphism reveal high amount of gene flow in Fennoscandian willow tits (*Parus montanus borealis*). *Hereditas* **128**, 133–143.

Kvist, L., Ruokonen, M., Lumme, J. & Orell, M. 1999*a* The colonisation history and present day colonisation structure of the European great tit (*Parus major major*). *Heredity* **82**, 495–502.

Kvist, L., Ruokonen, M., Lumme, J. & Orell, M. 1999*b* Different population structures in northern and southern populations of the European blue tit (*Parus caeruleus*). *J. Evol. Biol.* **12**, 798–805.

Kvist, L., Martens, J., Ahola, A. & Orell, M. 2001 Phylogeography of a Palaearctic sedentary passerine, the willow tit (*Parus montanus*). *J. Evol. Biol.* **14**, 930–941.

Kvist, L., Martens, J., Nazarenko, A. A. & Orell, M. 2003 Paternal leakage of mitochondrial DNA in the great tit (*Parus major*). *Mol. Biol. Evol.* **20**, 243–247.

Martens, J. 1993 Lautäusserungen von Singvögeln und die Entstehung neuer Arten. *Forsch. der Johannes Gutenberg-Universität Mainz* **9**, 34–44.

Martens, J. 1996 Vocalizations and speciation of Palearctic birds. In *Ecology and evolution of acoustic communication in birds* (ed. D. E. Kroodsma & E. H. Miller), pp. 221–240. Ithaca, NY: Cornell University Press.

Mayr, E. 1963 *Animal species and evolution*. Cambridge, MA: Harvard University Press.

Nazarenko, A. A., Valchuk, O. P. & Martens, J. 1999 Secondary contact and overlap of *Parus major* and *Parus minor* populations in the middle Amur river basin. *Zool. J. Moscow* **78**, 371–381. [In Russian with English summary.]

Nei, M. 1987 *Molecular evolutionary genetics*. New York: Columbia University Press.

Palumbi, S. R., Cipriano, F. & Hare, M. P. 2001 Predicting nuclear gene coalescence from mitochondrial data: the three times rule. *Evolution* **55**, 859–868.

Rozas, J. & Rozas, R. 1999 DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* **15**, 174–175.

Stepanyan, L. S. 1990 *Conspectus of the ornithological fauna of the USSR*. Moscow: Nauka. [In Russian.]

Tajima, F. 1989*a* Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595.

Tajima, F. 1989*b* The effect of change in population size on DNA polymorphism. *Genetics* **123**, 597–601.

Tajima, F. 1996 The amount of DNA polymorphism maintained in a finite population when the neutral mutation rate varies among sites. *Genetics* **143**, 1457–1465.

Tamura, K. & Nei, M. 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**, 512–526.

Tavaré, S. 1984 Line-of-descent and genealogical processes and their applications in population genetic models. *Theor. Popul. Biol.* **26**, 119–164.

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