Paternal Leakage of Mitochondrial DNA in the Great Tit (*Parus major*)

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Animal mitochondrial DNA is normally inherited clonally from a mother to all her offspring. Mitochondrial heteroplasmy, the occurrence of more than one mitochondrial haplotype within an individual, can be generated by relatively common somatic mutations within an individual, by heteroplasmy of the oocytes, or by paternal leakage of mitochondria during fertilization of an egg. This biparental inheritance has so far been reported only in mice, mussels, Drosophila, and humans. Here we present evidence that paternal leakage occurs in a bird, the great tit *Parus major*. The *major* and *minor* subspecies groups of the great tit mix in the middle Amur Valley in far-eastern Siberia, where we found a bird that possessed the very distinct haplotypes of the two groups. To our knowledge this is the first report of paternal leakage in birds.

Introduction

Mitochondrial heteroplasmy has been reported in a wide range of species, including insects (Drosophila: Solignac, Monnerot, and Mounolou 1983; Kondo et al. 1990), birds (gulls: Crochet and Desmarais 2000), fish (perch: Nesbø, Arab, and Jakobsen 1998), and mammals (mice: Gyllensten et al. 1991; bats: Wilkinson and Chapman 1991; dogs: Savolainen, Arvestad, and Lundeberg 2000; and humans: Schwartz and Vissing 2002). The benign heteroplasmic conditions mostly involve length variation caused by the variable number of tandem repeats in the noncoding control region of the mitochondrial genome (Lunt, Whipple, and Hyman 1998). This length variation is thought to have arisen through slipped strand mispairing during replication (Densmore, Wright, and Brown 1985). Pathogenic mitochondrial diseases, well documented in humans, are often caused by large deletions, and their clinical features reflect the frequency and tissue distribution of the mutant mitochondria (DiMauro and Schon 2001). Heteroplasmic single nucleotide polymorphisms have also been reported in several species (cattle: Hauswirth and Laipis 1982; human: DiMauro and Schon 2001; razorbill: Moum and Bakke 2001). All of these forms of heteroplasmy can be generated by somatic mutations within an individual or by heteroplasmy of the oocytes.

Another possibility is that heteroplasmy may arise through paternal leakage, which is to say that the paternal mitochondria are not always eliminated during fertilization of an egg. Paternal leakage has been reported to occur from time to time in Drosophila, mice, mussels, and humans (Kondo et al. 1990; Gyllensten et al. 1991; Zouros et al. 1992; Schwartz and Vissing 2002, respectively). One explanation proposed to account for the rare detection of biparental inheritance of mitochondria is that the two haplotypes should be dissimilar enough to be detected during usual screening, e.g., for population genetic studies. Studies of hybrid zones, where populations harboring relatively genetically distant mitochondrial haplotypes meet, provide an ideal opportunity to search for paternal mitochondrial leakage. Here we present evidence that introgression and paternal leakage of mitochondrial haplotypes occurs in a hybrid zone where two subspecies groups of the great tit, *Parus major*, meet. To our knowledge this is the first report of paternal leakage in birds.

Key words: heteroplasmy, hybrid zone, mitochondrial control region, *Parus major*.

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Materials and Methods

The great tit has been classified into four subspecies groups; *major* (occurring in Europe, Siberia, and northwest Africa), *minor* (occurring in China, Japan, and eastern Russia), *cinereus* (occurring from Iran east to India and southeast Asia), and *bokharensis* (occurring in central Asia), consisting of altogether about 30 subspecies (Cramp and Perrins 1993, pp. 145–281; Harrap and Quinn 1996, pp. 353–367). Some hybridization between these subspecies 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, where *major* occupies open agricultural and other human-associated habitats, but *minor* is observed mainly in semi-open hilly woodlands (Martens 1996).

We extracted DNA from blood samples using the standard phenol-chloroform procedure from a total of 27 great tits originating from four sampling sites in the middle Amur Valley. Of these birds, 15 were phenotypically *major* and 12 were *minor* birds or individuals close to those phenotypes. Amplification of the mitochondrial control region was performed with primers L16700 (5′ATCATAAATTCTCGCCGGGACTCT3′) and H636 (5′GAGATGAGGAGTATTCAACCGAC3′). The amplified region covered all of the first domain and part of the second domain of the control region. Polymerase chain reaction (PCR) was performed in 50 μl volume containing about 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 primer H636 with Big Dye Terminator Cycle Sequencing Kit version 2.0 and run with the ABI 377 automatic sequencer.

The sequence from one phenotypically *major* individual was repeatedly a mixture of *minor* and *major* haplotypes, making the sequence unreadable after a region where there were two indels (the one of a single and the other of two base pairs) between the different haplotypes. Therefore, new primer pairs were designed to amplify only the *minor* (L16700 + H328minor 5′GGGACATTATTCGTATACTGG3′ and L288minor 5′CGTACATACAAACTCCACCAG3′ + H636) or *major* (L16700 + H351major 5′CTTTAGGAGGTGGGCTTCATGC3′ and L288major 5′ACAAACTCCACTCTAGTATACGGA3′ + H636) haplotypes. The PCR conditions were the same as described above.

Sequencing reactions were performed with primers H328minor (5′ end of the *minor* control region), H351major (5′ end of the *major* control region), or H636 (central part of *major* or *minor* control region). These primers produced pure *major* (GenBank accession number AF537976) and *minor* (GenBank accession number AF537975) sequences from this individual, from which both haplotypes were sequenced (altogether 578 bp) four times from independent PCRs. A maximum likelihood tree of the 28 sequences (GenBank accession numbers AF537962–AF537989) was constructed using the program fastDNAml (Olsen et al. 1994), with a transition/transversion ratio of 11, empirical base frequencies, and 100 bootstraps.

In addition, to rule out a possible mixing of two samples in one, six polymorphic microsatellite loci (http://www.shef.ac.uk/misc/groups/molecol/Passerineprimers.xls) from the heteroplasmic bird were screened: *Pdo5* (Griffith et al. 1999), *Pocc6* (Bensch, Price, and Kohn 1997), *Esc6* (Hanotte et al. 1994), *PK12* (GenBank accession number AF041466), *Ppi2* (Martinez et al. 1999), and *Pca8* (Dawson et al. 2000). One specimen of a *minor* genotype and one of a *major* genotype were screened for comparison. *Pdo5*, *Esc6*, and *Ppi2* were amplified in a 10 μl PCR containing ~50 ng of template DNA, 0.4 μM of each primer, 0.1 mM of each dNTP, 1 μl of 10× PCR buffer (2.5 mM $MgCl_2$), and 0.16 units of Dynazyme (Finnzymes) using the following profile: 94 for 2 min followed by 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 45 s; and a final extension in 72°C for 2 min. *Pocc6*, *Pca8*, and *PK12* were amplified similarly, except the annealing temperature was 55°C, and $MgCl_2$ was 3.0 mM for *Pca8* and 1.5 mM for *PK12*.

Results and Discussion

Of the 27 birds sampled from the middle Amur Valley one bird of *major* phenotype had both *minor* and *major* mitochondrial haplotypes. All other samples proved to be phenotypically and genotypically from the same subspecies group. The difference between the two haplotypes within the heteroplasmic birds was 6.24% (31 transitions, 3 transversions, one 1-bp indel, and one 2-bp indel). From these substitutions 27 transitions, 2 transversions, and the indels are fixed differences between the *minor* and *major* haplotypes according to our larger body of unpublished data. The PCR products performed with *minor* or *major* specific primers were approximately of even quantity when judged from agarose gels. The possibility of amplifying a nuclear copy was ruled out, for two reasons: (1) all other amplifications did not reveal any traces of another haplotype and (2) in the phylogenetic tree, the haplotypes of the heteroplasmic bird were placed within the monophyletic clades of the respective haplotypes (fig. 1).

Five of the screened microsatellite loci were heterozygotes and one (*Pocc6*) was a homozygote. This finding is proof against an accidental mixing of samples of two individuals into one, which would have been revealed by at least some of these highly polymorphic loci (from 7 to 20 alleles according to the Bird microsatellite primer cross-utility database of the Sheffield Molecular Genetics Facility) having contained more than two alleles.

Obviously, introgression of mitochondrial haplotypes must occur from one group to another as a result of hybridization. Finding a heteroplasmic bird having the two very distinct haplotypes of *minor* and *major* was, however, a surprise, because this kind of heteroplasmy very likely occurred by paternal leakage, not somatic mutations. Heteroplasmic conditions thus far reported from other bird species may have been generated by two distinct somatic mutational processes. Variable numbers of heteroplasmic tandem repeats, likely produced through slipped strand mispairing (Densmore, Wright, and Brown 1985), have been documented at least in the shrike (*Lanius ludovicianus*: Mundy, Winchell, and Woodruff 1996), in some auks, gulls, and a wader (family Laridae and *Calidris maritima*: Berg, Moum, and Johansen 1995), and in other gulls and some terns (genera *Larus* and *Sterna*: Crochet and Desmarais 2000). To our knowledge, the only bird species which has been shown to be heteroplasmic due to a single-site base substitution is the razorbill (*Alca torda*: Moum and Bakke 2001). In the case reported here, the difference between the two haplotypes of the heteroplasmic great tit is too large to be explained by somatic mutations within an individual, and it would be highly unlikely that such somatic mutations would result in a haplotype of a sympatric subspecies group.

Whether the mitochondrial DNA has leaked from the father of the bird, or whether such leakage occurred in previous generations and was followed by maternal transmission of the heteroplasmy, cannot be determined, even though the fact that the phenotype is of a *major* type supports an older introgression from *minor* to *major*. Some authors have reported that the proportion of different mitochondrial haplotypes estimated from all the offspring is about the same as the proportion in their heteroplasmic mother, but the level of heteroplasmy varies among the offspring (Chinnery et al. 2000 and references therein). Often, heteroplasmic conditions are resolved within one or few generations through a bottleneck during oogenesis, a process analogous to strong genetic drift (e.g., Ashley, Laipis, and Hauswirth 1989; Gocke, Benko, and Rogan 1998). The level of heteroplasmy also varies in different tissues, as seen, for example, in humans, where clinical features in the affected tissues differ, depending on the relative amounts of pathogenic mitochondria and normal mitochondria (Chinnery 2002; Schwartz and Vissing 2002). Unfortunately, there are no data from which to examine the

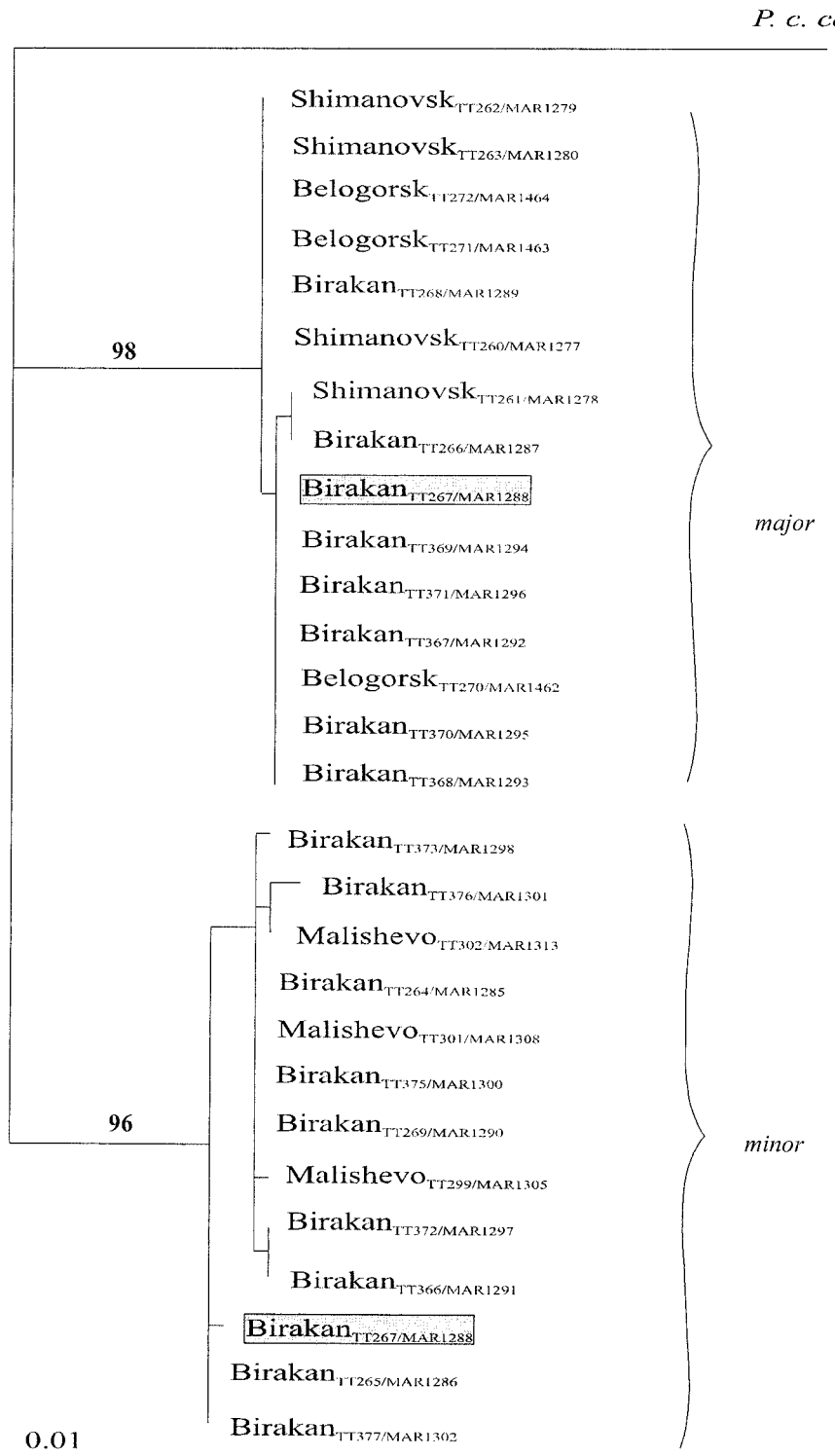

FIG. 1.—A maximum likelihood tree from the great tits from the hybrid zone in the middle Amur Valley. The number on each branch represents the bootstrap support of that branch. The haplotype names correspond to the names of the sampling sites, and the subscripts correspond to the sequence identification in the GenBank (accession numbers AF537962–AF537989) and the Martens (MAR) tissue collection. The haplotypes of the heteroplasmic bird are shaded.

transmission of mitochondria from parents to offspring in birds or to study the possible proportional differences of mitochondrial haplotypes in different tissues. In mice, there is some experimental evidence for persistent transmission of the leaked haplotype to subsequent generations (Gyllensten et al. 1991) and other evidence against ongoing transmission (Shitara et al. 1998). The proportion of leaked mitochondria in mice has been estimated from intraspecific crosses to be about 0.01% (Gyllensten et al. 1991). From crossing experiments of *Drosophila simulans* × *D. mauritiana*, the proportion of leaked paternal mtDNA per fertilization was estimated to be about 0.1%. In three of 331 lines, the maternal type was completely replaced by the paternal type of mitochondria, whereas a fourth line was heteroplasmic (Kondo et al. 1990).

Paternal leakage of mitochondria seems to be a widespread phenomenon among the animal phyla, being present at least in molluscs, insects, and vertebrates (mammals and birds). Further studies of hybrid zones could give more insight into the extent of paternal leakage in the animal kingdom, but estimation of the amount and persistence of leaked mitochondria would need controlled laboratory experiments. Paternal leakage of relatively distinct mtDNA also composes a framework for detection of possible mitochondrial recombination, a phenomenon which has recently been the subject of strong debate (Eyre-Walker and Awadalla 2001 and references therein).

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Literature Cited

Ashley, M. V., P. J. Laipis, and W. W. Hauswirth. 1989. Rapid segregation of heteroplasmic bovine mitochondria. Nucleic Acids Res. **17**:7325–7331.

Bensch, S., T. Price, and J. Kohn, 1997. Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler. Mol. Ecol. **6**:91–92.

Berg, T., T. Moum, and S. Johansen. 1995. Variable numbers of tandem repeats make birds of the order Ciconiiformes heteroplasmic in their mitochondrial genomes. Curr. Genet. **27**:257–262.

Chinnery, P. F. 2002. Modulating heteroplasmy. Trends Genet. **18**:173–176.

Chinnery, P. F., D. R. Thorburn, D. C. Samuels, S. L. White, H.-H. M. Dahl, D. M. Turnbull, R. N. Lightowlers, and N. Howell. 2000. The inheritance of mitochondrial DNA heteroplasmy: random drift, selection or both? Trends Genet. **16**:500–505.

Cramp, S., and C. M. Perrins. 1993. The birds of the Western Palearctic, Vol. 7. Flycatchers to shrikes. Oxford University Press, London.

Crochet, P.-A., and E. Desmarais. 2000. Slow rate of evolution in the mitochondrial control region of gulls (Aves: Laridae). Mol. Biol. Evol. **17**:1797–1806.

Dawson, D. A., O. Hanotte, C. Greig, I. A. K. Stewart, and T. Burke. 2000. Polymorphic microsatellites in the blue tit *Parus caeruleus* and their cross-species utility in 20 songbird families. Mol. Ecol. **9**:1941–1944.

Densmore, L. D., J. W. Wright, and W. M. Brown, 1985. Length variation and heteroplasmy are frequent in mitochondrial DNA from parthenogenetic and bisexual lizards (genus *Cnemidophorus*). Genetics **110**:689–707.

DiMauro, S., and E. A. Schon. 2001. Mitochondrial DNA mutations in human disease. Am. J. Med. Genet. **106**:18–26.

Eyre-Walker, A., and P. Awadalla. 2001. Does human mtDNA recombine? J. Mol. Evol. **53**:430–435.

Gocke, C. D., F. A. Benko, and P. K. Rogan. 1998. Transmission of mitochondrial DNA heteroplasmy in normal pedigrees. Hum. Genet. **102**:182–186.

Griffith, S. C., I. R. K. Stewart, D. A. Dawson, I. P. F. Owens, and T. Burk. 1999. Extra-pair paternity in mainland and island populations of a socially monogamous bird, the house sparrow (*Passer domesticus*): is there an "island effect"? Biol. J. Linnean Soc. **68**:303–316.

Gyllensten, U., D. Wharton, A. Josefson, and A. Wilson. 1991. Paternal inheritance of mitochondrial DNA in mice. Nature **352**:255–257.

Hanotte, O., C. Zanon, A. Pugh, C. Greig, A. Dixon, and T. Burke. 1994. Isolation and characterization of microsatellite loci in a passerine bird: the reed bunting *Emberiza schoeniclus*. Mol. Ecol. **3**:529–530.

Harrap, S., and D. Quinn. 1996. Tits, nuthatches & treecreepers. Christopher Helm Ltd, London.

Hauswirth, W. W., and P. J. Laipis. 1982. Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows. Proc. Natl. Acad. Sci. USA **79**:4686–4690.

Kondo, R., Y. Satta, E. T. Matsuura, H. Ishiwa, N. Takahata, and S. I. Chigusa. 1990. Incomplete maternal transmission of mitochondrial DNA in *Drosophila*. Genetics **126**:657–663.

Lunt, D. H., L. E. Whipple, and B. C. Hyman. 1998. Mitochondrial DNA variable number tandem repeats (VNTRs): utility and problems in molecular ecology. Mol Ecol. **7**:1441–1455.

Martens, J. 1996. Vocalizations and speciation of Palearctic birds. Pp. 221–240 *in* D. E. Kroodsma and E. H. Miller, eds. Ecology and evolution of acoustic communication in birds. Cornell University Press, Ithaca, N.Y.

Martinez, J. G., J. J. Soler, M. Soler, A. P. Møller, and T. Burke. 1999. Comparative population structure and gene flow of a brood parasite, the great spotted cuckoo (*Clamator glandarius*), and its primary host, the magpie (*Pica pica*). Evolution **53**:269–278.

Moum, T., and I. Bakke. 2001. Mitochondrial control region structure and single site heteroplasmy in the razorbill (*Alca torda*; Aves). Curr. Genet. **39**:198–203.

Mundy, N. I., C. S. Winchell, and D. S. Woodruff. 1996. Tandem repeats and heteroplasmy in the mitochondrial control region of the loggerhead shrike (*Lanius ludovicianus*). J. Hered. **87**:21–26.

Nesbø, C. L., M. O. Arab, and K. S. Jakobsen. 1998. Heteroplasmy, length and sequence variation in the control regions of three percid fish species (*Perca fluviatilis*, *Acerina cernua*, *Stizostedion lucioperca*). Genetics **148**:1907–1919.

Olsen, G. I., H. Matsuda, R. Hagstöm, and R. Overbeek. 1994. FastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. Comput. Applic. Biosci. **10**:41–48.

Savolainen, P., L. Arvestad, and J. Lundeberg. 2000. mtDNA tandem repeats in dogs and wolves: mutation mechanism studied by analysis of the sequence imperfect repeats. Mol. Biol. Evol. **17**:474–488.

Schwartz, M., and J. Vissing. 2002. Paternal inheritance of mitochondrial DNA. N. Engl. J. Med. **347**:576–580.

Shitara, H., J.-I. Hayashi, S. Takahama, H. Kaneda, and H. Yonekawa. 1998. Maternal inheritance of mouse mtDNA in interspecific hybrids: segregation of the leaked paternal mtDNA followed by the prevention of subsequent paternal leakage. Genetics **148**:851–857.

Solignac, M., M. Monnerot, and J.-C. Mounolou. 1983. Mitochondrial DNA heteroplasmy in *Drosophila mauritiania*. Proc. Natl. Acad. Sci. USA **80**:6942–6946.

Wilkinson, G. S., and A. M. Chapman. 1991. Length and sequence variation in evening bat D-loop mtDNA. Genetics **128**:607–617.

Zouros, E., K. R. Freeman, A. O. Ball, and G. H. Pogson. 1992. Direct evidence for extensive paternal mitochondrial DNA inheritance in the marine mussel *Mytilus*. Nature **359**:412–414.

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