Syst. Zool., 39(2):148-161, 1990

PATTERNS OF MITOCHONDRIAL DNA AND ALLOZYME EVOLUTION IN THE AVIAN GENUS *AMMODRAMUS*

ROBERT M. ZINK[1] AND JOHN C. AVISE[2]

[1]*Museum of Natural Science, Louisiana State University, Baton Rouge, Louisiana 70803 and;*
[2]*Department of Genetics, University of Georgia, Athens, Georgia 30602*

Abstract.—Analyses of mitochondrial DNA (mtDNA) and allozymes were used to estimate phylogenetic patterns in the avian genus *Ammodramus*. Levels of interspecific genetic differentiation were greater than most previous estimates for other congeneric avian taxa. Phenetic and phylogenetic patterns were highly concordant for these two genetically independent data sets, suggesting a robust estimate of the evolutionary history of these sparrows. However, the genetic pattern was not concordant with an estimate of variation in skeletal morphometrics produced by Robins and Schnell (1971); we suggest that ecological pressures effect convergence in skeletal morphology. Independent calibrations of mtDNA and allozyme distances suggest times of divergence that differ by a factor of two among the species assayed. [Phylogeny reconstruction; mitochondrial DNA; allozymes; cladistics; phenetics.]

A variety of molecular and morphological techniques yield information suitable for reconstruction of phylogenetic relationships. Both molecular and morphological data have advantages and disadvantages (Hillis, 1987; Sarich et al., 1989) as do various methods of analysis used to infer phylogenetic patterns. Generally speaking, a phylogeny is a genetic trace of a taxon's gene pool over evolutionary time. Molecular methods offer potentially powerful means to infer phylogenies because they expose genetic variation directly, and because alleles or haplotypes at unlinked loci are independent in genetic transmission. Furthermore, explicit models for the evolution of various molecular genetic characters are available (Nei, 1987). In contrast, many morphological characters are polygenic, and the degree to which they exhibit statistical independence is determined by the genetic correlations (Shaffer, 1986), which when studied are usually significant (Lynch, 1989; Schluter, 1984). Because both molecular and morphological characters are commonly analyzed as though statistically independent, this assumption becomes critical and would appear to favor molecular methods. However, it is clear that other problems, such as homoplasy, gene conversion, epistatic interactions, evolutionary rate heterogeneity, and the "shape" of evolutionary histories (Lanyon, 1988) can complicate phylogenetic conclusions derived from both molecular and morphological analyses. It is therefore of interest to compare the concordance and resolution of different procedures of data gathering and analysis on a common set of organisms.

In an earlier study (Avise and Zink, 1988) we analyzed mitochondrial DNA (mtDNA) and allozymes in avian sibling species and found that mtDNA offered consistently greater resolving power. We think that such comparisons offer useful insights because the data sets are genetically independent. Here we analyze patterns of variation in mtDNA and allozymes in the sparrow genus *Ammodramus*. We determine whether *Ammodramus* species exhibit conservative levels of genetic differentiation relative to other vertebrate congeners, as found previously for other birds (Avise and Aquadro, 1982; Kessler and Avise, 1985). We compare divergence times estimated from independent calibrations of mtDNA and allozymes. We determine if phenetic and phylogenetic analyses of interspecific variation in mtDNA and allozymes produce congruent estimates of evolutionary history for these species. Lastly, we compare the mtDNA and allozymic results to a phenetic analysis of skeletal measurements (Robins and Schnell, 1971).

MATERIAL AND METHODS

Study taxa.—The following species were used: Baird's sparrow (*Ammodramus bairdii*), grasshopper sparrow (*A. savannarum*), Henslow's sparrow (*A. henslowii*), LeConte's sparrow (*A. leconteii*), sharp-tailed sparrow (*A. caudacutus*), seaside sparrow (*A. maritimus*), yellow-browed sparrow (*A. aurifrons*), and grassland sparrow (*A. humeralis*); the savannah sparrow (*Passerculus sandwichensis*) was used as an outgroup for rooting networks. Specimen localities are available from the senior author. For mtDNA analysis, our samples of grassland sparrow were inadequate.

Protein electrophoresis.—Tissue samples were collected and stored under various field conditions, but upon return to the Louisiana State University Museum of Natural Science (LSUMNS) were held at −70°C. Samples of liver and muscle were minced with a razor blade, combined with 0.1 ml of deionized water, and centrifuged for 30 min at 30,000 × *g*. Protein extracts were frozen at −70°C until used for electrophoresis. Gels were made of 11% (Sigma) starch. Each locus (see below) was examined using standard starch gel procedures (Selander et al., 1971; Johnson et al., 1984; Zink, 1986), on at least two gel-buffer combinations to detect hidden variation (Hackett, 1989). Electromorphs are assumed to have a genetic basis, and we refer to them as alleles. Alleles were coded by their mobility from the origin, with the most anodal alleles coded as "a," and successively more cathodal alleles as b, c, and so on.

Genotypes at each locus were entered into the computer program BIOSYS-1 (Swofford and Selander, 1981), which computed percent heterozygosity, Nei's (1978) and Rogers' (1972) genetic distances, and a UPGMA phenogram. The computer program PHYLIP, written by J. Felsenstein (1986), was used to construct a tree from the matrix of Rogers' distance values following the approach of Fitch and Margoliash (1967; hereafter "FM" tree); order of input of taxa was varied to assure that the minimum length tree was found (Felsenstein, 1986). Given the controversy over distance analyses (Farris, 1986; Felsenstein, 1986), we also performed a parsimony analysis using loci as characters and alleles as unordered character states; we agree with Buth (1984) that coding the presence/absence of individual alleles is inappropriate. For each phylogenetically informative locus, the state assigned was that of the most common allele, thereby ignoring allelic frequency information and shared polymorphisms (a disadvantage we fully acknowledge). To test the robustness of this coding scheme, we recoded loci to reflect shared polymorphisms (noncommon alleles); the results were no different than those apparent from comparisons of the different trees (see below). The computer program HENNIG86, written by James S. Farris, was used to perform a phylogenetic analysis using the principle of maximum parsimony (option "ie"), and to produce a consensus tree (option "nelsen").

Mitochondrial DNA.—Tissues were placed into MSB-EDTA buffer at 4°C (Lansman et al., 1981) within an hour after specimen collection. MtDNA was prepared following the procedures outlined in Avise and Zink (1988). In brief, intact circular mtDNA from each individual was localized in a cesium chloride density equilibrium gradient, recovered, purified of cesium and ethidium bromide via dialysis, and stored at −20°C. Aliquots of mtDNA were digested with 16 restriction endonucleases. MtDNA fragments were end labeled with ^{35}S radionuclides, separated on 1.0, 1.2 or 1.8% agarose gels and visualized by autoradiography as bands in gel profiles. Fragment sizes were compared against a 1-kilobase ladder standard purchased from Bethesda Research Labs. Only fragments 400 base pairs and longer were scored.

Each animal was assigned a composite mtDNA genotype based on the restriction fragment profiles across all restriction enzymes. The composite data were also summarized in a presence/absence matrix of all mtDNA fragments, which was then employed to compute p (the average number of substitutions per nucleotide site) between genotypes using the approach de-

TABLE 1. Allelic frequencies for variable allozyme loci.

Locus (E.C. No.)	Sample/(N)[a] LeConte's Sparrow (3)	Sharp-tailed Sparrow (3)	Seaside Sparrow (6)	Henslow's Sparrow (8.5)	Baird's Sparrow (2)	Grasshopper Sparrow (5)	Grassland Sparrow (4.2)	Yellow-browed Sparrow (3.0)	Savannah Sparrow (5.1)
FUMH (4.2.1.2)	A	A	A	B	B	C	C	C	B (.83) D (.17)
ME (1.1.1.40)	A	A	A	A	A	B	B	B	C
PNP (2.4.2.1)	A	A (.67) B (.33)	B (.93) C (.07)	D (.91) E (.09)	F (.75) G (.25)	H	H (.83) I (.17)	J	K
LA-2 (3.4.-.-)	A (.67) B (.17) C (.16)	A (.67) D (.33)	C (.67) E (.33)	C	F	G (.83) H (.17)	H (.17) I (.83)	H (.17) I (.83)	J
sMDH (1.1.1.37)	A	A	A	A	A	B	A	A	A
LAP (3.4.-.-)	A	A	A	A	A	B	A	A	A
EAP (3.1.3.2)	A	A (.83) B (.17)	A	A	A	B	B	B	A
HK (2.7.1.1)	A	A (.83) B (.17)	A	A	C	D	E	F	A (.67) C (.33)
GPI (5.3.1.9)	A (.67) B (.16) G (.17)	A	A	A (.91) C (.09)	A	A	D (.83) E (.17)	D (.83) F (.17)	A
ESTD (3.1.1.-)	A (.67) B (.17) C (.16)	A (.83) D (.17)	A	A	A	A	A	A	A
PGM-2 (5.4.2.2)	A (.83) B (.17)	A	A (.94) B (.06)	A	A	A	A (.90) C (.10)	A	A (.93) D (.07)
GPGD (1.1.1.44)	A (.83) B (.17)	A (.67) B (.33)	B (.83) C (.17)	B	B	B	B	B	B (.83) D (.17)
ADA (3.5.4.4)	A	A (.67) B (.17) C (.16)	A	D	A	E	E (.83) F (.17)	E	A (.83) G (.17)
sACOH (4.2.1.3)	A	A	A	A	A	B	C	D	E (.83) F (.17)
sIDH (1.1.1.42)	A	A	B (.93) C (.07)	A	A	A	A	A	A
αGPD (1.1.1.8)	A	A	A	A	A (.25) B (.75)	A	A	A	A
CK (2.7.3.2)	A	A	A	A	A	B	B	B	A
LGG (3.4.-.-)	A	A	A (.87) B (.13)	A (.91) E (.05) F (.04)	A	A (.83) C (.09) D (.08)	A	A	A
LA-1 (3.4.-.-)	A	A	A	A (.93) D (.07)	A	A (.83) B (.09) C (.08)	A	A	A (.83) E (.17)
sGOT (2.6.1.1)	B	A	A	A	A	A	A (.33) C (.50) D (.17)	A	A
SDH (1.1.1.14)	A	A	A	B	B	B	B	B	C
SOD-1 (1.15.1.1)	B	A	A	A	A	A	A	A	A
LDH-1 (1.1.1.27)	A	A	A	A	B	C	D	D	E
MPI (5.3.1.8)	A	A	A	A (.73) B (.18) C (.09)	A	A	A	A	A

[a] Sample size is mean number of individuals examined per locus.

scribed by equation [20] and fig. 1 of Nei and Li (1979). A UPGMA phenogram and an FM network (from PHYLIP) were generated from the matrix of *p*-values.

Because of the large number of taxa examined and the high mtDNA diversity observed, it proved infeasible to map restriction sites, and we limited further analyses to the fragments themselves. Using restriction fragments instead of sites involves loss of information, because clones can share sites but (sometimes) not fragments. However, shared fragments are characters that qualify as synapomorphies in phylogenetic hypotheses. We used the bootstrap approach in PHYLIP on the presence/absence fragment (not site or enzyme) data and produced a consensus tree derived from 100 maximum parsimony trees, each based on a replicate (bootstrapped) sample of the characters (fragments). Because the bootstrap approach requires independent characters to estimate statistical confidence intervals about nodes, and because mtDNA fragments are not always independent, we view the bootstrap results merely as a description of the data. In addition, the fragment data for one specimen per species were entered into HENNIG86 to find the most parsimonious tree(s) representing the data.

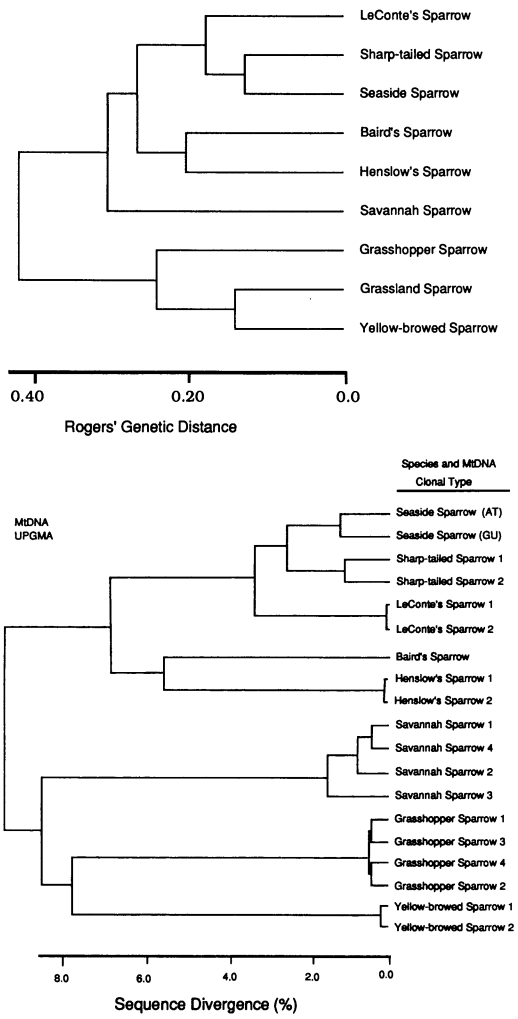

FIG. 1. a. UPGMA phenogram of Rogers' genetic distances derived from allozyme frequencies. Cophenetic correlation coefficient equals 0.92. b. UPGMA phenogram based on matrix of *p*-values derived from patterns of mtDNA restriction fragments. The two seaside sparrow samples represent the most common genotypes in the Atlantic (AT) and Gulf Coast (GU) localities surveyed by Avise and Nelson (1989).

RESULTS

Allozymes

Although sample sizes were relatively small (mean sample size per locus ranged from 2 [Baird's sparrow] to 8.5 [Henslow's sparrow]; Table 1), mean direct-count heterozygosity was typical of that observed for birds (range 0.02 [yellow-browed sparrow] to 0.09 [LeConte's sparrow]; Corbin 1987). Of the 30 loci examined, 6 (20%) were monomorphic and fixed for the same allele in all samples (*CK-2* [Enzyme Commission 2.7.3.2]; *mMDH-2* [E.C. 1.1.1.37], *GDA* [E.C. 3.5.4.3], *mIDH-2* [E.C. 1.1.1.42], *LDH-2* [E.C. 1.1.1.27], *SOD-2* [E.C. 1.15.1.1]). The other loci exhibited patterns of within- or among-taxon differentiation (Table 1). Each species has at least one diagnostic allele (autapomorphy).

Mean genetic distance (Nei, 1978) across all taxa was 0.392 ± 0.176 (SD); the range was 0.078 (sharp-tailed versus LeConte's sparrows) to 0.777 (LeConte's versus grasshopper sparrow). The matrix of D-values is available from the authors or can be computed from the data in Table 1.

The UPGMA phenogram (Fig. 1a) re-

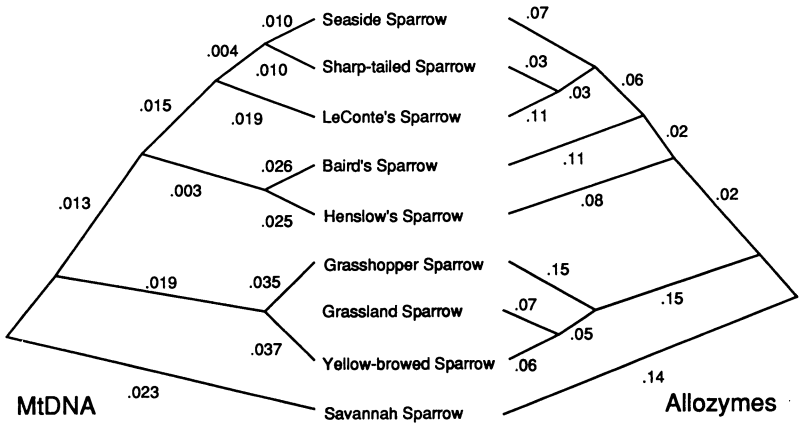

FIG. 2. Trees (rooted at savannah sparrow) based on the Fitch-Margoliash algorithm applied to the matrix of mtDNA *p*-values (left; %SD = 7.79) and Rogers' genetic (allozyme) distances (right; %SD = 2.71).

veals several clusters of taxa: 1) LeConte's, sharp-tailed, and seaside sparrows, the latter two sister taxa; 2) Henslow's and Baird's sparrows, these forming a sister-taxon relationship to the first group; 3) savannah sparrow, most similar to groups 1 and 2; and 4) grasshopper, grassland, and yellow-browed sparrows, the latter two sister taxa, and the entire cluster quite distinct from groups 1, 2, and 3. The phenetic placement of the savannah sparrow (*P. sandwichensis*) raises the question of whether the *Ammodramus* are a natural (monophyletic) group.

The FM tree (Fig. 2, right) and the phenogram differ in two respects. First, sharp-tailed and LeConte's sparrows are sister taxa, not sharp-tailed and seaside sparrows. Second, Baird's and Henslow's sparrows are not sister taxa (in terms of overall genetic similarity). The placement of the savannah sparrow is consistent in both diagrams, because the FM tree is arbitrarily rooted. In other details, the two branching diagrams are consistent.

The HENNIG86 parsimony analysis of loci resulted in six equally parsimonious

TABLE 2. *p*-values among individuals surveyed for mtDNA variation. The values for the yellow-browed sparrows are based on all enzymes except *Hinc* II, which could not be reliably resolved for these individuals.

1. Seaside sparrow 1							
2. Seaside sparrow 2	0.0110						
3. Sharp-tailed sparrow 1	0.0209	0.0209					
4. Sharp-tailed sparrow 2	0.0250	0.0192	0.0086				
5. Baird's sparrow 1	0.0533	0.0532	0.0677	0.0621			
6. Henslow's sparrow 1	0.0554	0.0549	0.0577	0.0575	0.0507		
7. Henslow's sparrow 2	0.0614	0.0573	0.0627	0.0626	0.0475	0.0028	
8. LeConte's sparrow 1	0.0351	0.0288	0.0317	0.0262	0.0662	0.0573	0.0620
9. LeConte's sparrow 2	0.0345	0.0282	0.0312	0.0258	0.0656	0.0567	0.0614
10. Grasshopper sparrow 1	0.0950	0.0860	0.0967	0.0923	0.0951	0.1090	0.1062
11. Grasshopper sparrow 2	0.0961	0.0870	0.0977	0.0931	0.0959	0.1063	0.1036
12. Grasshopper sparrow 3	0.0988	0.0894	0.0967	0.0923	0.0996	0.1090	0.1062
13. Grasshopper sparrow 4	0.1022	0.0863	0.0970	0.0925	0.0952	0.1086	0.1035
14. Savannah sparrow 1	0.0758	0.0757	0.0684	0.0745	0.0592	0.0614	0.0702
15. Savannah sparrow 2	0.0711	0.0708	0.0688	0.0749	0.0619	0.0688	0.0746
16. Savannah sparrow 3	0.0754	0.0749	0.0764	0.0769	0.0552	0.0611	0.0670
17. Savannah sparrow 4	0.0779	0.0777	0.0707	0.0772	0.0577	0.0639	0.0731
18. Yellow-browed sparrow 1	0.0891	0.0819	0.0861	0.0852	0.1008	0.1017	0.1026
19. Yellow-browed sparrow 2	0.1001	0.0819	0.0959	0.0952	0.0898	0.0928	0.0932

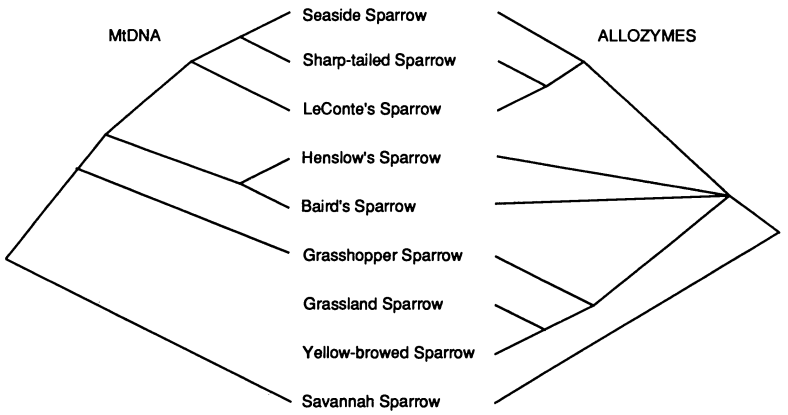

FIG. 3. Maximum parsimony trees based on patterns of mtDNA fragments (left; length 296, consistency index = 0.75) and allozymes (right; length = 42; consistency index = 0.95). The allozyme tree is a Nelson consensus tree (see text). Both trees are rooted at the savannah sparrow.

trees (not shown) of 41 steps, which differ only in the placement of Baird's and Henslow's sparrows. A Nelson consensus tree (Fig. 3, right; length 42 steps), derived from the six trees, is less resolved than either the phenogram or FM trees, and portrays the major groups descended from a polychotomy. The consensus tree is not topologically equivalent to any of the six equally parsimonious trees, and we do not consider it a phylogeny. LeConte's and sharp-tailed sparrows are sister taxa, as in the FM tree, and together with the seaside sparrow form a clade, as in all analyses. The relationships among grasshopper, grassland, and yellow-browed sparrows were consistent with the phenogram and FM tree.

Mitochondrial DNA

Estimates of mitochondrial DNA differentiation were based on a total of 253 observed fragments. The average p-value (Table 2) was 0.006 within species ($n = 7$), and 0.073 between species ($n = 28$ comparisons; range 0.021 [seaside versus sharp-tailed

TABLE 2. Continued.

0.0014										
0.1063	0.1059									
0.1068	0.1063	0.0040								
0.1063	0.1059	0.0031	0.0056							
0.1026	0.1058	0.0026	0.0045	0.0041						
0.0667	0.0660	0.0704	0.0712	0.0704	0.0706					
0.0732	0.0725	0.0678	0.0685	0.0678	0.0679	0.0059				
0.0853	0.0846	0.0672	0.0678	0.0672	0.0703	0.0117	0.0116			
0.0725	0.0718	0.0732	0.0739	0.0732	0.0733	0.0038	0.0063	0.0122		
0.1089	0.1089	0.0727	0.0687	0.0759	0.0732	0.0926	0.0753	0.0884	0.0881	
0.1089	0.1089	0.0652	0.0653	0.0684	0.0653	0.0751	0.0648	0.0718	0.0713	0.0065

TABLE 3. Clonal designations for mtDNA genotypes observed in the assayed *Ammodramus* sparrows. Letters in the designations, from left to right, refer to multi-fragment mtDNA profiles produced by digestion with *Ava* I, *Ava* II, *Bam*H I, *Bcl* I, *Bgl* I, *Bgl* II, *Cla* I, *Eco*R I, *Hinc* II, *Hind* III, *Nde* I, *Pst* I, *Pvu* II, *Spe* I, *Sst* II, and *Stu* I. Different letters designate distinct mtDNA profiles, but their proximity in the alphabet implies nothing about genetic relationships.

MtDNA clone	Designation															No. of birds
Seaside sparrow 1	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	11
Seaside sparrow 2	C	G	D	C	C	C	C	D	C	C	C	C	G	C	C	14
Sharp-tailed sparrow 1	B	E	E	D	D	C	E	D	G	G	C	C	F	D	C	6
Sharp-tailed sparrow 2	B	E	E	D	D	C	E	D	E	G	D	C	G	E	C	3
Baird's sparrow 1	E	P	C	H	H	C	C	B	M	M	C	E	G	J	C	H	2
Henslow's sparrow 1	B	M	G	J	L	C	C	B	K	Q	B	E	A	H	C	J	7
Henslow's sparrow 2	B	N	G	J	M	C	C	B	K	Q	B	E	A	H	C	J	1
LeConte's sparrow 1	B	K	D	F	E	C	I	A	I	O	B	C	G	F	C	B	2
LeConte's sparrow 2	B	J	D	F	E	C	I	A	I	O	B	C	G	F	C	B	1
Grasshopper sparrow 1	B	R	I	O	F	A	M	D	O	S	Z	A	I	M	C	L	2
Grasshopper sparrow 2	B	S	I	O	F	A	M	D	P	S	Z	A	I	L	C	L	1
Grasshopper sparrow 3	B	R	I	O	G	A	M	D	O	S	Z	A	I	N	C	L	1
Grasshopper sparrow 4	B	Q	I	O	F	A	M	D	O	S	Z	A	I	L	C	L	1
Savannah sparrow 1	G	U	K	M	B	B	G	B	R	K	A	A	A	O	C	F	1
Savannah sparrow 2	G	V	K	L	B	B	G	B	R	K	A	A	A	O	C	F	1
Savannah sparrow 3	G	W	K	L	A	B	G	B	S	K	A	A	A	P	C	F	1
Savannah sparrow 4	G	X	K	M	B	D	G	B	R	K	A	A	A	O	C	G	1
Yellow-browed sparrow 1	I	Y	M	Q	J	E	K	D	—	U	A	G	K	R	C	N	1
Yellow-browed sparrow 2	J	Z	M	Q	J	E	K	D	—	U	A	G	K	R	C	O	1

sparrows]) to 0.109 [Henslow's versus grasshopper sparrows]). Each species had a diagnostic genetic profile (Table 3); examples of fragment profiles for *Bcl* I and *Hind* III are shown in Figure 4. Various gel profiles united pairs of species (Table 4).

The UPGMA phenogram (Fig. 1b) summarizing the matrix of pair-wise *p*-values

TABLE 4. Single-enzyme mtDNA gel profiles shared between assayed species of *Ammodramus* sparrows. In parentheses are the numbers of scored mtDNA fragments in the respective gel patterns.*

Species pair	Endonuclease pattern shared (no. of fragments)
Seaside/Sharp-tailed	*Bgl* II (2); *Eco*R I (2); *Nde* I (4); *Pst* I (3) *Pvu* II (3) and (2); *Stu* I (8).
Seaside/Baird's	*Bam*H I (3); *Bgl* II (2); *Nde* I (4); *Pvu* II (2).
Seaside/Henslow's	*Bgl* II (2).
Seaside/LeConte's	*Bam*H I (2); *Bgl* II (2); *Pst* I (3); *Pvu* II (2).
Seaside/Grasshopper	*Eco*R I (2).
Seaside/Yellow-browed	*Eco*R I (2).
Sharp-tailed/Baird's	*Bgl* II (2); *Nde* I (4); *Pvu* II (2).
Sharp-tailed/Henslow's	*Ava* I (3); *Bgl* II (2).
Sharp-tailed/LeConte's	*Ava* I (3); *Bgl* II (2); *Pst* I (3); *Pvu* II (2).
Sharp-tailed/Grasshopper	*Ava* I (3); *Eco*R I (2).
Sharp-tailed/Yellow-browed	*Eco*R I (2).
Baird's/Henslow's	*Bgl* II (2); *Pst* I (4).
Baird's/LeConte's	*Bgl* II (2); *Pvu* II (2).
Henslow's/LeConte's	*Ava* I (3); *Bgl* II (2); *Nde* I (3).
Henlow's/Grasshopper	*Ava* I (3).
LeConte's/Grasshopper	*Ava* I (3).
Grasshopper/Yellow-browed	*Eco*R I (2).
Savannah/Yellow-browed	*Nde* I (4).

* Patterns with only zero or one restriction site are not included. In addition to the endonuclease patterns listed above, all *Ammodramus* species shared a 2-band *Sst* II profile.

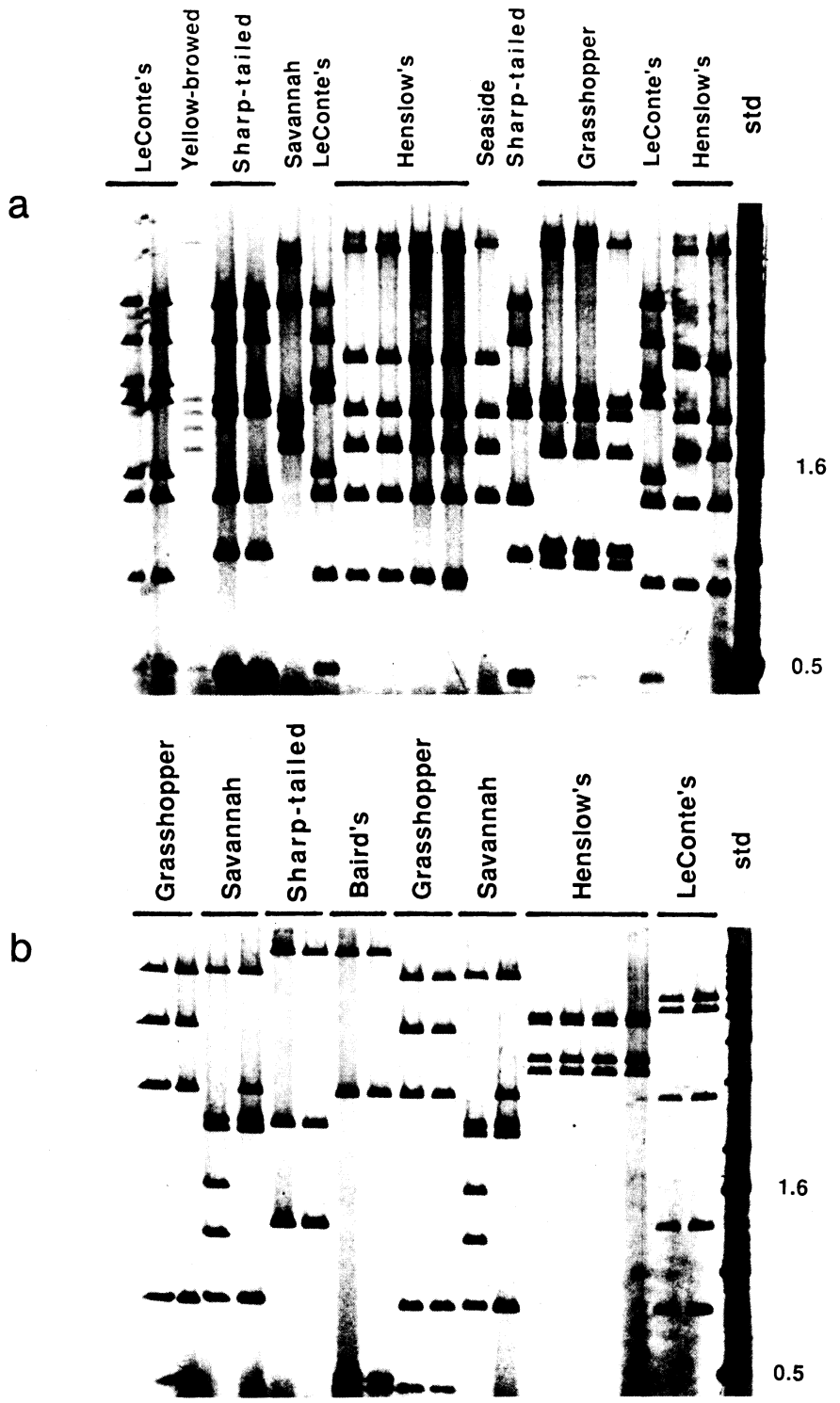

FIG. 4. a. Example of genetic variation in mtDNA when digested with *Hind* III. Numbers on right hand side represent kilobase pairs of DNA. b. Example of genetic variation in mtDNA when digested with *Bcl* I.

shows four basic groups: 1) seaside, sharp-tailed, and LeConte's sparrows; 2) Henslow's and Baird's sparrows; 3) grasshopper and yellow-browed sparrows; and 4) the savannah sparrow. In the bootstrapped consensus tree (not shown), only one node was identified in over 95% of the trees (other than the clustering of conspecific individuals)—that uniting the seaside, sharp-tailed, and LeConte's sparrows as a trichotomy. In the following analyses, a single specimen of each species was used because the conspecific individuals clustered with other conspecifics in all of 100 bootstrapped replicate trees. The FM tree (Fig. 2) is topologically consistent with the phenogram (Fig. 1b) as is the parsimony analysis of fragments (Fig. 3; unfortunately, the yellow-browed sparrow could not be scored for every enzyme and is not included in this analysis).

DISCUSSION

Level and Calibration of Genetic Differentiation

Many studies have documented the conservative nature of allozymic differentiation among avian taxa (Avise and Aquadro, 1982). The mean protein genetic distance separating the species in this study, D = 0.39, is 6.5 times higher than the average of 0.06 reported by Zink (1982) in a comparable study of sparrows in the genera *Melospiza* and *Zonotrichia*, and for birds in general (Avise and Aquadro, 1982). The difference in levels of allozymic differentiation might involve taxonomic comparability (Johnson et al., 1988): *Ammodramus* might be old by avian standards, or polyphyletic. Until recently, several species currently in *Ammodramus* were placed in other genera (A.O.U., 1957), and the genus might be too inclusive relative to other avian genera. Although several of the interspecific allozyme distances in *Ammodramus* are relatively high by avian standards, they remain low or moderate compared to values for many other vertebrate congeners (Avise and Aquadro, 1982). In any case, genetic distances alone should not be used to decide taxonomic rank.

The mean level of mtDNA differentiation in *Ammodramus*, although lower than that in other vertebrate groups of comparable taxonomic rank, is also somewhat larger than values previously obtained in limited comparisons of other avian congeners: 0.07 versus an average of ca. 0.04 (Kessler and Avise, 1985; Shields and Helm-Bychowsky, 1988). Kessler and Avise (1985) estimated a percentage sequence divergence of 0.05 among the mtDNAs of some of the same species for which Zink (1982) estimated a genetic (allozyme) distance of 0.14. However, mtDNA divergence in *Ammodramus* is only about 2-fold higher (i.e., 0.07 vs. 0.04) than previous reports for certain other congeneric sparrows, compared to our finding of a 6.5-fold difference (0.39 vs. 0.06) for allozymes. One possibility is that the mtDNA data could reflect a more rapid saturation of observable changes (via homoplasy in gel bands) at these levels of divergence. In any event, both mtDNA and protein comparisons reveal that the *Ammodramus* group of sparrows is genetically differentiated to a greater degree than most avian congeners previously studied (Avise and Aquadro, 1982; Avise and Zink, 1988).

Geographic variation within species surveyed could affect our phylogenetic analysis. There was little evidence of intraspecific allozymic variation, consistent with most surveys of birds (Barrowclough, 1983). Few studies of geographic variation in mtDNA exist for birds (see Avise and Zink, 1988). Our mtDNA analysis uncovered evidence of some intraspecific differentiation. In the seaside sparrow, samples from the Atlantic and Gulf coasts differ markedly (Avise and Nelson, 1989). MtDNAs of some savannah sparrows also differed, but because our sample included only wintering specimens, we do not know whether the variation represents geographic differentiation or within-population polymorphism. Overall however, in comparison to between-species differences in mtDNA, within-species distances in *Ammodramus* appear low (mean p = 0.006), and geographic variation is not therefore a likely bias in our interspecific comparisons.

A supposed virtue of molecular-system-

atic analysis is the approximately time-dependent nature of molecular evolution (Nei, 1987). Few attempts have been made to calibrate avian protein distances for estimation of divergence dates (Gutiérrez et al., 1983; Marten and Johnson 1986). Using the calibrations proposed in these papers (26.3 and 19.7 million years, respectively, per unit of Nei's [1978] genetic distance), the average time of divergence of *Ammodramus* species is roughly 10.3 to 7.7 MYBP. For various vertebrates, including birds (Shields and Wilson, 1987), mtDNA divergence between a pair of lineages has been calibrated at about 2% per million years. Moritz et al. (1987) noted values as low as 1% sequence divergence per million years, although they warned that this calibration might only be applicable for primates. Given these rate estimates for mtDNA, the interspecific mtDNA difference of $p = 0.07$ yields an average divergence date of 3.5 to 7.0 MYBP for *Ammodramus* species, lower than the estimate based on protein differences, but depending on the calibration used, potentially similar (e.g., 7.7 vs. 7.0). These comparisons represent the first attempts to compare avian divergence dates based on comparisons of both mtDNA and proteins, and we note that at least some independently proposed calibrations (of different taxa and genomic regions) seem to converge. Further comparisons of independently calibrated, genetically independent data sets such as mtDNA and allozymes might help clarify rates of molecular evolution.

Comparison of mtDNA and Allozymic Patterns of Variation

There is a high degree of congruence between the mtDNA and allozyme trees, as well as between different methods of analysis within each data set (Figs. 1–3). We next evaluate the significance of the observed congruence. Taxa missing from some mtDNA analyses (grassland and yellow-browed sparrows) complicate direct comparison; hence, we concentrate on the phenograms, the analyses with most taxa in common. The general approach is outlined by Simberloff (1987) who calculated the probability that independent branching diagrams match by chance alone. This approach seems useful because our data sets are genetically independent. There are seven "phylogenetically" informative nodes in the allozyme phenogram and six nodes in the mtDNA phenogram (Fig. 1a, b). If we exclude the node in the protein data set that subtends the grassland sparrow (not in the mtDNA analysis), the two branching diagrams share 5 of 6 nodes. This degree of topological congruence is far greater than that expected by chance (Simberloff, 1987). For instance, with eight species (in the mtDNA analysis), there are 135,135 possible bifurcating unrooted trees (Felsenstein, 1978). A perfect match would occur by chance with a probability of 7×10^{-6} (Simberloff, 1987). If we excluded the savannah sparrow, which causes the conflict between mtDNA and allozyme data sets, the two phenograms would agree perfectly. Agreement at this level would occur by chance once in 10,395 times (9.6×10^{-5}). Thus, the similarity of the phenograms is likely not spurious.

The different methods used to infer branching diagrams yielded generally concordant results. However, comparison of the allozyme phenogram and FM tree reveals differences that might be due to rate heterogeneity. The FM and parsimony trees, which are not as biased by rate heterogeneity, also show high levels of congruence. The agreement among Figures 1–3 suggests that rate heterogeneity and other causes of homoplasy, although present, are not extensive in our data sets. Our data thus appear to be robust to differences in assumptions between phenetic and parsimony methods. This should not, of course, necessarily be viewed as a general result. Therefore, the congruence among our mtDNA and protein branching diagrams is not spurious, and we suggest that the reason for congruence is that both data sets reflect phylogenetic relationships.

Neigel and Avise (1986) have shown that the phylogenetic relationship in particular gene genealogies (such as those provided by mtDNA) typically changes through time from a condition of polyphyly to paraphy-

ly to monophyly, with reference to the history of fragmentation of the populations through which the genes have been transmitted. The agreement between the mtDNA and protein data sets in these sparrows suggests that sufficient internodal times have elapsed for the two types of phylogenies, one based on a single organellar gene (mtDNA) and one based on many nuclear genes (allozymes), to have reached a state of congruence (monophyly). It is likely that species less differentiated than those studied here, or those separated by shorter internodal times, would yield increasingly discordant mtDNA and allozyme phylogenies (as well as discordances among the haplotype genealogies of unlinked nuclear loci), simply as a result of the stochastic process of lineage sorting and extinction during speciation (Neigel and Avise, 1986).

Considerable attention has focused on estimating the robustness of phylogenetic trees (e.g., Nei et al., 1983; Lanyon, 1985; Felsenstein, 1985), and bootstrapping offers an important statistical approach. The bootstrapped mtDNA (fragment) tree of all individuals included only one node with significant phylogenetic support. However, bootstrapping requires independent characters (Felsenstein, 1985), which restriction fragments often are not. Bootstrapping across restriction sites is preferred, but in our study not feasible. Nonetheless, the mtDNA and allozyme data sets yielded highly congruent estimates of relationships, which in our opinion shows that the fragment data set contains phylogenetic information. In addition to nonindependence of characters, other factors could compromise bootstrap analyses, including ours. Closely-spaced speciation events will not often have many molecular synapomorphies, especially if these events occurred relatively early in a clade's history (Lanyon, 1988); consequently, bootstrapping will indicate, correctly, low levels of confidence in a phylogenetic hypothesis. Perhaps when many nodes are more or less uniformly distributed along a tree uniting many taxa (e.g., Fig. 1b), certain nodes may not receive bootstrap support even though the majority of pairs of taxa are clearly distinguishable. For example, suppose that only the grasshopper, Henslow's and Baird's sparrows had been included in our survey. The Henslow's-Baird's cluster is clearly highly distinct from the grasshopper cluster, though each cluster is connected to others by intermediate nodes, which complicates the overall picture, and leads to little structure in the bootstrap consensus tree. There can be much more information present in the data than the bootstrap consensus tree indicates. Comparison of trees derived from genetically independent data sets provides a powerful means to assess confidence in a particular branching topology (Simberloff, 1987), especially in the situations in which bootstrapping might be too conservative (Sanderson, 1989), or inappropriate if the characters are not independent.

Systematic Relationships

Systematic relationships in this group of sparrows are poorly resolved (e.g., compare classifications in A.O.U. [1957] and A.O.U. [1983]). The protein and mtDNA trees support the distinctness of the seaside, sharp-tailed, and LeConte's sparrow cluster. The arrangement of taxa in this group is unclear, although seaside and sharp-tailed sparrows seem to be sister species. In contrast, Murray (1968) concluded (termed convincing by Mayr and Short [1970]) that LeConte's and sharp-tailed sparrows are sister species. Based on the protein evidence, the grassland and yellow-browed sparrows are sister species and form a sister-group relationship with the grasshopper sparrow, as suggested by Short (in Mayr and Short, 1970); this group is genetically distinct from the others. The protein and mtDNA data suggest that Baird's and Henslow's sparrows are a relatively old sister-species pair. Given the relationships suggested by the phenogram, the genus *Ammodramus* (sensu A.O.U., 1983) is possibly not monophyletic. Additional analyses using outgroups to the savannah sparrow plus *Ammodramus* are needed to document limits of monophyletic groups (and clarify intrageneric ge-

netic distances). Previous generic limits (A.O.U., 1957) seem better to reflect phylogeny than current taxonomy (A.O.U., 1983).

A criterion used by some (e.g., Mayr and Short, 1970) for merging genera, including those studied here, is hybridization. As discussed previously (Zink, 1982), hybridization is not limited to sister taxa, and can reflect retention of an ancestral condition (McKitrick and Zink, 1988). If the specimen referred to by Dickerman (1968) as a hybrid between the savannah and grasshopper sparrows is indeed a hybrid, it indicates only that hybridization can occur between genetically distinct species, and not that these species are sister taxa or congeneric. Given that the occurrence of hybridization is suspect as a sole reference point for phylogenetic analysis and classification, we advocate using a classification system that reflects a phylogeny based on a broader set of genetic characters.

In contrast with Buth's (1984) assertion, we suggest that protein electrophoresis is a viable systematic tool at the genus and family levels for groups such as birds exhibiting conservative molecular evolution (Lanyon and Zink, 1987).

Molecules and Morphometrics

Morphometric analyses portray the pattern of overall similarity in a hierarchical branching diagram, most often a phenogram. The degree to which such diagrams are phylogenetically informative depends on the degree to which patterns of overall similarity reflect genetic divergence. Many of the standard measurements usually subjected to phenetic analysis exhibit significant genetic correlations (Schluter, 1984). Hence, the actual number of characters may be less than the number of measurements coded. Phenograms derived from morphometric comparisons might reflect a history of adaptive responses, which may or may not be coincident with phylogenetic history (Endler, 1982).

Robins and Schnell (1971) obtained a series of measurements of skeletal characters from species of *Ammodramus*; their analysis was exemplary of its kind. The phenogram

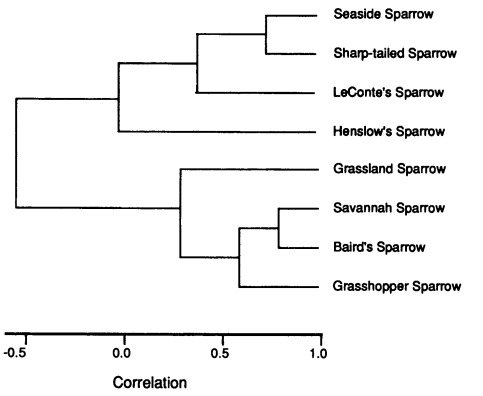

FIG. 5. UPGMA phenogram based on correlation coefficients among skeletal measurements (from Robins and Schnell, 1971).

in Figure 5 represents what Robins and Schnell (1971) termed their best phenogram. We have standardized nomenclature to facilitate comparison with our trees, and we only show the taxa in common between the two studies. Considerable conflict exists between the genetic and morphometric results. We input the topology of Figure 5 into HENNIG86, and found that the allozyme data required 52 steps, 11 more than the most parsimonious trees derived from the allozyme data. Consistent with the genetic data is the grouping of LeConte's, sharp-tailed and seaside sparrows, although there are essentially no other points in common. The morphometric results led Robins and Schnell (1971) to advocate two genera, *Ammodramus* (with yellow-browed, savannah, Baird's, and grasshopper sparrows; the grassland sparrow would presumably be included here), and *Ammospiza* (with LeConte's, sharp-tailed, seaside, and Henslow's sparrows). They referred to *Ammodramus* as the grassland sparrows and *Ammospiza* as the marshland sparrows. Our genetic data support the monophyly of the marshland group, excluding Henslow's sparrow, but the grassland group seems to be a conglomerate of different genetic lineages, which have likely converged in (or retained the primitive) skeletal morphology. A cladistic analysis of morphological patterns might better reveal phylogenetic patterns. Given the lack of a strong phy-

logenetic signal in the morphometric pattern, we suggest that in this example, skeletal morphometrics is a better index to habitat or ecological association (e.g., grassland) than to phylogeny.

ACKNOWLEDGMENTS

We are grateful to S. W. Cardiff for collecting many of the specimens used in this study. We are grateful to G. F. Barrowclough, J. M. Bates, K. J. Burns, J. Cracraft, S. J. Hackett, M. S. Hafner, T. W. Reeder, J. V. Remsen, Jr., and an anonymous reviewer for comments on the manuscript. B. Nelson and M. Ball provided excellent technical assistance. RMZ was supported by a grant from the Louisiana Board of Regents (LEQSF #86-LBR-(048)-08), and JCA by grants from NSF.

REFERENCES

AMERICAN ORNITHOLOGISTS' UNION. 1957. Check-list of North American birds. Fifth ed. Amer. Ornithol. Union, Washington, D.C.

AMERICAN ORNITHOLOGISTS' UNION. 1983. Check-list of North American birds. Sixth ed. Amer. Ornithol. Union, Washington, D.C.

AVISE, J. C., AND C. F. AQUADRO. 1982. A comparative summary of genetic distances in the vertebrates. Evol. Biol., 15:151–185.

AVISE, J. C., AND W. S. NELSON. 1989. Molecular genetic relationships of the extinct dusky seaside sparrow. Science, 243:646–648.

AVISE, J. C., AND R. M. ZINK. 1988. Molecular genetic divergence between avian sibling species: King and Clapper rails, Long-billed and Short-billed dowitchers, Boat-tailed and Great-tailed grackles, and Tufted and Black-crested titmice. Auk, 105:516–528.

BALL, R. M., JR., S. FREEMAN, F. C. JAMES, E. BERMINGHAM, AND J. C. AVISE. 1988. Phylogeographic population structure of Red-winged Blackbirds assessed by mitochondrial DNA. Proc. Natl. Acad. Sci. USA, 85:1558–1562.

BARROWCLOUGH, G. F. 1983. Biochemical studies of microevolutionary processes. Pages 223–261 *in* Perspectives in ornithology (A. H. Brush and G. A. Clark Jr., eds.). Cambridge Univ. Press, New York.

BUTH, D. G. 1984. The application of electrophoretic data in systematic studies. Ann. Rev. Ecol. Syst., 15: 501–522.

CORBIN, K. W. 1987. Geographic variation and speciation. Pages 321–353 *in* Avian genetics (F. Cooke and P. A. Buckley, eds.). Academic Press, London.

DICKERMAN, R. W. 1968. A hybrid Grasshopper Sparrow × Savannah Sparrow. Auk, 85:312–315.

ENDLER, J. A. 1982. Problems in distinguishing historical from ecological factors in biogeography. Am. Zool., 22:441–452.

FARRIS, J. S. 1986. Distances and statistics. Cladistics, 2:144–157.

FELSENSTEIN, J. 1978. The number of evolutionary trees. Syst. Zool., 27:27–33.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach utilizing the bootstrap. Evolution, 39:783–791.

FELSENSTEIN, J. 1986. Distance methods: A reply to Farris. Cladistics, 2:130–143.

FITCH, W. M., AND E. MARGOLIASH. 1967. Construction of phylogenetic trees. Science, 155:279–284.

GUTIÉRREZ, R. J., R. M. ZINK, AND S. Y. YANG. 1983. Genic variation, systematic, and biogeographic relationships of some galliform birds. Auk, 100:33–47.

HACKETT, S. J. 1989. Effects of varied electrophoretic conditions on detection of evolutionary patterns in the Laridae. Condor, 91:73–90.

HILLIS, D. M. 1987. Molecular versus morphological approaches to systematics. Ann. Rev. Ecol. Syst., 18: 23–42.

JOHNSON, N. K., R. M. ZINK, G. F. BARROWCLOUGH, AND J. A. MARTEN. 1984. Suggested techniques for modern avian systematics. Wilson Bull., 96:543–560.

JOHNSON, N. K., R. M. ZINK, AND J. A. MARTEN. 1988. Genetic evidence for relationships in the avian family Vireonidae. Condor, 90:428–445.

KESSLER, L. G., AND J. C. AVISE. 1985. A comparative description of mitochondrial DNA differentiation in selected avian and other vertebrate genera. Mol. Biol. Evol., 2:109–125.

LANSMAN, R. A., R. O. SHADE, J. F. SHAPIRA, AND J. C. AVISE. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations III. Techniques and potential applications. J. Mol. Evol., 17:214–226.

LANYON, S. M. 1985. Detecting internal inconsistencies in distance data. Syst. Zool., 34:397–403.

LANYON, S. M. 1988. The stochastic mode of molecular evolution: What consequences for systematic investigations? Auk, 105:565–573.

LANYON, S. M., AND R. M. ZINK. 1987. Genetic variation in piciform birds: Monophyly and generic and familial relationships. Auk, 104:724–732.

LYNCH, M. 1989. Phylogenetic hypotheses under the assumption of neutral quantitative-genetic variation. Evolution, 43:1–17.

MARTEN, J. A., AND N. K. JOHNSON. 1986. Genetic relationships of North American cardueline finches. Condor, 88:409–420.

MAYR, E. AND L. L. SHORT. 1970. Species taxa of North American birds. Pages 1–127 *in* Publication of the Nuttall Ornithology Club, No. 9 (R. A. Paynter, Jr., ed.). Cambridge, Massachusetts.

MCKITRICK, M. C., AND R. M. ZINK. 1988. Species concepts in ornithology. Condor, 90:1–14.

MORITZ, C., T. E. DOWLING, AND W. M. BROWN. 1987. Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. Ann. Rev. Ecol. Syst., 18:269–292.

MURRAY, B. G., JR. 1968. The relationships of sparrows in the genus *Ammodramus*, *Passerherbulus*, and *Ammospiza*, with a description of a hybrid LeConte's × Sharp-tailed Sparrow. Auk, 85:586–593.

NEI, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89:583–590.

NEI, M. 1987. Molecular evolutionary genetics. Columbia Univ. Press., Cambridge.

NEI, M., AND W. H. LI. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA, 76: 5269-5273.

NEI, M., F. TAJIMA, AND Y. TATENO. 1983. Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. J. Mol. Evol., 19:153-170.

NEIGEL, J. E., AND J. C. AVISE. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. Pages 513-534 *in* Evolutionary processes and theory (E. Nevo and S. Karlin, eds.). Academic Press, New York.

ROBINS, J. D., AND G. D. SCHNELL. 1971. Skeletal analysis of the *Ammodramus-Ammospiza* grassland sparrow complex: A numerical taxonomic study. Auk, 88:567-590.

ROGERS, J. S. 1972. Measures of genetic similarity and genetic distance. Univ. Texas Stud. Genet., 7: 145-153.

SANDERSON, M. J. 1989. Confidence limits on phylogenies: The bootstrap revisited. Cladistics, 5:113-129.

SARICH, V. M., C. W. SCHMID, AND J. MARKS. 1989. DNA hybridization as a guide to phylogenies: A critical analysis. Cladistics, 5:3-32.

SCHLUTER, D. 1984. Morphological and phylogenetic relations among the Darwin's finches. Evolution, 38:921-930.

SELANDER, R. K., M. H. SMITH, S. Y. YANG, W. E. JOHNSON, AND J. B. GENTRY. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). Univ. Texas Publ. Genetics, 7103: 49-90.

SCHAFFER, H. B. 1986. Utility of quantitative genetic parameters in character weighting. Syst. Zool., 35: 124-134.

SHIELDS, G. F., AND K. M. HELM-BYCHOWSKI. 1988. Mitochondrial DNA of birds. Pages 273-295 *in* Current ornithology. Volume V (R. F. Johnston, ed.). Plenum, New York.

SHIELDS, G. F., AND A. C. WILSON. 1987. Calibration of mitochondrial DNA evolution in geese. J. Mol. Evol., 24:212-217.

SIMBERLOFF, D. 1987. Calculating probabilities that cladograms match: A method of biogeographic inference. Syst. Zool., 36:175-195.

SIMBERLOFF, D. 1988. Effects of drift and selection on detecting similarities between large cladograms. Syst. Zool., 37:56-59.

SWOFFORD, D. L., AND R. K. SELANDER. 1981. A computer program for the analysis of allelic variation in genetics. J. Hered., 72:281-283.

ZINK, R. M. 1982. Patterns of genic and morphologic variation among sparrows in the genera *Zonotrichia, Melospiza, Junco,* and *Passerella*. Auk, 99:632-649.

ZINK, R. M. 1986. Patterns and evolutionary significance of geographic variation in the Schistacea group of the fox sparrow (*Passerella iliaca*). Ornithol. Monographs. Volume 40. Amer. Ornithol. Union, Washington, D.C.

Received 19 May 1989; accepted 12 December 1989