MITOCHONDRIAL DNA VARIATION AND THE PHYLOGENY OF *ZONOTRICHIA*

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ABSTRACT.—We used 19 restriction endonucleases to analyze patterns of cleavage site variation in the mitochondrial DNA (mtDNA) of the five recognized species of *Zonotrichia*. Each species possesses a unique mtDNA profile. Relative to most congeneric avian comparisons, these species are closely related, with an average percent nucleotide divergence of 4.1%. The Golden-crowned Sparrow (*Z. atricapilla*) and White-crowned Sparrow (*Z. leucophrys*) differ by a single restriction site (out of 122), which yields an estimate of 0.11% sequence divergence. These species are sister species in all phylogenetic analyses (Wagner and Dollo parsimony, bootstrapping, distance analyses), and White-throated Sparrow (*Z. albicollis*) is the sister to these, followed by Harris' Sparrow (*Z. querula*) and Rufous-collared Sparrow (*Z. capensis*). The phylogeny based on mtDNA restriction sites differs from that based on allozymes (Zink 1982) in that Harris' and White-throated sparrows are reversed in this sequence. In general, however, the allozyme and mtDNA phylogenies are highly concordant. Similarities in song between White-throated and Golden-crowned sparrows can be interpreted as ancestral retentions. The song of the White-crowned Sparrow is derived relative to other north temperate congeners, and song dialects appear to have evolved independently in White-crowned and Rufous-collared sparrows. Based on the mtDNA data, patterns of hybridization are inconsistent with phylogenetic relationships, and we suggest that hybridization should not be used in taxonomic decisions. *Received 4 September 1990, accepted 26 December 1990.*

A PHYLOGENY not only provides a trace of a lineage's evolutionary diversification, but it also serves as a framework for the interpretation of the evolution of characters and suites of characters. Molecular methods have been widely heralded because they provide a set of characters that have a simple, known genetic basis, and are genetically independent. Furthermore, it is likely that molecular characters are selectively neutral (e.g. Barrowclough et al. 1985), and thus their evolution can be predicted by explicit evolutionary models (Nei 1987) and can serve as an approximate molecular clock. Although molecular characters have disadvantages for phylogeny inference (Hillis 1987), they are used with increasing frequency as a sort of "null" phylogenetic hypothesis, both for estimation of evolutionary history and interpretation of character evolution. Whether molecular analyses actually produce superior estimates of phylogeny remains to be documented, and tests will likely involve comparison of independently derived phylogenies (including other molecular estimates) of common sets of organisms.

In avian systematics, protein electrophoresis has been used to produce a number of phylogenetic estimates. In many interspecific comparisons of avian protein evolution (e.g. Avise et al. 1980, Johnson and Zink 1983), there was little variation partitioned among species. This does not inspire confidence in any pattern (without independent corroboration from other data sets). Other studies of allozyme variation that used "phylogenetic" and phenetic methods often produced results that surprised many taxonomists, including the biochemical systematists themselves (e.g. Johnson et al. 1988, Dittmann et al. 1989). However, few protein-based phylogenetic estimates for birds were tested for robustness or confidence with data-resampling techniques such as bootstrapping (Felsenstein 1985b). Therefore, the confidence in these protein-based branching diagrams as phylogenies is unknown.

One method of assessing confidence is to compare phylogenetic estimates derived from independent data sets. Zink and Avise (1990) compared allozyme and mitochondrial DNA (mtDNA) evolution in the genus *Ammodramus* and found a high degree of congruence. Zink and Dittmann (1991) found a generally high correspondence between allozymes and mtDNA in towhees (*Pipilo*). In these examples, the phylogenetic signal appeared in both of the genetically independent data sets. However, in *Ammodramus* and *Pipilo*, unlike many avian studies, there was considerable allozymic dif-

ferentiation, which rather strongly supported a particular phylogeny. Congruence of mtDNA and allozyme results implies confidence in phylogenetic conclusions, whereas confidence in a single data set is difficult to assess (Felsenstein 1985b).

The genus *Zonotrichia* contains five species, several of which have been studied extensively by behaviorists and ecologists. For example, vocal dialects in the White-crowned Sparrow (*Z. leucophrys*) have been studied thoroughly (Kroodsma et al. 1985). Vocalizations and dialects have also been studied in the Rufous-collared Sparrow (*Z. capensis*; Handford and Nottebohm 1976). Interpretation of the evolution of vocal as well as morphological and ecological conditions in *Zonotrichia* requires an independently derived phylogeny (Felsenstein 1985a, Mickevich and Weller 1990).

Zink (1982) used allozymes and morphometrics to assess the systematic status of species in *Zonotrichia*, and he concluded White-crowned and Golden-crowned (*Z. atricapilla*) sparrows were sister taxa, followed by Harris' Sparrow (*Z. querula*), the White-throated Sparrow (*Z. albicollis*), and the Rufous-collared Sparrow. This arrangement conflicted with previous opinions (Paynter 1964, Short and Simon 1965). For example, Mayr and Short (1970) stated that Golden-crowned and White-throated sparrows were sister species, and together with White-crowned Sparrows formed a species group. By default, Harris' and Rufous-collared sparrows would be outliers to this group. Evidence cited for the close relationship between White-throated and Golden-crowned sparrows was an essentially allopatric distribution; these species also exhibit similarities in male primary song. Mayr and Short (1970) suggested that hybridization between the White-throated Sparrow and Dark-eyed Junco (*Junco hyemalis*) revealed a close relationship between these taxa.

For birds, mtDNA has been shown to provide more characters for systematic analysis than allozymes (Avise and Zink 1988, Zink 1991). This corresponds with the relatively fast rate of mtDNA evolution observed in birds (Shields and Helm-Bychowski 1988) and other vertebrates (Avise 1986, Moritz et al. 1987). We used restriction endonuclease analysis of purified mtDNA to provide information for the inference of phylogenetic relationships among species of *Zonotrichia*. In so doing, we test the allozyme-based tree of Zink (1982) and the taxonomic conclusions of others, provide data on evolution of this organellar piece of DNA, and comment on the evolution of behavioral and distributional characteristics in the genus.

Methods

The following samples were used: Harris' Sparrow ($n = 2$), White-throated Sparrow (5), White-crowned Sparrow (6), Golden-crowned Sparrow (6), Rufous-collared Sparrow (3—two from Bolivia ["BO"] and one from Costa Rica ["CO"]), and a single specimen of Dark-eyed Junco as an outgroup for rooting trees. Collecting localities are available from the authors upon request. We collected and preserved tissues, and analyzed mtDNA according to established protocols (Lansman et al. 1981, Avise and Zink 1988, Zink 1991). We isolated intact mitochondria from frozen tissue via differential centrifugation, lysed mitochondrial membranes and removed them, and purified intact mtDNA in cesium chloride density equilibrium gradients via ultracentrifugation. After dialysis, mtDNA samples were stored at −20°C, and digestions were carried out according to manufacturers' specifications. Mitochondrial DNA fragments produced by restriction endonucleases were end-labeled with ^{35}S, separated in horizontal 0.8–1.5% agarose gels, and visualized by autoradiography. A molecular size standard was used to determine the sizes of fragments. The fragment profile for each endonuclease was assigned a letter, and each specimen is represented by the appropriate letter for all endonucleases surveyed. The letter code constitutes an individual's haplotype designation (a haplotype or clone is equivalent in genetic transmission to an allele, and the entire mtDNA molecule is inherited as a single gene locus). In addition, by comparing fragment profiles among individuals in reference to the molecular size standard, we were able to infer the distribution of restriction sites. The presence/absence of each site was scored for each individual. We used the computer programs HENNIG86 (option "ie") and PHYLIP (option "MIX") to infer a phylogeny based on site data according to the principle of maximum parsimony. Also in PHYLIP, we used the bootstrap programs BOOT and DOLBOOT, which use maximum (Wagner) and Dollo parsimony, respectively, to assess confidence in the data. Bootstrapping involves resampling data with replacement, in our study 100 times (replicates), and inferring a cladogram for each replicate data set. The end result is a majority-rule consensus tree with percentages that indicate the frequency of particular groups of taxa in the 100 trees. Dollo parsimony favors gains over losses, which is likely to be appropriate for restriction-site data where it is easier to lose than to gain a site (Moritz et al. 1987). In both bootstrap analyses, we coded the sites by endonuclease, and the bootstrap resampling (of restriction site presence/absence) was therefore random with

TABLE 1. Haplotypes for sparrow mtDNA profiles. Letters signify different digestion profiles, but position of letters in alphabet do not equate one-to-one to restriction site differences. The common patterns for each species are shown. Underlining indicates polymorphisms (and fragments) unique to the species (and the fragment profile was only one step removed from the letter listed for the species). The sequence of restriction enzymes is: *AvaI*, *AvaII*, *BamHI*, *BanII*, *BclI*, *BglI*, *BglII*, *EcoRI*, *HindIII*, *KpnI*, *NciI*, *NcoI*, *PstI*, *PvuII*, *SstII*, *StuI*, *XbaI*, *NdeI*, and *SalI*. For *Z. capensis*, "BO" refers to Bolivia and "CO" to Costa Rica.

Species	Haplotype																		
Z. querula	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Z. atricapilla	A	B	B	B	B	B	A	B	A	A	A	A	A	B	B	B	B	B	A
Z. leucophrys	A	B	B	B	B	C	A	B	A	A	A	A	A	B	B	B	B	B	A
Z. albicollis	A	B	B	C	C	B	A	C	B	A	B	A	B	C	B	C	C	A	A
Z. capensis BO	B	C	C	D	D	A	A	C	A	B	E	B	C	E	B	D	A	A	B
Z. capensis CO	C	D	C	D	D	B	A	C	A	B	F	C	D	E	B	F	A	A	C
J. hyemalis	D	E	D	E	E	D	B	D	C	C	G	A	E	F	B	G	D	C	A

respect to endonuclease, which reduces potential bias caused by some endonucleases with more sites.

We also used the site data to compute the percentage nucleotide difference between common haplotypes for each species using the formula $p = -\ln(F)/N$, where F is the proportion of shared restriction sites between two haplotypes (Nei and Li 1979). We input the resulting values into the FITCH and KITSCH programs, which are "distance" analyses, in PHYLIP to infer branching networks. We compared these with the discrete-character analyses. FITCH partitions distances with a least-squares approach and permits "rates" of evolution to differ. KITSCH constrains branch lengths of sister taxa to be equal, which approximates a uniform rate of evolutionary change.

RESULTS

The 19 endonucleases revealed considerable differentiation (Table 1) among all taxa except *Z. leucophrys* and *Z. atricapilla*, which differ by only a single restriction site (*BglI*). The 122 sites (Appendix) analyzed represent approximately 3.5% of the mtDNA genome. In *Zonotrichia* the mtDNA genome averaged 16,700 bases, approximately average for passerine birds (Shields and Helm-Bychowski 1988). The percentage nucleotide differentiation (Table 2) averaged 4.1% ± 0.019 (SD), with the range 0.11% (*Z. leucophrys* vs. *Z. atricapilla*) to 6.75% (*J. hyemalis* vs. *Z. capensis* from Costa Rica). There was little variation within species (Table 1).

All methods used to infer phylogenetic trees gave the same branching structure (Fig. 1). The bootstrap analyses reveal considerable confidence in this particular branching order. The maximum parsimony tree from both HENNIG86 and PHYLIP was 86 steps and the consistency index was 0.91. The Dollo parsimony tree required 20 reversions (which are maximized in this procedure). The Fitch tree (Fig. 1) reveals some evidence of rate differences, especially regarding *Z. albicollis*, but of insufficient magnitude to influence branching order.

DISCUSSION

Variation and dates of divergence.—As various molecular methods are used in systematic studies, it is of interest to compare results. We used Mantel's (1967) test to compare the Nei (1978) allozyme distances (in Zink, 1982) and mtDNA differences (Table 2). Mantel's test, which tests

TABLE 2. Nucleotide differentiation between taxa.

Taxon	Taxon					
	1	2	3	4	5	6
1. *Z. querula*	0.0000					
2. *Z. atricapilla*	0.0231	0.0000				
3. *Z. leucophrys*	0.0241	0.0011	0.0000			
4. *Z. albicollis*	0.0277	0.0177	0.0188	0.0000		
5. *Z. capensis* BO	0.0374	0.0430	0.0441	0.0461	0.0000	
6. *Z. capensis* CO	0.0430	0.0457	0.0468	0.0490	0.0100	0.0000
7. *J. hyemalis*	0.0593	0.0628	0.0639	0.0627	0.0612	0.0675

the hypothesis that two distance matrices are independent, revealed that the two matrices are significantly congruent ($t = 2.29$, df = infinite, $P < 0.05$).

The mtDNA divergence among *Zonotrichia* species is typical of that observed for other avian congeners (Kessler and Avise 1985, Avise and Zink 1988, Shields and Helm-Bychowski 1988), although the average is somewhat low. The mtDNA data reveal that speciation events among north temperate *Zonotrichia* were relatively recent. Using a calibration of 2% sequence divergence per million years (Shields and Wilson 1987), *leucophrys* and *atricapilla* split very recently, *albicollis* arose 750,000 yr before present, and *querula* originated 1.2 MYBP. The dates of average interspecific divergences based on allozyme distances (Zink 1982) are roughly equivalent to the mtDNA estimates, as found for mtDNA and allozyme studies of other sparrows (Zink and Avise 1990).

Variation within *Z. capensis* needs to be assessed before predicting its divergence date, because of the mtDNA distance between the Bolivia and Costa Rica samples (Table 2). Allozyme data suggest that *capensis* (formerly in a monotypic genus, *Brachyspiza*) is very different from north temperate congeners, but not especially distinct in level of mtDNA differentiation (it is, however, distinct for 12 of 20 restriction-fragment profiles). The value of 0.11% sequence divergence between *leucophrys* and *atricapilla* appears to be the lowest mtDNA distance recorded between avian species (Avise and Zink 1988). Because of the similarity of *leucophrys* and *atricapilla*, variation within the polytypic *leucophrys* should be examined. It is possible that mtDNA comparisons could identify a particular group of *leucophrys* from which *atricapilla* arose; our specimens were of the form *gambelii*.

Phylogeny.—For species of *Zonotrichia* the overall estimates of phylogeny based on allozymes (Zink 1982) and mtDNA (Fig. 1) are highly congruent, more so than one would expect by chance (Simberloff 1987). The allozyme tree (Zink 1982) differs from the mtDNA tree in that the positions of *Z. querula* and *Z. albicollis* are reversed. The tree topology from Zink's (1982) study in the HENNIG86 program required 90 steps to explain the mtDNA data, versus 86 steps for the most parsimonious mtDNA tree. The significance of this difference is unclear. Given the consistency of our analyses of mtDNA, we suggest that the tree most likely to be correct

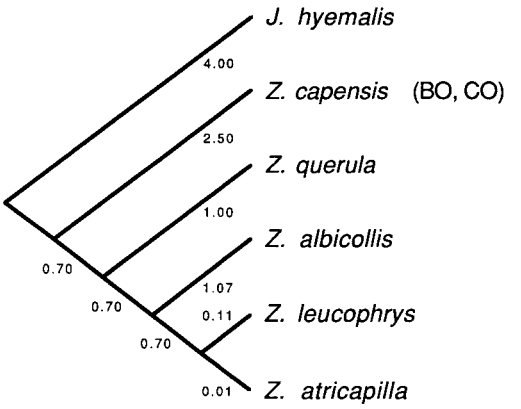

Fig. 1. Phylogenetic tree obtained by all methods of analysis. Branch lengths are in units of percent nucleotide differentiation derived from the FITCH analysis (%SD = 1.82, sum of squares = 0.0132). For the KITSCH analysis, the %SD = 6.24 and the sum of squares = 0.156. The numbers of times out of 100 each node occurred in a bootstrap analysis using Wagner parsimony were as follows (from top to bottom): 100, 97, 100, and 94, respectively. For the bootstrap analysis using Dollo parsimony, the corresponding nodes occurred 100, 90, 92, 99, and 99.

is that in Figure 1. The mtDNA molecule, inherited as a single gene, provides only one gene genealogy, however, and could be discordant with the organismal phylogeny (Avise 1986). In Zink's (1982) allozyme study there were 39 presumably independent gene loci analyzed. Discordance between mtDNA and allozyme results could be explained by "random noise" in the single mtDNA gene lineage. Such a proposition is testable only by recourse to additional data sets, especially those that expose variation in nuclear genes.

The allozyme and mtDNA results reveal close similarity of *Z. atricapilla* and *Z. leucophrys*. These two species are generally similar morphologically, especially in immature plumages. The near absence of hybrids between these species (Payne 1979) suggests that cross-species transfer of mtDNA cannot account for the extreme mtDNA similarity (Moritz et al. 1987). We consider the evidence overwhelming for the sister-species relationship between these taxa, contrary to opinions expressed by other authors (Short and Simon 1965, Mayr and Short 1970, Paynter 1964). Thus, the evolution of plumage, especially crown pattern, and morphometric (Zink 1982) differences in either *leucophrys* or *atricapilla* (or both), has proceeded rapidly, given the mtDNA

similarity, and has provided an example of mosaic evolution.

Our data are consistent with the hypothesis that *capensis* is a sister taxon to the north temperate *Zonotrichia* (which our data consistently unite), although other taxa should be examined to test the monophyly of *Zonotrichia* (Paynter 1964, Zink 1982). Paynter (1964) suggested that *capensis* was phylogenetically intermediate between *Melospiza* and *Zonotrichia*, and members of these genera apparently have hybridized (Dickerman 1961). Kessler and Avise (1985) estimated that *Z. albicollis* differed from the three species of *Melospiza* by an average *p*-value of 6.7%. In our study, *Z. capensis* differed from *Z. albicollis* by an average *p*-value of 4.8%. If mtDNA divergence occurs at an approximately uniform rate, *Melospiza* cannot be closer than *capensis* to north temperate *Zonotrichia*. Furthermore, inspection of restriction fragment profiles of *M. melodia* (Zink 1991) reveals no close relationship between *M. melodia* and *Z. capensis*. We suggest that *Zonotrichia* including *capensis* likely constitutes a monophyletic group. If this is true, *Z. capensis* is the oldest living member of *Zonotrichia*, which implies that the genus originated in the Neotropics. Furthermore, our small sample of *capensis* suggests considerable intraspecific variation, which may be typical of tropical birds (Capparella 1988), or simply a correlate of the fact that *capensis* is one of the most polytypic species in the New World.

Tracing traits on the phylogeny.—The phylogeny of *Zonotrichia* provides a context for interpretation of aspects of the organismal phenotype. The primary songs of *albicollis* and *atricapilla* are similar, but the song of *Z. querula* is also generally similar (Zink pers. obs.). Therefore, we propose that the simple whistled songs of the genus are primitive within at least the north temperate members of the genus and are not evidence of a sister-taxon relationship between *albicollis* and *atricapilla*. The song(s) of *leucophrys* is(are) therefore derived and "autapomorphous."

The phylogeny (Fig. 1) also allows the inference that song dialects in *leucophrys* and *capensis* most likely evolved independently. That is, if the song dialects evolved in the ancestor of modern *Zonotrichia* were subsequently lost and regained in *leucophrys*, a minimum of three evolutionary events was required. If song dialects were gained independently in *capensis* and *leucophrys*, only two events were required. Because *leucophrys* and *atricapilla* diverged recently, song dialects in *leucophrys* must have evolved rapidly. Study of ecological reasons for the evolution of song dialects in *leucophrys* is justified now that common ancestry is ruled out. For example, both *leucophrys* and *capensis* have large, often fragmented ranges. Dialect boundaries, however, do not necessarily correlate with geographic and genetic breaks in *leucophrys* (Kroodsma et al. 1985), and *albicollis* has a relatively large range apparently without dialect formation.

Previous considerations of relationships among members of *Zonotrichia* involved breeding distributions. In particular, the wide-ranging species, such as *leucophrys*, might be considered as relatively primitive to the allopatric *atricapilla* and *albicollis*, which were viewed as sister taxa. However, the phylogeny reveals that either *leucophrys* was always widely distributed, and *atricapilla* recently isolated, or that *leucophrys* very recently expanded its range. Thus, although allopatric differentiation is likely the common mode of avian speciation, sister taxa of *Zonotrichia* are not currently allopatric.

In avian taxonomy, hybridization has been used as evidence for making taxonomic arrangements. In *Zonotrichia*, there are apparently only three intrageneric hybrids known: *leucophrys* × *atricapilla* (Miller 1940), *leucophrys* × *querula*, and *atricapilla* × *albicollis* (Payne 1979). Hybridization between *Z. albicollis* and *J. hyemalis* has been interpreted to mean that the genera should be merged (Short and Simon 1965, Paynter 1964), although this suggestion was not formally adopted (AOU 1983). If one considers that hybridization is an ancestral condition (Rosen 1979, Cracraft 1983), then these hybrids are irrelevant to classifications. Patterns of hybridization have no consistent relationship to phylogenetic relationships (the proper foundation of classifications) and therefore cannot aid in constructing a classification. Hybrid *Zonotrichia* do reveal that hybridization is not limited to sister taxa, and clearly one cannot recover the phylogeny from instances or pattern of hybridization. In fact, evidence on hybridization in *Zonotrichia* is most consistent with Banks and Johnson's (1961) viewpoint that intergeneric hybridization is more likely than intrageneric. The hybrids between *Zonotrichia* and *Junco* similarly cannot be used as evidence that the taxa are congeneric. These hybrids support only the rather nebulous concept that the taxa are "re-

lated," but they do not permit reconstruction of even the basal phylogenetic limits of the group of species to which *Zonotrichia* and *Junco* might be aligned. That is, the absence of hybrids does not mean that two species are necessarily phylogenetically closer or more distant than two species that do hybridize. We suggest that hybridization has no important role in constructing phylogenies and deriving subsequent classifications for these sparrows.

ACKNOWLEDGMENTS

We thank S. W. Cardiff, members of various Louisiana State University expeditions, and G. F. Barrowclough for collecting some specimens used in this study. We thank J. V. Remsen for helpful discussions, and J. M. Bates, A. H. Brush, K. J. Burns, S. J. Hackett, J. V. Remsen, K. V. Rosenberg, and an anonymous reviewer for useful comments on the manuscript. Funds were provided by NSF grant BSR-8906621 and Louisiana Board of Regents grant LEQSF No. 86-LBR-(048)-08 to Zink. We thank the following for providing computer programs: K. W. Corbin (Mantel's test), J. S. Farris (HENNIG86), J. Felsenstein (PHYLIP), and R. M. Ball (*p*-values).

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APPENDIX. Presence or absence of 122 restriction sites for species *Zonotrichia* and *Junco*.

Zonotrichia querula
1110001111111000111100111111111100001111000111010101100011111001111111100010011111001111110111100110111110001001111111110001

Z. atricapilla
1110001111110000110000111111110000011111001110001011100111100111111110001001111110111111011110110110111011111101000100 0

Z. leucophrys
1110001111110000110000111111110000011111001110010111001111100111111110001001111110111111011110110110111011111101000100 0

Z. albicollis
1110001111110000110000101111110100001110101110001011110111111011111111100100111110011111001110011011010111111100000000

Z. capensis—Bolivia
0111001110010000100000111111100011011000001110101011110111110011011110010110111110001111100111011011010001101101111100 00

Z. capensis—Costa Rica
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J. hyemalis
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