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## Anomalous Variation in Mitochondrial Genomes of White-crowned (*Zonotrichia leucophrys*) and Golden-crowned (*Z. atricapilla*) Sparrows: Pseudogenes, Hybridization, or Incomplete Lineage Sorting?

JASON D. WECKSTEIN,<sup>1,3</sup> ROBERT M. ZINK,<sup>1</sup> RACHELLE C. BLACKWELL-RAGO,<sup>1</sup> AND DOUGLAS A. NELSON<sup>2</sup>

<sup>1</sup>*Bell Museum of Natural History, and Department of Ecology, Evolution and Behavior, 100 Ecology Building, University of Minnesota, St. Paul, Minnesota 55108-6097, USA; and*

<sup>2</sup>*Borror Laboratory of Bioacoustics, and Department of Zoology, 1735 Neil Avenue, The Ohio State University, Columbus, Ohio 43210-1293, USA*

The White-crowned Sparrow (*Zonotrichia leucophrys*) is a common breeding bird in scrubby habitat of the Pacific coast, montane, and boreal regions of North America; five subspecies are recognized (Fig. 1A). The Golden-crowned Sparrow (*Z. atricapilla*) has a more restricted breeding range and is not subdivided into subspecies (Fig. 1B). The full species status of these two sparrows is undisputed by ornithologists. Despite considerable breeding season sympatry, there are few hybrids known. Because a previous restriction site analysis (Zink et al. 1991) of mitochondrial DNA (mtDNA) suggested that these two species are very closely related, we compared mtDNA sequences and allozyme data from those species and the other North American congeners (*Z. querula*, *Z. albicollis*).

Partial sequences from two mitochondrial genes in Golden-crowned and White-crowned sparrows were virtually identical. Neither species is reciprocally monophyletic in a haplotype tree, and two haplotypes are shared between several White-crowned and Golden-crowned sparrows. Considered together, *Z. leucophrys* and *Z. atricapilla* mtDNA sequences possess less variation than that found in single populations of passeriform bird species. In contrast, one fixed allozyme difference and several frequency differences (Zink 1982) indicated that *Z. leucophrys* and *Z. atricapilla* have been evolving independently for a considerable period of time. In this paper, we evaluate four possible explanations that could account for that anomalous lack of mtDNA differentiation between *Z. leucophrys* and *Z. atricapilla*: (1) accidental amplification of a nuclear pseudogene, (2) hybrid origin of either *Z. leucophrys* or *Z. atricapilla*, (3) incomplete lineage sorting, or (4) past introgressive hybridization.

*Materials and Methods.*—We sequenced 985 base pairs (bp) from the mtDNA genome in each of 13 Golden-crowned and 22 White-crowned sparrows

that encompassed all described subspecies. Of these 985 bp, 433 bp are from a coding gene, cytochrome-*b*, and 552 bp are from the non-coding mtDNA control region. DNA was extracted from muscle using standard methods (Ellegren 1992, Lansman et al. 1981). We used the polymerase chain reaction (Kocher et al. 1989) and standard thermocycling regimes to amplify a 433 bp segment of the mtDNA cytochrome-*b* gene with primers L14841 (Kocher et al. 1989), LCBKLICKA (5'-CCTTTACTATGGCTCATACC, designed by the authors), and H15299 (Hackett 1992), and a 1,000 bp segment of the mtDNA control region with primers LCR4 and HPHE-1 (Tarr 1993). We used the above-listed primers and standard dideoxy DNA sequencing methods (Hillis et al. 1990) to obtain 433 bp of sequence from the cytochrome-*b* gene and 552 bp of the mtDNA control region. The extreme similarity of the sequences allowed us to align them visually. Each unique sequence was considered a haplotype, and only one representative of each haplotype was used for phylogenetic analysis. Harris' Sparrow (*Z. querula*) and White-throated Sparrow (*Z. albicollis*), the closest relatives to White-crowned and Golden-crowned sparrows (Zink and Blackwell 1996), were used as outgroups to root the crowned sparrow haplotype tree. We used the computer programs PAUP (Swofford 1990) for phylogenetic analysis, MEGA (Kumar et al. 1993) to compute pairwise Kimura (1980) two-parameter distances, and Arlequin (Schneider et al. 1997) to compute nucleotide diversity ( $\pi$ ). Collecting localities, collection dates, specimen voucher numbers, and sequences are associated with the GenBank accession numbers (U40173, U40175, U40185, U40186, AF305744–AF305776, AF308245–AF308281, AY008087–AY008123). We used allozyme data from Zink (1982), who analyzed 39 presumptive loci using standard starch gel electrophoresis.

*Results and Discussion.*—Four salient findings emerged: (1) we found extraordinarily low levels of sequence divergence between *Z. leucophrys* and *Z. atricapilla*, despite distinct plumage, song, and allozymes (Zink 1982); (2) some individuals of *Z. leucophrys* and *Z. atricapilla* share mtDNA haplotypes; (3) nucleotide diversity within the pool of *Z. leucophrys*

<sup>3</sup> Current Address: Department of Biological Sciences and Museum of Natural Science, 119 Foster Hall, Louisiana State University, Baton Rouge, Louisiana 70803-3216, USA. E-mail: jweckst@unix1.sncc.lsu.edu.

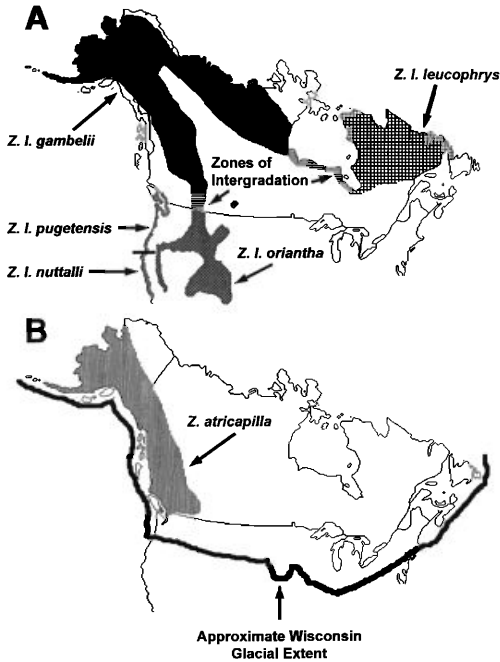


FIG. 1. Maps showing (A) distribution of five *Z. leucophrys* subspecies and (B) distribution of *Z. atricapilla* and approximate extent of glaciation during the Wisconsin glacial age.

and *Z. atricapilla* sequences is extremely low; and (4) *Z. leucophrys* and *Z. atricapilla* are not reciprocally monophyletic.

First, allozyme distance (Rogers 1972) between these two undisputed species was 0.0448 (Zink 1982), whereas Kimura (1980) two-parameter distances computed from mtDNA sequences ranged from 0 to 0.62% and averaged 0.24% over all individuals. Comparison of relative mtDNA and allozyme distances places the discrepancy in perspective (Fig. 2). MtDNA distance between White-crowned and Golden-crowned sparrows is very short relative to their congeners, whereas the allozyme distance is relatively longer. MtDNA evolves as rapidly or more rapidly than allozymes (Brown et al. 1979); therefore, given the observed allozyme distance (0.0448), divergence between mtDNA haplotypes of the two sparrow species should be an order of magnitude greater than that observed. In a comparison (not shown) of 122 pairs of passeriform mtDNA and allozyme distances, we found no pairs of species that showed a similar pattern.

Second, some individuals of three White-crowned Sparrow subspecies, including *Z. l. oriantha*, *Z. l. pugetensis*, and *Z. l. nuttalli*, share two haplotypes with *Z. atricapilla*; however, none of those subspecies currently exists sympatrically with *Z. atricapilla* during the breeding season (Fig. 1).

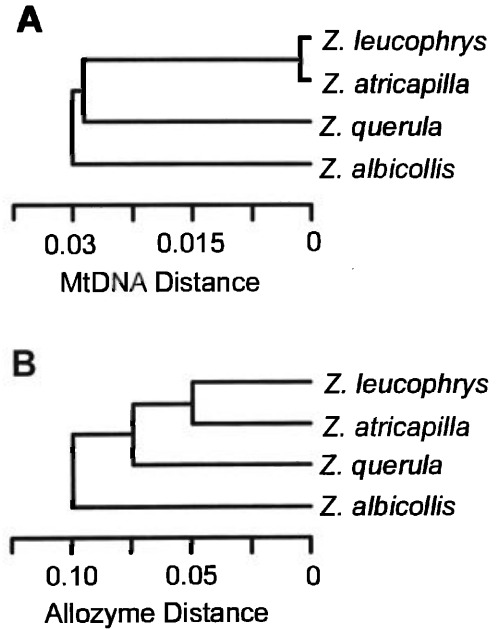


FIG. 2. UPGMA phenograms based on distances calculated using (A) mtDNA sequences (Kimura [1980] two-parameter distance) and (B) allozymes (Rogers' [1972] D). In comparison with distances depicted in the allozyme tree, the mtDNA phenogram shows a much shorter distance between *Z. leucophrys* and *Z. atricapilla* relative to their distances from *Z. querula* and *Z. albicollis*.

Third, calculations of nucleotide diversity ( $\pi \pm$  SD) for White-crowned Sparrow ( $0.0019 \pm 0.0013$ ), Golden-crowned Sparrow ( $0.0016 \pm 0.0011$ ), and both sparrows pooled ( $0.0021 \pm 0.0013$ ) are at the low extreme of ranges that are typical for many populations of Song Sparrow (*Melospiza melodia*) and Red-winged Blackbird (*Agelaius phoeniceus*) which range from 0.00095 to 0.008, and 0.00132 to 0.00507, respectively (Fry and Zink 1998). Observed blindly, such low nucleotide diversity values from pooled White-crowned and Golden-crowned sparrow mtDNA sequences would suggest that we sampled individuals from one population of a single species.

Fourth, White-crowned and Golden-crowned sparrows are not reciprocally monophyletic (Fig. 3). Thus, instead of two monophyletic lineages with relatively divergent and phylogenetically distinct mtDNA genomes, *Z. leucophrys* and *Z. atricapilla* share a set of genomes as similar as those typically found in a single avian population.

Several explanations could account for the anomalously low mtDNA distances, low nucleotide diversity ( $\pi$ ), paraphyly, and sharing of identical mtDNA sequences among individuals of two undisputed species. We may have amplified and sequenced a

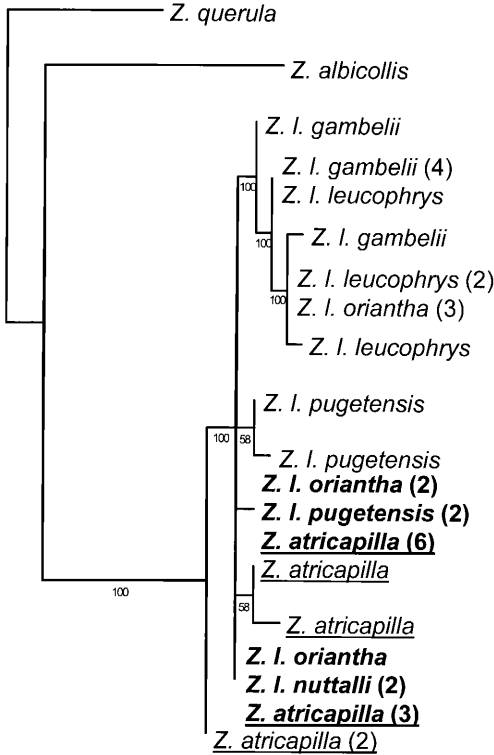


FIG. 3. Phylogram of a 50% majority rule consensus of the 19 shortest trees generated using the Branch-and-bound algorithm in PAUP (Swofford 1990) (tree length = 53 steps, CI = 0.89, RI = 0.81). Haplotypes shared by both *Z. leucophrys* and *Z. atricapilla* are in bold. Two haplotypes shared by *Z. leucophrys* and *Z. atricapilla* differ by 0.2% Kimura (1980) two-parameter sequence divergence. All haplotypes carried by *Z. atricapilla* are underlined. Nodes shared by more than 50% of the 19 shortest trees are labeled with a percentage. Relative branch lengths represent the number of characters mapped onto the particular internode. The uppermost clade containing all *Z. l. leucophrys*, *Z. l. gambelii*, and three *Z. l. oriantha* is supported by one unambiguous synapomorphy.

slowly evolving nuclear copy of targeted mitochondrial genes (Zhang and Hewitt 1996). Second, either *Z. leucophrys* or *Z. atricapilla* could be a hybrid species with an ancestral female parent of the other species. Third, *Z. leucophrys* and *Z. atricapilla* could be extremely recently separated from their common ancestor. Lastly, limited hybridization between *Z. leucophrys* and *Z. atricapilla* could have resulted in capture and introgression of the mtDNA genome of one species into the other (Wilson and Bernatchez 1998).

Genes from the mtDNA genome can be integrated into the nuclear genome (Arctander 1995, Zhang and Hewitt 1996). Nuclear copies of mitochondrial genes evolve much more slowly than their mitochondrial homologs (Zhang and Hewitt 1996) and could yield a pattern of extreme sequence similarity much like the one we have documented. However, it is unlikely that we sequenced a nuclear copy of the targeted mtDNA genes because our sequence data come in part from ultrapurified mtDNA samples (Zink et al. 1991). With nuclear copies, one expects relatively few haplotypes, whereas we observed a large number of haplotypes among individuals surveyed. Furthermore, protein coding sequence from cytochrome-*b* translates without stop codons or frameshift mutations, and we sequenced two separate regions of the mtDNA genome. Finally, our sequence data are consistent with restriction site analysis (Zink et al. 1991), in which nuclear homologs of mitochondrial genes are not a factor.

The Golden-crowned Sparrow might be a hybrid taxon, produced by a mating between a White-crowned Sparrow female (hence, identical mtDNA) and a male from a closely related congener, the White-throated Sparrow. Hybrid taxa are morphologically intermediate and polymorphic at allozyme loci diagnostic for parental species, and also carry the mtDNA of the female parental species (DeMarais et al. 1992). The hybrid origin hypothesis is effectively ruled out for *Z. leucophrys* or *Z. atricapilla* by lack of loci polymorphic for alleles fixed in other *Zonotrichia*. Furthermore, one fixed difference between *Z. leucophrys* and *Z. atricapilla* at the SDH locus and frequency differences at 13 out of 39 loci suggest that White-crowned and Golden-crowned sparrows have been evolving independently for a considerable period (Zink 1982).

Recent speciation and a lack of time for the phylogenetic sorting of lineages can result in individuals from different species sharing ancestral mtDNA haplotypes (Avice et al. 1990). For example, Klicka et al. (1999) showed that (recent) speciation of the Timberline Sparrow (*Spizella taubereri*) is as yet unaccompanied by complete lineage sorting. The White-crowned and Golden-crowned sparrow haplotype tree (Fig. 3) also does not reflect species limits, which is a signature of either recent isolation of species or recent transfer of the mtDNA genome from one species into another. Although recent speciation is consistent with our mtDNA data, it is unlikely given the magnitude of morphological and allozymic differences (Fig. 2) between these two species. Because nuclear genes require four times as long to coalesce than mitochondrial genes, owing to differences in effective population size (Avice 2000), if nuclear loci suggest that species are well differentiated, it follows that mitochondrial comparisons should be even more differentiated. In all known examples, significant allozyme differentiation is accompanied by sub-

stantial and diagnostic mtDNA differences, unless hybridization is suspected.

Although we cannot entirely rule out the recent speciation hypothesis, we believe that anomalously low mtDNA distances, low nucleotide diversity ( $\pi$ ), and sharing of identical mtDNA sequences concomitant with each species having its own distinctive phenotype and allozymes is consistent with a past limited hybridization event between *Z. leucophrys* and *Z. atricapilla*. Two modern specimen records of *Z. l. gambelii*  $\times$  *Z. atricapilla* hybrids (Miller 1940, Morton and Mewaldt 1960) suggest that those species have the ability to hybridize; however, they are not hybridizing extensively today (on the basis of few documented hybrid individuals), or in the past (on the basis of the fixed allozyme difference). Northern subspecies of White-crowned Sparrow including *Z. l. gambelii*, which is currently sympatric with Golden-crowned Sparrow, and *Z. l. leucophrys* do not share identical mtDNA sequences with *Z. atricapilla*, and they differ from it by 1–6 bp. Curiously, three of the western subspecies (*Z. l. pugetensis*, *Z. l. nuttalli*, and *Z. l. oriantha*), which do not currently overlap in breeding range with *Z. atricapilla* (Fig. 1), have individuals with sequence identical to many Golden-crowned Sparrows (Fig. 3).

We hypothesize that limited hybridization occurred when Golden-crowned Sparrows were forced into sympatry with *Z. l. oriantha*, *Z. l. pugetensis*, and *Z. l. nuttalli* during one of the most recent Pleistocene glaciations (Fig. 1), when many North American bird populations were displaced into refugia south of the ice (Rand 1948). As species become rare (Avice and Saunders 1984, Wayne et al. 1992) and environmental disturbances increase (Lamb and Avice 1986, Lehman et al. 1991), hybridization becomes more frequent. Hybridization was probably sporadic because it did not result in the breakdown of the fixed allozymic difference (Carr et al. 1986, Tegelström 1987; see Fig. 4). We suggest that time frame for the hybridization because individuals assayed differ by an average of 0.24%, indicating a recent origin of extant haplotypes. That *gambelii* and *leucophrys* were not involved in the hybridization is suggested by the single base pair they share, which differs from most *oriantha* and from *nuttalli* and *pugetensis*. Thus, the extremely low level of variation found in *Z. leucophrys* and *Z. atricapilla* is consistent with limited hybridization in the Late Pleistocene, which likely resulted in the capture, introgression, and replacement of the mtDNA genome of one of those species into the other.

Similar anomalous patterns have been discovered in the skuas (Stercorariidae). Based on mtDNA sequences, nuclear genetic variation, and the distribution of ectoparasitic chewing lice, *Stercorarius pomarinus* is more closely related to the *Catharacta* skuas than it is to the other two other congeneric species of *Stercorarius* (*parasiticus* and *longicaudus*) (Cohen et al.

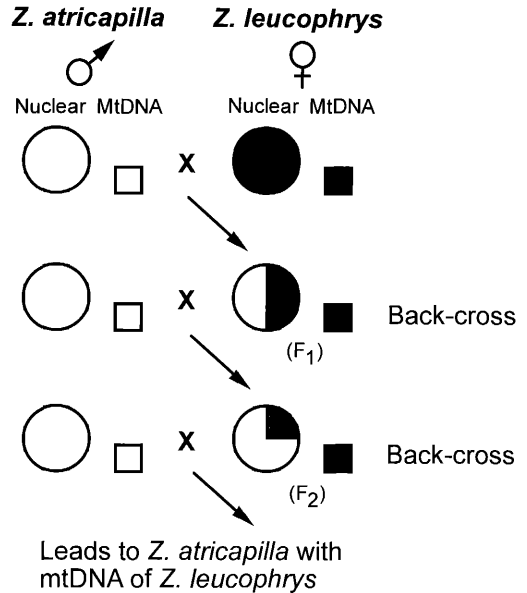


FIG. 4. Diagram of a hypothetical hybridization process showing how back-crossing to the paternal donor could ultimately yield a population with *Z. leucophrys* mtDNA and a *Z. atricapilla* nuclear genome. Nuclear genomes are represented by circles and mtDNA genomes are represented by squares. *Zonotrichia leucophrys* DNA is represented by blackening of the circle or square (e.g. a half blackened circle contains one half *Z. leucophrys* and one half *Z. atricapilla* nuclear DNA).

1997), the latter of which share many plumage and body size characteristics with *S. pomarinus* (Andersson 1999b). Andersson (1999a, b) suggests that hybridization between *S. pomarinus* and *C. skua* explains apparent conflict between molecular and morphological results.

A small amount of interspecific gene flow permits transmission of one species' mtDNA genome into another species (Takahata and Slatkin 1984). Moreover, several mechanisms can accelerate the rate of mtDNA replacement. First, an adaptive mutation in the mtDNA genome of the female parent can lead to a "selective sweep," and ultimate fixation of one haplotype in two hybridizing populations (Rand 1996, Ballard and Kreitman 1994). Second, population bottlenecks during glaciation (Hewitt 1996) and resultant genetic drift (Arnold 1993) could accelerate the rate of mtDNA replacement. Distinguishing between those alternatives is difficult because each leaves similar genetic imprints on subsequent population structure. For example, bottlenecks and natural selection during a selective sweep, can yield significant values of Tajima's *D*-statistic (Rand 1996, Tajima 1989), which tests for departures from neu-

tral evolution expectations. Tajima's D-value for Golden-crowned Sparrow was  $-0.16$  and that for White-crowned Sparrow was  $0.48$ ; neither value is statistically significant. However, negative values are expected if haplotypes have been under selection or populations went through a bottleneck. Thus, values of Tajima's test are of the sign expected if mtDNA transmission went from White-crowned into Golden-crowned sparrows. The much larger range of the former species also suggests this direction of transmission. Hence, the surviving mtDNA genome could be that of *Z. leucophrys*, but this merits further consideration.

Our results have implications for molecular clocks. Past hybridization, and reliance solely on mtDNA sequence data (a uniparentally transmitted marker), can lead to incorrect inferences of time since separation and could lead to reconstruction of incorrect patterns of evolutionary history. *Zonotrichia leucophrys* and *Z. atricapilla* are almost certainly not as recently isolated as comparison of their mtDNA genomes would suggest. In fact, on the basis solely of mtDNA, one would hypothesize an extremely rapid rate of morphological and song evolution, including the complex system of song dialects that exists in the White-crowned Sparrow. In our study, the phylogenetic pattern of the *Zonotrichia* sparrows was not obscured, because the mtDNA genome transfer occurred between sister taxa. However, reliance solely on mtDNA could lead to incorrect phylogenetic inferences when hybridization is not limited to nearest relatives, as is often the case in birds (Zink and McKittrick 1995).

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